IDENTIFICATION AND CHARACTERIZATION OF SOME BIOACTIVE COMPONENTS IN SELECTED LOCAL WHEAT (*TRITICUM* SPP.) VARIETIES

SEÇİLEN YEREL BUĞDAY (*TRİTİCUM* SPP.) ÇEŞİTLERİNDE BAZI BİYOAKTİF BİLEŞENLERİN TANIMLANMASI VE KARAKTERİZASYONU

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ABSTRACT

Identification and Characterization of Some Bioactive Components in Selected Local Wheat (*Triticum* spp.) Varieties

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This study was carried out to determine some bioactive compounds and their antioxidant properties in whole wheat grains of selected on-farm conserved Turkish wheat genotypes (including 4 monococcum, 3 dicoccum, 4 durum, and 7 aestivum varieties). The chemical composition of whole wheat grains has been extensively studied, yet the literature on the bioactive compounds of Turkish local wheat genotypes is limited. For this purpose, 18 different wheat varieties including 4 different genotypes were supplied from TAGEM, Ankara. The total phenolics (including soluble-free, soluble-conjugated, and insoluble-bound phenolics), individual phenolic acid composition, total flavonoids, fatty acid composition, phytosterol profile, and steryl ferulate contents were determined in all whole wheat samples by UV/Vis Spectrophotometer, GC-FID, GC-MS, and HPLC-DAD. Overall, a comprehensive analysis of all results was performed using statistical tools such as Heatmap.

Sünter was selected as a representative wheat for advanced purification (TLC, PTLC, and MPLC) and characterization due to its high phenolic content in local wheat varieties. Metabolites such as phenolic acids, fatty acids, sterol/stanols, steryl ferulates, α-tocopherol, phospholipids, sugars, sugar alcohols, organic acids etc., were identified in

different whole and isolated extracts of Sünter wheat with GC-MS, GC-FID, and HPLC-DAD, whereas characterization of purified compounds was achieved with the help of ^{1}H and ^{13}C NMR.

The results indicated that the dicoccum genotypes had the highest soluble-free and soluble-conjugated phenolics, whereas aestivum was rich in insoluble-bound, total phenolic, and flavonoid contents. The antioxidant capacity of monococcum and dicoccum wheat was slightly higher compared to the durum and aestivum genotypes.

The major fatty acids in wheat lipids were identified as linoleic, oleic, and palmitic acids. Phytosterol/stanols included campesterol, campestanol, stigmasterol, β-sitosterol, β-sitostanol, and Δ5-avenasterol, whereas campesteryl, campestanyl & sitosteryl, and sitostanyl ferulate were identified as steryl ferulates. The monococcum wheat varieties were considered healthier for human consumption due to the presence of low amounts of saturated fatty acids. Also, monococcum varieties were found rich in terms of total phytosterol and steryl ferulate contents as compared to other genotypes.

The analyses lead to the conclusion that local wheat genotypes, particularly dicoccum and aestivum, serve as valuable sources of both phenolic and antioxidant compounds. The local wheat varieties, such as Sünter, Zerun, AK-702, Köse 220/33, Spelt S. başak Siyez-4, and Karakılçık, contained higher amounts of beneficial bioactive compounds, e.g., phenolics, flavonoids, ferulic acid, unsaturated fatty acids, phytosterols, and steryl ferulates, making them a better choice over the commercial ones.

This study offered a quick approach to obtain the important information regarding bioactive compounds in local wheat varieties. Furthermore, it could also provide valuable insights into the selection of on-farm conserved wheat genotypes for future wheat breeding programs. This study is expected to make important contributions to the limited literature on the bioactive composition of local Turkish wheat genotypes.

Keywords: Wheat bioactive compounds, phenolics, fatty acid composition, phytosterols, steryl ferulates, antioxidant capacity.

ÖZET

Seçilen Yerel Buğday (*Triticum* spp.) Çeşitlerinde Bazı Biyoaktif Bileşenlerin Tanımlanması ve Karakterizasyonu

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Bu çalışma, koruma programı kapsamındaki bazı yerel buğday genotiplerinin (4 monococcum, 3 dicoccum, 4 durum, 7 aestivum çeşitler) biyoaktif bileşikleri ve antioksidan özelliklerini belirlemek amacıyla yapılmıştır. Tam buğdayların bileşimi ayrıntılı araştırılmış olsa da yerel buğday genotiplerinin biyoaktif içerikleri hakkındaki literatür sınırlıdır. Bu amaçla, TAGEM (Ankara)'den sağlanan 4 farklı genotipi içeren 18 buğday çeşidinde toplam fenolik madde (çözünür-serbest, çözünür-bağlı ve çözünmez-bağlı fenolik madde), fenolik asit kompozisyonu, toplam flavonoidler, yağ asidi içeriği, fitosterol ve steril ferulat profili, UV/Vis Spektrofotometre, GC-FID, GC-MS ve HPLC-DAD kullanılarak belirlenmiştir. Genel olarak, tüm sonuçların kapsamlı bir analizi, Heatmap gibi istatistiksel araçlar kullanılarak gerçekleştirilmiştir.

Tüm yerel buğday örneklerini temsil etmek üzere ileri saflaştırma (TLC, PTLC ve MPLC) ve tanımlama analizleri için fenolik madde içeriği yüksek olan Sünter çeşidi seçilmiştir. Yapılan saflaştırma ve tanımlama sonucunda fenolik asitler, yağ asitleri, sterol/stanoller, steril ferulatlar, α-tokoferol, fosfolipitler, şekerler, şeker alkolleri,

organik asitler vb. gibi metabolitler GC-MS, GC-FID, HPLC-DAD, ^{1}H ve ^{13}C NMR ile tanımlanmıştır.

Dicoccum genotipi buğdaylarda çözünür-serbest ve çözünür-bağlı fenolik bileşikler yüksek miktarlarda bulunurken, aestivum genotiplerinin çözünmeyen-bağlı fenolik ve toplam fenolik madde ile flavonoid bakımından zengin olduğu gözlenmiştir. Monococcum ve dicoccum buğdaylarının antioksidan kapasitesi durum ve aestivum genotiplerinden daha yüksek bulunmuştur.

Tüm buğday çeşitlerinde linoleik, oleik ve palmitik asitler temel yağ asitleri olarak saptanmış olup, fıtosterol/stanol olarak; kampesterol, kampestanol, stigmasterol, β-sitosterol, β-sitostanol, Δ5-avenasterol ile steril ferulat olarak da; kampesteril, kampestanil & sitosteril, ve sitostanil ferulat belirlenmiştir. Monococcum buğday genotipleri, diğer genotiplere göre daha az doymuş yağ asidi içerirken, fitosterol ve steril ferulat bakımından da daha zengin bulunmuştur.

Genel olarak sonuçlar, dicoccum ve aestivum'un hem fenolik hem de antioksidan bileşikler açısından önemli bir kaynak olduğunu göstermiştir. Sünter, Zerun, AK-702, Köse 220/33, Spelt S. başak Siyez-4 ve Karakılçık gibi bazı yerel çeşitlerin fenolik madde içerikleri, flavonoidleri, ferulik asitleri, çoklu-doymamış yağ asidi içerikleri ve fitosterol/stanol, steril ferulat içerikleri bazı ticari buğday çeşitlerinden yüksek bulunmuştur. Bu nedenle, belirtilen yerel çeşitlerin ticarileştirilme şansı yüksektir.

Bu çalışmada uygulanan prosedür yerel buğday çeşitlerindeki biyoaktif bileşiklerle ilgili bir izleme programında kullanılabilecek bir protokol ve gelecekteki buğday ıslah programları için önemli bir temel kaynak oluşturabilir. Ayrıca tez sonuçları yerel Türk buğdaylarının biyoaktif madde kompozisyonu konusunda literatüre önemli katkılar sağlayabilir.

Anahtar Kelimeler: Buğdayın biyoaktif bileşikleri, fenolikler, yağ asidi bileşimi, fitosteroller, steril ferulatlar, antioksidan kapasite.

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SYMBOLS AND ABBREVIATIONS

Symbols

α	Alpha
β	Beta
μ	Micro
λ	Lambda
Δ	Delta

Abbreviations

AACC	American Association of Cereal Chemists
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACA	Aldehyde/Carboxylic Acid Assay
ANOVA	Analysis of Variance
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
BSTFA	Bis-trimethylsilyltrifluoroacetamide
CE	Catechin Equivalent
CUPRAC	Cupric Reducing Antioxidant Capacity
DAD	Diode Array Detector
DM	Dry Matter
DPPH	2,2-diphenyl-1-picrylhydrazyl
ESI-MS	Electrospray Ionization Mass Spectrometry
ESI-TOF-MS	Electrospray Ionization and Time of Flight Mass Spectrometry
FAB-MS LOD	Fast Atom Bombardment Mass Spectrometry
FAMEs	Fatty Acid Methyl Esters

FAO	Food and Agriculture Organization
FID	Flame Ionization Detector
FLD	Fluorescence Detector
FOX	Ferrous Oxidation−Xylenol Orange Assay
FRAP	Ferric Reducing Antioxidant Power Assay
FTC	Ferric Thiocyanate Assay
GAE	Gallic Acid Equivalent
GC	Gas Chromatography
HEI	Healthy Eating Index
HPLC	High performance liquid chromatography
LDL	Low Density Lipoprotein
LOD	Limit of Detection
LOQ	Limit of Quantification
LPO	Lipid Peroxidation Inhibitory
MA/GC	Malonaldehyde/Gas Chromatography Assay
MA/HPLC	Malonaldehyde/High-Performance Liquid Chromatography Assay
MALDI–MS	Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry
MPLC	Medium Pressure Liquid Chromatography
MS	Mass Spectrometry
MSTFA	N-methyl-N-trimethylsilylfluoroacetamide
MTT	[3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide]
MUFAs	Monounsaturated Fatty Acids
NIR	Near-Infrared
NIST	National Institute of Standards and Technology
NMR	Nuclear Magnetic Resonance
PDA	Photodiode Array Detector

PTFE	Polytetrafluorethylene Membrane Filter
PTLC	Preparative Thin Layer Chromatography
PUFAs	Polyunsaturated Fatty Acids
QE	Quercetin Equivalent
QUENCHER	QUick, Easy, New, CHEap and Reproducible
SFA	Saturated Fatty Acids
TAGEM	Ministry of Agriculture and Forestry, Field Crops Central Research Institute
TBA	Thiobarbituric Acid Assay
TBHQ	t-butyl hydroquinone
TEAC	Trolox Equivalent Antioxidant Capacity
TFC	Total Flavonoid Contents
TIC	Total Ion Chromatogram
TKW	Thousand Kernal Weight
TLC	Thin Layer Chromatography
TMS	Trimethylsilyl
TPC	Total Phenolic Contents
UFA	Unsaturated Fatty Acids
UPLC	Ultra-Performance Liquid Chromatography
UV/Vis	Ultraviolet/Visible

1. INTRODUCTION

Wheat, initially domesticated in the Fertile Crescent of the Middle East (including Turkey, Syria, Iran, Iraq, Palestine, Lebanon and Jordan), has evolved into a dietary staple for the worldwide human population [1]. The adaptability of this crop enables it to thrive in diverse climatic conditions and geographical regions, resulting in its extensive distribution around the globe. Turkey benefits from its good ecological conditions and its status as a significant center of origin for wheat, which provides it with unique advantages in the production of high-quality wheat varieties.

Anatolia is home to 23 wild and more than 400 cultivated wheat varieties [2, 3]. Common bread wheat (*Triticum aestivum* L., 2n=42) comprises of approximately 90 to 95% of global wheat production. The remaining portion, approximately 35–40 million tons, consists of durum wheat (*T. turgidum* var. durum, 2n=28) and is primarily cultivated in the Mediterranean region [4]. However, ancient wheats such as emmer (*T. dicoccum*, 2n=28) and einkorn (*T. monococcum*, 2n=14) may yield less than modern ones, but their perceived sustainability and superior nutritional profiles have attracted consumer and market interest [5].

Modernization of agriculture along with traditional breeding for high yields have led to a loss of genetic diversity. However, wheat landraces evolved through a combination of natural and farmer-driven selection, typically have a broader genetic base, making them valuable sources for breeding programs [6]. The local wheat varieties were also included within a special support framework initiated by the Ministry of Agriculture and Forestry, Field Crops Central Research Institute (TAGEM) Ankara, Turkey, with the involvement of universities in monitoring and reporting on the conservation system project [7].

The local wheat varieties have gained attention from both researchers and farmers recently, because of their health-promoting benefits. Although a number of studies have been carried out on bioactive compounds of commercial wheat [8-10], the investigation has remained limited to the local genotypes [11].

Generally, wheat is considered to have numerous health-beneficial bioactive compounds, which are mainly located in the bran and germ fractions [12, 13]. However, the availability of these compounds has been affected by many factors including wheat genotypes, growing conditions, and processing techniques [14]. It is beneficial to study Turkish local wheat genotypes, which are considered significant sources of healthy

bioactive compounds, such as phenolics, fatty acids, phytosterols, steryl ferulates and other antioxidant compounds. Therefore, these local wheat varieties are valuable not only in terms of their commercial potential but also in their suitability for future breeding programs.

The health beneficial role of phenolic acids is already supported by many epidemiological studies against the prevention of chronic diseases e.g., diabetes, cardiovascular disease, aging, cancer etc. [15, 16]. In addition to phenolic compounds, the omega fatty acids such as linoleic (n-6) and α-linolenic (n-3) are essential fatty acids that enhance the nutritional value of wheat lipids [17]. A diet rich in n-3 fatty acids has a protective effect on a variety of autoimmune illnesses in Western population [18].

Phytosterols and steryl ferulates exhibit bioactive properties like antioxidant, anti-fungal, anti-inflammatory, anti-atherogenicity, and anti-ulcerative activities [19, 20]. In wheat, the phytosterols are present in esterified form with sugars and ferulic acid, commonly known as steryl glycosides and steryl ferulates, respectively [21]. Moreover, phytosterols are also valuable in lowering blood pressure and preventing various types of cancer [22].

The investigation of local wheat varieties has the potential to raise the awareness of local farmers regarding the bioactive properties of these varieties and facilitate their incorporation into future breeding initiatives. In addition, this approach offers an easy and reliable alternative to evaluate and quantify the bioactive compounds in local wheat genotypes, as part of an ongoing conservation programs.

2. LITERATURE REVIEW

2.1. Wheat

History and Genetics

Wheat (*Triticum* spp.) is considered a staple cereal food and has a huge importance all over the world. The cultivation of wheat has long history and can be traced back to a period exceeding 10,000 years (during the Neolithic era) in the Eastern Fertile Crescent, covering present-day Turkey Syria, Iraq, and North Africa (Figure 2.1) [23].

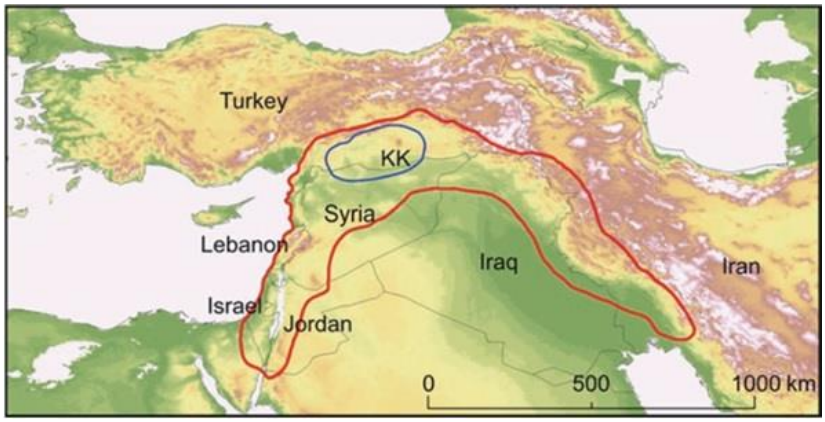


Figure 2.1. Map of Fertile Crescent area (red line) and Karacadağ mountain range (blue line), (adapted from [23]).

Archaeological excavations and genetic research have revealed that Karacadağ mountain region of Diyarbakır city in southeastern Turkey was identified as the geographical origin of Einkorn (*Triticum boeoticum*) and emmer wheat, which are the wild ancestor of modern commercial wheat varieties [24, 25]. Einkorn and emmer wheats are also among the eight primary founder crops that played an important role in early-stage agricultural development. The global distribution of wheat is believed to have occurred through the first farmers who facilitated the adaptation of domesticated wheat populations to diverse environmental conditions [26].

The wheat plant is classified taxonomically as a member of the genus Triticum, which falls within the Triticeae tribe of the Poaceae family. This Triticeae tribe exhibits a global distribution of more than 15 genera and 300 species [27]. In 1918, wheat species has been recognized for its characteristic chromosome number of n = 1× = 7 [28]. The chromosomes ranging from 1 to 7 in different diploid genomes (A, B, and D) have been proposed to have a connection with the evolutionary history of wheat. The einkorn (*T. monococcum* ssp.

monococcum) has diploid (A^mA^m, 2n = 2× = 14) chromosomes; emmer (*T. dicoccum*) and durum (*T. durum*) have tetraploid (A^uA^uBB, 2n = 4× = 28) chromosomes; and spelt (*T. spelta*) and bread wheat (*T. aestivum* L.) have hexaploid (A^uA^uBBDD, 2n = 6× = 42) chromosomes [1].

The evolution of modern bread wheat can be attributed to natural hybridization between its wild and cultivated progenitors through various polyploidization events (Figure 2.2). In the first event of association between genomes of two diploid species, A genome was provided by wild species *Triticum urartu* (A^uA^u, 2n = 2× = 14), while B genome was supplied by an unknown species *Aegilops speltoides* from Sitopsis [29]. This led to the development of allotetraploid wild emmer wheat (*Triticum turgidum* ssp. *dicoccoides*, A^uA^uBB, 2n = 4× = 28), which is generally more dynamic as compared to its wild parents, producing higher yields and adaptable to a wider range of climatic conditions. Subsequently, cultivated diploid einkorn (*T. monococcum* ssp. *monococcum*, A^mA^m, 2n = 2× = 14), tetraploid emmer (*T. turgidum* ssp. *dicoccum*, A^uA^uBB, 2n = 4× = 28) and tetraploid durum wheat (*T. turgidum* ssp. *durum*, A^uA^uBB, 2n = 4× = 28) have derived from their wild ancestor during a long period of domestication [30]. The second hybridization occurred between cultivated emmer wheat (*T. turgidum* ssp. *dicoccum)* and a wild diploid species *Ae. tauschii* (DD, 2n = 2x = 14), resulting in the formation of the allohexaploid early spelt (*T. aestivum* subsp. *spelta*, A^uA^uBBDD, 2n = 6× = 42). Moreover, the evolutionary origin of hexaploid bread wheat (*Triticum aestivum* L., A^uA^uBBDD, 2n = 6× = 42) can be attributed to a complex hybridization event involving cultivated emmer wheat and the diploid *Ae. tauschii* Coss. [31]. This suggests that wild emmer wheat played a pivotal role as the immediate progenitor to all commercially significant cultivated wheat varieties.

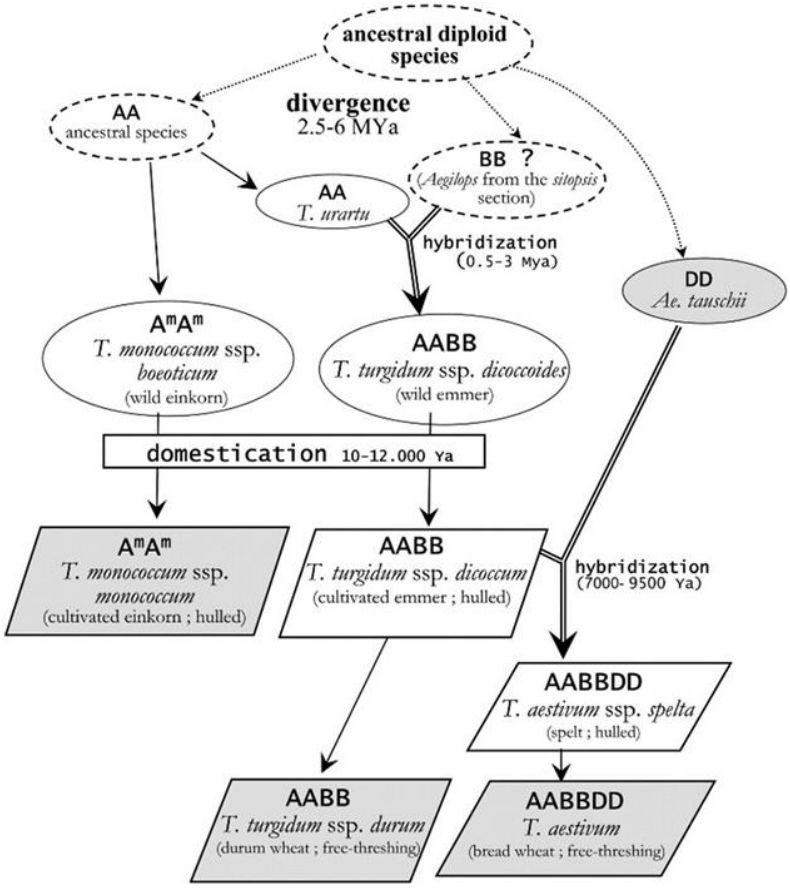


Figure 2.2. Schematic diagram for the evolution of wheat (adapted from [29]).

Economic importance of wheat: from global to local perspective

Wheat plays a significant role in agri-food systems on a worldwide scale and holds considerable importance in ensuring global food security. Wheat production is continuously increasing to meet the nutritional requirements of the growing world population. Recently, wheat cultivation utilized more land than any other commercially grown crop, making it the main source of cereal grains for human consumption [32]. According to the Food and Agriculture Organization (FAO), global cereal production has reached a volume of 2,819 million tons (mt) in the year 2023. However global wheat production accounts for 783.3 mt, still 18.4 mt less than 2022 [33]. Wheat production is anticipated to expand by approximately 60% by the year 2050 as a result of the growing demand brought on by the increasing global population [14].

India, China, and European countries are the major wheat-producing countries, with 40% to 50% of global wheat production. Nevertheless, they are also the top three wheat consuming countries, with at least 40% of global consumption. On the other hand, Russia and Ukraine have contributed 10% and 3%, respectively, to the average global wheat production over the

past five seasons. In addition, Russia is the largest wheat exporter in the world, contributing in 20% of total exports, while Ukraine ranks fifth, accounting for 10% of global wheat exports. War between Russia and Ukraine, which began on February 24, 2022, has significant impact on individual production capacity of Ukraine. Similarly supplies of fertilizer, which is primary export commodity of Russia, has been disturbed through trade restrictions of several countries. These situations have also affected global wheat prices and supply, notably in the Near East and North Africa [34].

Turkey has been among the top ten wheat-producing countries globally, owing to its higher wheat production with an annual production of 19.8 mt in 2022 [35]. The Turkish map displays a percentage distribution of wheat production across various regions of the country (Figure 2.3). Turkey is one of the few countries that are self-sufficient in terms of wheat production and consumption [36], whereas annual wheat consumption per capita is estimated to be 179.3 kg [37]. Turkey also imports wheat primarily for the purpose of producing flour, which are subsequently exported, accounting for 19.04% of global wheat flour in terms of value and 22.45% in terms of quantity [38].

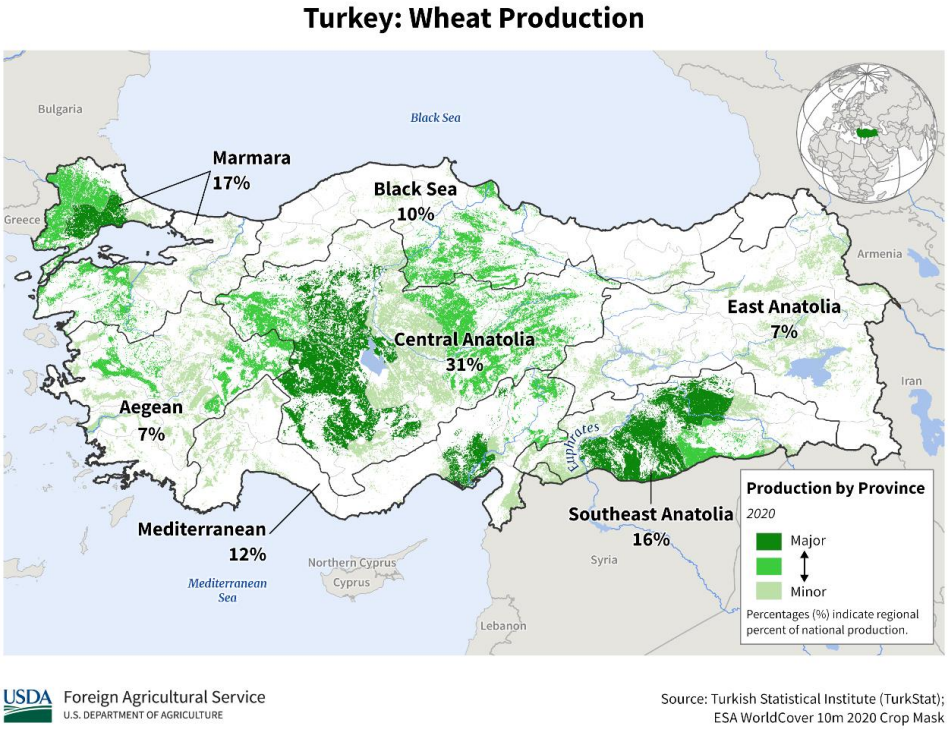


Figure 2.3. Distribution of wheat production in Turkey (adapted from [39]).

2.2. Composition of Wheat Grain

The wheat grain is composed of three primary fractions, namely the endosperm, germ (embryo), and outer coverings (pericarp, seed coat, and aleurone). The endosperm constitutes approximately 82% of the grain on dry basis (Figure 2.4) [40]. Whole wheat grain generally contains carbohydrates (70%) including dietary fibers (10.6%), water (13.2%), protein (11.5%), lipids (1.9%), and minerals (1.9%) [41].

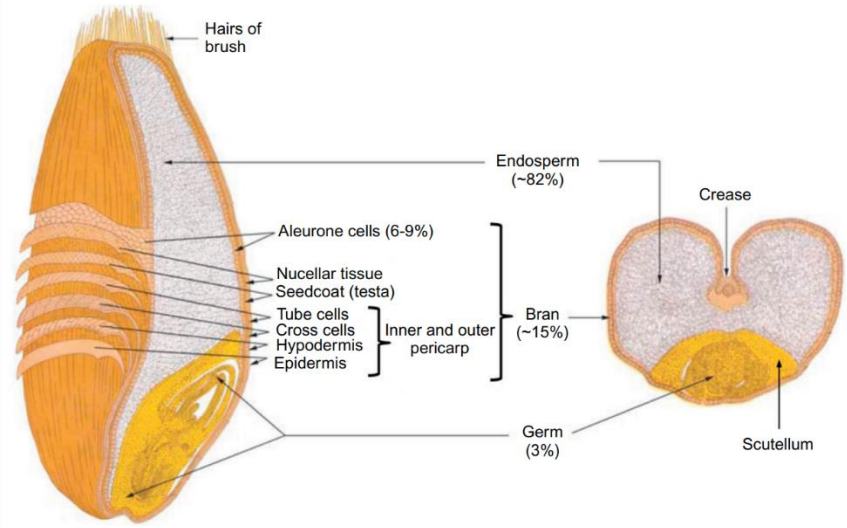


Figure 2.4. The main fractions of whole wheat kernel (adapted from [40])

Wheat has been essential source of complex carbohydrates, mainly found in starchy endosperm fraction of grain. These complex carbohydrates are known for the control release of energy according to the need of human body, thus associated with health beneficial applications.

The proteins are mostly located inside the endosperm, germ, and aleurone layer of whole wheat grain [42]. Modern wheat has comparatively reduced protein contents to that of ancient wheat, probably due to their high thousands kernel weight (TKW) with increased share of starchy endosperm [43].

Lipids comprise a comparatively minor but valuable fraction in the wheat grain due to its bioactive properties. Whole wheat grains possess a diverse range of lipids, such as fatty acids, sphingolipids, terpenes, phospholipids, and other lipid compounds [44]. Generally, ancient wheat varieties (einkorn, emmer, and spelt) exhibit different lipid profiles, tend to have higher lipid content compared to common wheat. In addition, einkorn is rich in monounsaturated fatty acids and reduced saturated fatty acids in comparison to bread wheat, having a positive effect on human health [11]. Furthermore, apart from the main components, wheat grain also serves

as a significant source of other bioactive compounds, antioxidants, as well as micronutrients, all of which play crucial roles in maintaining human health [45, 46].

2.3. Bioactive Compounds in Wheat

The distribution of bioactive compounds inside the grain is not uniform, and the germ and bran fractions tend to have elevated levels of these compounds. Many bioactive compounds along with minerals, vitamins and dietary fibers are rich in bran fraction of whole wheat grain, however milling of wheat grains results in the reduction of their amount in flour [13]. The bran fraction refers to the outermost portion of the wheat kernel, consisting of several layers such as the outer pericarp, intermediate layers (which includes inner pericarp, testa, and hyaline layer), and the inner aleurone layer (Figure 2.3). In the large-scale European HEALTHGRAIN project, bioactive components in cereals were investigated to improve industrial use of whole grain and a novel wheat grain fractionation model was created to enhance the amount of beneficial compounds into the flour [47-49].

The bioactive compounds found in wheat can be subdivided into several primary classes, which include phenolic acids, carotenoids, tocopherols, alkylresorcinols, as well as many additional components such as phytosterols, steryl ferulates, benzoxazinoids, and lignans etc. Extensive research has been conducted on the composition of wheat, yielding significant findings that have been summarized in Table 2.1.

Table 2.1. Bioactive compounds in wheat genotypes.

	Monococcum	**Dicoccum**	**Durum**	**Spelta**	**Aestivum**	**Ref**
Total Phenolic contents (µg/g DM)	615	779	699	579	664	[50]
Soluble-free (µg/g DM)	10	9	13	7	11	[50]
Soluble-conjugated (µg/g DM)	229	172	267	138	162	[50]
Insoluble-bound (µg/g DM)	376	599	418	433	492	[50]
4-hydroxybenzoic acid (µg/g DM)	5.3	11.7	12.9	8.8	7.6	[50]
Vanillic acid (µg/g DM)	12.2	15.7	20.4	15.4	20.9	[50]
Syringic acid (µg/g DM)	5.1	7.9	9.3	11.2	17.6	[50]
Caffeic acid (µg/g DM)	0.8	0.6	-	0.9	0.4	[50]
Sinapic acid (µg/g DM)	121.7	88.5	114	70.8	80.9	[50]
Ferulic acid (µg/g DM)	302	478.8	403.3	368.4	398.8	[50]
ρ-coumaric acid (µg/g DM)	8.2	8.5	10.6	8.6	15.8	[50]
2-hydroxycinnamic acid (µg/g DM)	4.6	7.6	7.8	6.5	6.23	[50]
Total flavonoids (mg QE/g)	141.58	122.83	117.83		122	[51]

Total carotenoids (µg/g DM)	2.26	8.23	3.58	2.16	2.36	[52]
Lutein (µg/g DM)	7.28	2.72	2.81	1.68	1.55	[52]
Zeaxanthin (µg/g DM)	0.20	0.19	0.21	0.12	0.13	[52]
Total Tocols	72.53	46.38	55.78	-	89.03	[53]
α-tocopherol **(**µg/g DM**)**	9.82	6.04	5.06	-	16.46	[53]
β-tocopherol **(**µg/g DM**)**	0.26	0.36	0.25	-	1.02	[53]
α-tocotrienol **(**µg/g DM**)**	12.67	7.78	7.68	-	7.75	[53]
β-tocotrienol **(**µg/g DM**)**	49.78	32.20	42.79	-	63.80	[53]
Alkylresorcinols (µg/g DM)	595	581	-	605	432	[54]
Fatty acids						
Palmitic acid (%)	13.39	15.84	13.98	15.33	17.68	[55]
Steric acid (%)	0.85	1.19	1.34	1.10	0.92	[55]
Oleic acid (%)	29.22	21.96	18.72	22.98	14.36	[55]
Linoleic acid (%)	50.73	55.51	58.94	55.99	61.57	[55]
α-linolenic acid (%)	4.15	4.44	5.88	3.52	4.67	[55]
Arachidic acid (%)	0.19	0.26	0.21	0.17	0.15	[55]
Eicosenoic acid (%)	1.47	0.81	0.93	0.90	0.65	[55]
Total saturated fatty acids (%)	14.43	17.29	15.53	16.6	18.75	[55]
Total unsaturated fatty acids (%)	85.57	82.72	84.47	83.39	81.25	[55]
Total sterols/stanols (µg/g DM)	1054	857	987	928	864	[56]
Campesterol **(**µg/g DM**)**	195	134	159	133	129	[56]
β-sitosterol **(**µg/g DM**)**	500	391	438	457	459	[56]
Stanols **(**µg/g DM**)**	229	228	271	247	199	[56]
Others **(**µg/g DM**)**	130	103	119	91	78	[56]
Total steryl ferulates (µg/g DM)	136.3	82.2	63.6	87.1	91.2	[57]
Campesteryl ferulate (%)	16.6	10.6	9.0	11.9	14.0	[57]
Campestanyl & sitosteryl ferulates (%)	51.7	55.1	59.9	49.6	53.4	[57]
Sitostanyl ferulate (%)	31.6	34.3	31.1	38.5	32.6	[57]

2.3.1. Phenolic compounds

Phenolic compounds are secondary metabolites in plants and produced as a defense mechanism against pathogens and adverse climatic conditions. One prominent class of phenolic compounds is the group known as cell wall phenolics. These compounds are commonly insoluble and coexist with a range of other cellular constituents [58].

These phenolics have a key structural property, characterized by the existence of an aromatic ring having one or more hydroxyl groups. Generally, they are found in the form of soluble-free, soluble-conjugate (esters with low molecules and/or sugar molecules), and insoluble-bound, attached with cell wall via covalent cross linking (Table 2.1). The latter form of phenolics is abundant in wheat grains and responsible for higher antioxidant capacity than free phenolics [59]. These compounds are often combined with monosaccharides or polysaccharides in the form of derivatives. Thus, their solubility varies based on molecular weight of attached molecule and their degree of glycosylation, acylation, or esterification [60, 61]. Various studies have reported alkaline hydrolysis as effective method to release free phenolics, hence contributing to higher antioxidant values. Phenolics are further grouped into five main classes, namely phenolic acids, flavonoids, lignans, stilbenes and other polyphenols [62].

Phenolic acids

Phenolic acids are a class of aromatic acid compounds characterized by the presence of a phenolic ring and an organic carboxylic acid group. They are sub-divided into two groups such as hydroxybenzoic and hydroxycinnamic acids (Figure 2.5) [63]. The hydroxybenzoic acids consist of C1-C6 chain and phenolic acids like gallic, vanillic, protocatechuic, 4-hydroxybenzoic, and syringic acids are some of the main examples of this group. On the other hand, hydroxycinnamic acids have C3-C6 chain in their structure and mainly include ferulic, sinapic, ρ-coumaric, chlorogenic and caffeic acids [64].

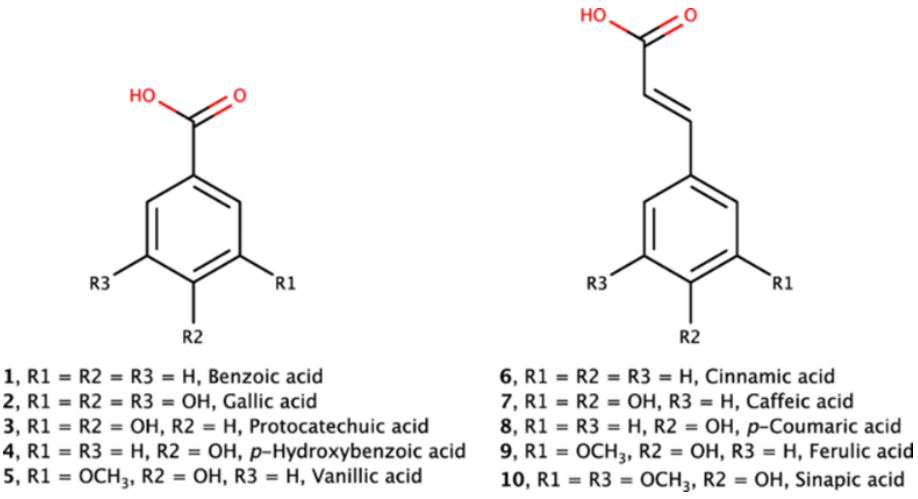


Figure 2.5. Structure of phenolic acids (adapted from [63])

In general, the ferulic acid is most abundant phenolic acid in whole wheat grains (Table 2.1), whereas other phenolic acids such as syringic, ρ-hydroxybenzoic, vanillic, ρ-coumaric, o-

coumaric, sinapic, and salicylic acids were present in minor amounts [65]. Phenolic acids have been observed to demonstrate antioxidant properties that include scavenging free radicals, chelating metal ions, and blocking prooxidant enzymes [66, 67].

Ancient, landrace and hybrid wheat genotypes were compared with the modern bread and durum wheat cultivars and their total phenolic contents ranged from 17.2 to 48.3 mg GAE/g wheat extract [68]. In a study, Zhang, Wang, Yao, Yan and He [16] observed that 37 Chinese winter wheat cultivars had an average bound phenolic concentration of 661 µg/g of DM, making 97.5% of the total phenolic acids.

Flavonoids

Flavonoids contain a 15-carbon structure consisting of two phenyl rings (A and B) and a heterocyclic ring (C). These flavonoids can be divided into six main subgroups based on their shared C6-C3-C6 carbon framework [69]. The group of flavonoids includes a range of subclasses, such as flavones, flavonols, isoflavonols, anthocyanins, anthocyanidins, proanthocyanidins, and catechins [66].

Similar to phenolic compounds, flavonoids are also found in wheat grains [70]. The study conducted by Brewer, Kubola, Siriamornpun, Herald and Shi [71] reported the total flavonoid content ranging from 177.05 to 206.74 µg/g in the wheat bran layers. Furthermore, Leoncini, Prata, Malaguti, Marotti, Segura-Carretero, Catizone, Dinelli and Hrelia [72] revealed that the free flavonoid content of ancient Italian wheat genotypes varied between 9.90 and 31.96 mg CE/100 g, while the bound flavonoids were found to be in between 10.24 to 35.73 mg CE/100 g of grain. In another study, the flavonoid concentration of the bran/germ fraction was reported to be from 740 to 940 µmol of CE/100 g [73].

2.3.2. Fatty acids

The lipids are present as minor components and their amount varied from 0.9% to 3.3% in different wheat genotypes. The major fatty acids in wheat are reported to be linoleic acid (C18:2), palmitic acid (C16:0), and oleic acid (C18:1), whereas linolenic acid (C18:3) and stearic acid (C18:0) were present in lower quantities, as shown in Table 2.1 [55]. The amounts of C18:2, C18:1, C16:0, and C18:3 in whole wheat kernels were found to be varying from 48.5% to 53.1%, 23.4% to 28.6%, 12.9% to 15.4%, and 3.1% to 4.0%, of total fatty acids, respectively [74]. In another study, Narducci, Finotti, Galli and Carcea [10] have reported fatty acid profiles of 10 Italian durum wheat cultivars in decreasing order of linoleic

(C18:2), palmitic (C16:0), oleic (C18:1), linolenic (C18:3), stearic (C18:0), and palmitoleic (C16:1).

Most of the fatty acids in wheat lipids (C18:2, C18:1, C18:3, and C16:1) are unsaturated, and two of them (linoleic and linolenic) are essential fatty acids. The presence of linoleic (n-6) and α-linolenic (n-3) acids as omega fatty acids enhances the nutritional value of the wheat lipids [17]. Various studies have reported the biochemical and clinical significance of dietary n-6 and n-3 polyunsaturated fatty acids on the prevention of cardiovascular and various autoimmune diseases [18, 75].

In a study, Kan [6] performed fatty acid analysis by GC-MS and found oleic acid (n-9), linoleic acid (n-6), and linolenic acid (n-3) as primary fatty acids in different Turkish wheat varieties. Among these, linoleic acid had the highest concentration, with 59.10% in common wheat than 52.16% in durum wheat. Nevertheless, when it comes to the overall unsaturated fatty acid content, durum wheat exhibited a higher percentage at 78.14%, while common wheat closely followed with 77.97%.

The variations in lipid content and fatty acid composition of wheat are influenced by various factors, including genetic factors (wheat species and cultivar) [76], and environmental factors, such as agronomic practices, pedoclimatic factors, and the maturity level of the wheat kernels [10, 77]. Nejadsadeghi, Maali-Amiri, Zeinali, Ramezanpour and Sadeghzade [78] reported the effect of cold stress in durum and bread wheat genotypes, which causes the reduction the saturated fatty acids.

2.3.3. Phytosterols

Plant sterols are bioactive compounds that are present in several plant-derived food sources. These molecules are composed of alcohols with either 28 or 29 carbon atoms and have a structural similarity to cholesterol, characterized by a steroid nucleus, a hydroxyl group at position 3, and a double bond at positions 5 and 6 (Figure 2.7). Phytosterols exhibit the presence of supplementary methyl or ethyl groups, along with double bonds in their side chains. Most of these side chains consist of 9 to 10 carbon atoms. The phytosterols are also classified as 4-desmethyl sterols within the cholestane series, and uniformly possess double bonds at the C-5 position of the ring [79].

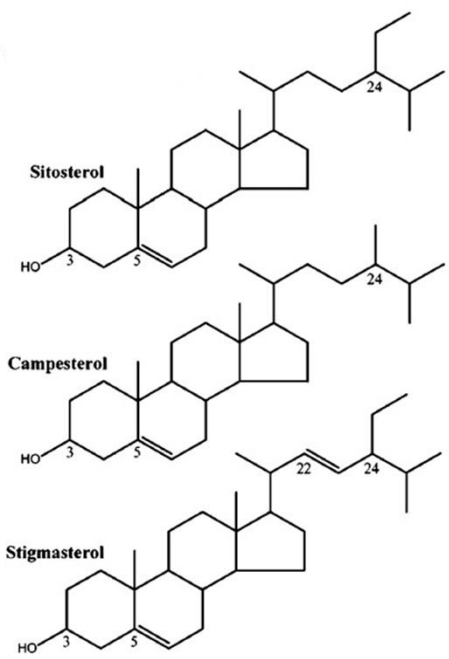


Figure 2.6. Chemical structure of major phytosterols (adapted from [79])

A wide range of phytosterols, exceeding 200 in number, have been identified across many plant species. Among them, the most prevalent ones include β-sitosterol, campesterol, and stigmasterol (Table 2.1). On the other hand, phytostanols, which are a type of saturated phytosterols, don't have the double bonds in their structures [48].

Total phytosterol contents in whole wheat grains have been determined in between 763 and 818 mg/kg on DM basis [56]. Wheat generally contains β-sitosterol, campesterol, and stigmasterol as the main phytosterols [80, 81], whereas the major phytostanols include stigmastanol and sitostanol [48]. On the other hand, brassicasterol, Δ5-avenasterol, cycloartenol, Δ7-stigmastenol, Δ7-avenasterol, and 24-methylenecycloartanol were present in minor amounts [82]. The study conducted by Erdem, Tosun, Akkbik and Hazer [8] revealed the presence of various phytosterols, including stigmasterol, campesterol, and β-sitosterol, in Turkish bread wheat varieties, ranging from 4 to 23 mg/kg, 15 to 76 mg/kg, and 303 to 682 mg/kg, respectively. In another study, Nyström, Paasonen, Lampi and Piironen [49] reported the sterol content of various wheat fraction with germ exhibiting the highest sterol concentration at 492.3 mg/100 g, which was roughly 2.5 times greater than the bran and flour, measuring 207.5 mg/100 g and 200.8 mg/100 g, respectively. Similar to fatty acids, the composition of phytosterols in wheat can be influenced by genetic and environmental variations [82].

Phytosterols rich or supplemented foods can reduce serum cholesterol and low-density lipoprotein (LDL) by inhibiting the absorption of dietary or bile-induced cholesterol from the gastrointestinal tract [83]. In addition to these effects, phytosterols have also been reported to possess anti-inflammatory, antibacterial, antifungal, antiulcer, and antitumor properties [84]. According to Nurmi, Lampi, Nyström, Hemery, Rouau and Piironen [47], the phytosterol-rich fractions could be utilized in cereal foods to increase the consumption of health-promoting compounds from natural sources.

2.3.4. Steryl ferulates

The phytosterols are also present in esterified form with sugars and ferulic acid, commonly known as steryl glycosides and steryl ferulates, respectively (Figure 2.8) [21, 85]. γ-oryzanol in rice bran is the best known steryl ferulate mixture. It consists of cycloartenol ferulate, 24-methylene cycloartenol ferulate, campesteryl ferulate and sitosteryl ferulate [45]. Although oryzanol in wheat was not reported in any studies, but steryl ferulates such as campesteryl and sitosteryl along with sitostanyl ferulate were identified in Japanese wheat varieties [86].

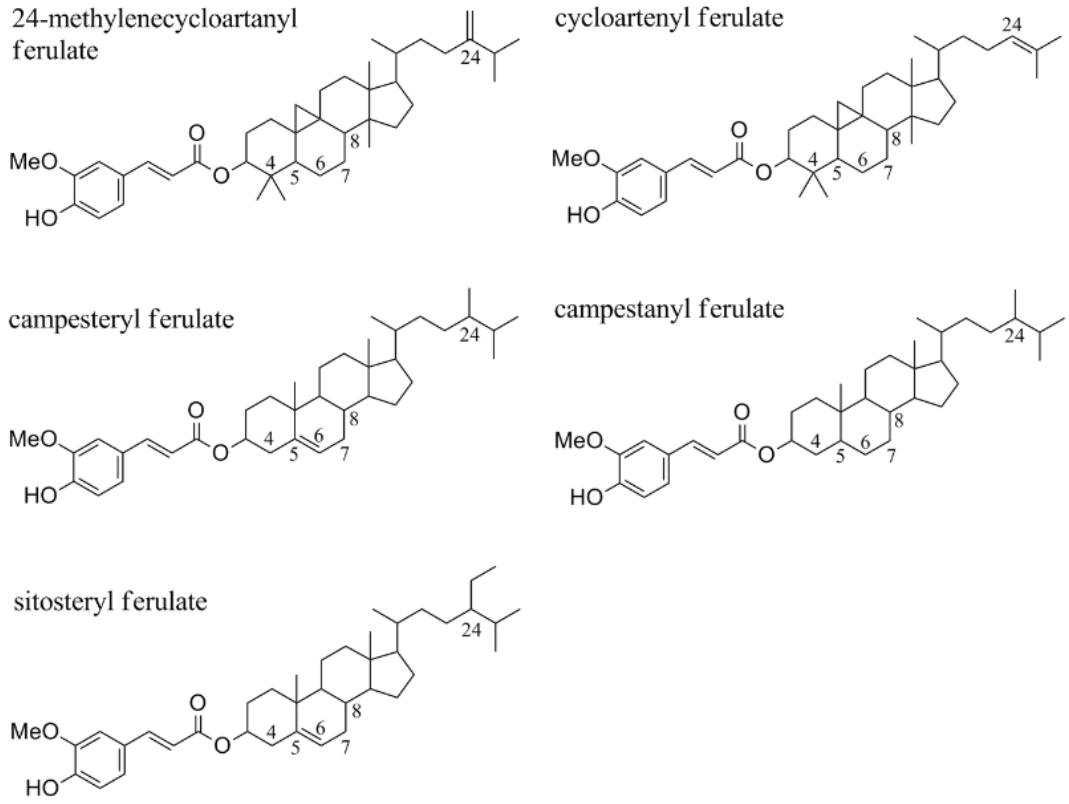


Figure 2.7. Chemical structure of steryl ferulates (adapted from [85])

Total steryl ferulate contents were found in between 6.6 and 12.6 mg/100 g of the whole wheat grains, which is about 6 to 10% of total phytosterols [86]. Nurmi, Lampi, Nystrom and Piironen [48] also reported the steryl ferulate content of 26 different wheat genotypes in the range of 75 to 114 µg/g of DM. In another study, einkorn was reported to have the highest value of 136.3 µg/g DM, whereas bread wheat, spelt, emmer and durum were found to have 91.2 µg/g, 87.1 µg/g, 82.2 µg/g and 63.6 µg/g DM of steryl ferulates [57]. Similar to phytosterol, steryl ferulates are also important for human health, exhibiting bioactive properties like antioxidant, anti-fungal, anti-atherogenic, anti-inflammatory, and anti-ulcerative activities [19, 20].

2.4. Extraction of Bioactive Compounds in Wheat

Bioactive compounds exist in both soluble and insoluble forms. Therefore, the efficient extraction of bioactive compounds with appropriate solvent system is of extreme importance for their accurate recovery from food and raw materials. The choice of extraction solvents, including water, acetone, ethyl acetate, alcohols (propanol, methanol, and ethanol), pure or mixtures, significantly effects the amount of extracted bioactive compounds [87]. Also, time and temperature have significant impacts on the extraction of bioactive compounds from plant-derived foods [88]. On the other hand, prolonged extraction and higher temperatures have been observed to increase the solubility of the analyte. However, the extended extraction periods and elevated temperatures might result in degradation or unfavorable oxidation of bioactive compounds [89].

The extraction of bioactive compounds from solid food matrices is generally achieved by utilizing conventional techniques such as Soxhlet extraction, heated reflux extraction, and maceration. Extraction by the maceration method is normally carried out at room temperature for several days, whereas Soxhlet and heated reflux extraction can be carried out within a few hours at 90 °C. However, despite their simplicity, affordability, and satisfactory results in extracting bioactive compounds, these methods have significant limitations, including the use of large quantities of harmful organic solvents, require prolonged extraction, and susceptibility to interference, degradation, and external factors such as light, air, high temperatures, and enzymatic reactions [90].

Solid-liquid extraction is a separation technique that relies on the selective dissolving of one or more components of a solid mixture in a liquid solvent. The process of solid-liquid extraction is commonly referred to as "elution" or "leaching", especially when it involves the extraction of precipitated solute from adsorbent material [91]. Another example of solid-liquid extraction

is sequential extraction, in which multiple extractions were carried out on a solid sample aliquot using more than one solvent to isolate specific compound groups based on their varying polarity. [92].

Liquid-liquid extraction, also known as solvent extraction or partitioning, is a technique used to separate substances based on their different solubilities in two immiscible liquids, typically an organic solvent (non-polar) and water (polar) [93].

Furthermore, Folch and Bligh & Dyer techniques are based on two-step such as solid-liquid and then liquid-liquid extraction methods. These techniques effectively transfer hydrophobic lipids into an organic solvent-rich phase, while hydrophilic compounds are collected in the water-rich phase [94].

Besides the conventional methods, several emerging technologies, such as ultrasound-assisted extraction, microwave-assisted extraction, pressurized liquid extraction, subcritical fluid extraction, solid-phase extraction, and enzyme-assisted extraction were generally used to enhance the extract yield of bioactive compounds [90]. Although these new techniques have abilities to increase extraction yield, they also lead to excessive breakage of bonding forces in targeted phenolic compounds [95, 96].

2.4.1. Extraction of phenolic compounds

The phenolic extraction from plant materials is substantially influenced by the content of the sample matrix and the particle size. Phenolics have abilities to establish chemical interactions with other constituents of the sample. The cleavage of these bonds can be achieved by the introduction of enzymes or alkaline conditions in order to release free phenolic compounds [97, 98]. Accordingly, it can be shown that around 90% of phenolics exist in the insoluble-bound form, whereas less than 9% and 1% are present in the soluble-conjugated and soluble-free forms, respectively [45]. The extraction of free phenolics is generally carried out using suitable solvent system including pure water or organic solvents, or their mixture. Afterwards, the remaining solid residue is subjected to hydrolysis in the presence of strong alkali. The mixture is subsequently acidified, and phenolic fraction was then isolated using mixture of ethyl acetate and diethyl ether solvents. The resulting extract is utilized for the quantification of bound phenolic compounds [99].

A number of studies have already reported different extraction methods for total phenolic compounds of whole wheat grain and methanol, ethanol and acetone are among the commonly used solvents for the extraction of free phenolics [100]. However, extraction of soluble-

conjugated and insoluble-bound phenolics has been achieved with the help of alkaline hydrolysis techniques [97].

2.4.2. Phytosterol and steryl ferulate extraction

The total phytosterol content of a food sample is a combination of the free, fatty acyl ester, glycoside, and fatty acyl glycoside forms of phytosterol. In general, there are three steps to measure phytosterols: (a) hydrolysis (acidic and alkaline), (b) derivatization, and (c) quantification. The trimethylsilyl (TMS) derivatization could be used immediately after alkaline hydrolysis to measure the free and fatty acyl esters forms of phytosterols, whereas acid hydrolysis was required to measure glycosidic phytosterols [56, 101]. Generally, agents like N-methyl-N-trimethylsilylfluoroacetamide (MSTFA) or bis-trimethylsilyltrifluoroacetamide (BSTFA) were used for derivatization of plant extracts [102].

Extraction of sterile ferulates from wheat can also be accomplished using organic solvents as an effective choice. Solvents such as acetone, ethyl methyl ketone, and ethyl acetate have exhibited the highest efficiency in extraction of 24-methylene cycloartanyl ferulate, cycloartenyl ferulate, and sitosteryl ferulate, respectively [85].

2.5. Identification of Bioactive Compounds

The identification and determination of bioactive compounds in whole grains are undergoing continuous improvement within the analytical approach, mostly due to the rapid advancements in technology. The utilization of high-performance liquid chromatography (HPLC) and gas chromatography (GC) has been widely preferred in the field of analytical chemistry for isolating, identifying, and quantifying phytochemical substances present in wheat samples [103]. Among the available options, thin layer chromatography (TLC), medium pressure liquid chromatography (MPLC), preparative thin layer chromatography (PTLC), HPLC-DAD (diode array detector), FID (flame ionization detector), MS (mass detector) have emerged as the most extensively utilized techniques. Besides chromatography, nuclear magnetic resonance (NMR) and near-infrared (NIR) detection are crucial for investigating structural characteristics and conducting both qualitative and quantitative assessments of bioactive compounds [45].

Liquid chromatography

TLC is a simple, inexpensive, sensitive, fast, and widely used technique that separates the multiple compounds in the crude extract at different retention times (Rf) based on their polarities [104]. It is performed as a preliminary analysis to determine the optimum working

conditions in column chromatography. After finding the appropriate mobile phase system, MPLC is applied as a general technique for the isolation of bioactive molecules in large sample using a combination of different solvents (gradient elution). The mixture is eluted over the stationary phase with the help of the mobile phase and each substance is separated according to its polarities. However, PTLC can be substituted for column chromatography when purification of small quantities of extracts is required [105].

In HPLC, many factors including sample purification, mobile phase selection, column types, and detection techniques have impact on the analysis of phenolic compounds [90]. Typically, purified phenolic samples are isolated and detected with the help of HPLC device equipped with a reversed-phase C18 column (RP-C18) and DAD. The integration of MS, including electrospray ionization mass spectrometry (ESI-MS), matrix-assisted laser desorption/ionization mass spectrometry (MALDI–MS), and fast atom bombardment mass spectrometry (FAB-MS), has also been used to analyze and confirm the structural characteristics of various classes of phenolic compound. In addition, novel techniques such as HPLC-NMR and ultra-performance liquid chromatography (UPLC) have emerged as effective systems for identifying bioactive compounds within natural products [106].

A comprehensive examination of phenolic compounds including phenolic acids, flavonoids, coumarins, proanthocyanidins, stilbenes, and lignans, was carried out on both ancient and modern Italian durum wheat varieties utilizing HPLC in conjunction with electrospray ionization and time of flight mass spectrometry (ESI-TOF-MS) [107]. In another study by Moheb, Ibrahim, Roy and Sarhan [108], 40 phenolic compounds were detected in wheat leaves by using HPLC-ESI-MS. Most of these compounds were coumarin, phenolic, hydroxycinnamoyl amides, and flavonoid derivatives.

HPLC-UV based method was developed for quantitative analysis of total phytosterol. Here concentration of phytosterol was determined at 254 nm after derivatization with benzoyl chromophore. This innovative approach analyzed phytosterols with recovery of more than 95% [109].

On the other hand, the separation of steryl ferulates was accurately achieved using both HPLC-DAD and HPLC-MS based analyses, revealing the individual steryl ferulates (sitosteryl ferulate, sitostanyl ferulate, and campesteryl ferulate) in wheat samples [21, 110]. In a study, Ziegler, Schweiggert, Würschum, Longin and Carle [57] used HPLC-PDA-FLD for the determination of steryl ferulate contents in einkorn, emmer, durum, spelt, and bread. Similarly,

the steryl ferulate contents of Japanese and Canadian wheat varieties were determined using HPLC-UV [86].

Gas chromatography

GC is employed for the analysis of volatile bioactive compounds that are not sensitively determined using HPLC methods [111]. The most common columns used in GC to quantify bioactive compounds are fused silica capillary columns (30 m length with internal diameters ranging from 25 to 32 µm and stationary phase particles size of 0.25 µm). Recently, MS has gained in popularity and significantly improved the sensitivity and selectivity of GC and replaced the conventional FID detector as the method of choice for detecting volatile bioactive compounds [112].

Analytical techniques using GC equipment have been developed and validated in response to the growing interest about the composition and quantification of fatty acids in foods. The fatty acids are generally analyzed by GC-FID or GC-MS techniques after derivatizing them into fatty acid methyl esters (FAMEs) [113]. The detection of FAMEs using GC-FID offered improved resolution for saturated fatty acids, whereas GC-MS exhibited enhanced sensitivity for unsaturated fatty acids [114]. The fatty acid composition of Italian durum wheat was examined, and GC-FID was used to identify several fatty acids, such as linoleic, palmitic, oleic, linolenic, stearic, and palmitoleic acids [10]. The study conducted by Pastor, Ilić, Vujić, Jovanović and Ačanski [115] introduced a novel approach to utilize the fatty acid composition with GC-MS analysis, for establishing authentication techniques in industrial crops, including corn, wheat, barley, and oat, as well as their corresponding food products.

The profiling and composition of individual phytosterols were also achieved with the help of GC-MS or GC-FID equipment. The phytosterol concentrations of the wheat fractions were determined using GC-FID, whereas the steryl ferulate amounts were analyzed using HPLC-UV [47].

Nuclear magnetic resonance

NMR spectroscopy is of great importance in the determination of the molecular structure of biologically active phytochemicals and has been increasingly employed in metabolomic investigations [116]. NMR normally uses magnetic properties of certain specific nuclei for the characterization of pure compounds [117].

The main idea of NMR usually involves three steps: nuclear spins are aligned under constant magnetic field (B0), a radiofrequency (RF) pulse used to disrupt this nuclear spin alignment,

and detection of signal during RF pulse [118]. NMR analysis utilizes the interaction between externally applied radiofrequency radiation and atomic nuclei in order to characterize the structure of chemical compounds.

Different types of NMR are being used for characterization of pure compounds, including ^{1}H NMR, ^{13}C NMR, ^{15}N NMR, ^{19}F NMR, ^{31}P NMR with both 1D and 2D NMR techniques [119]. In comparison to MS, NMR spectroscopy offers the advantage of providing quantitative data without requiring additional separation or derivatization steps [120].

NMR was generally used for characterization of bioactive compounds such as phenolics, flavonoids and phytosterols etc. [121, 122]. In a study, the identification and characterization of ρ-hydroxybenzoic acid, vanillic acid, syringic acid, ρ-coumaric acid, ferulic acid, cinnamic acid, and lignin were conducted in wheat straw using NMR and NIR techniques [123]. Barison, Pereira da Silva, Campos, Simonelli, Lenz and Ferreira [124] used NMR to study fatty acids. The fact that all fatty acid chains are esterified to a common moiety (glycerol) makes it easy to figure out the fatty acid composition of edible oils using ^{1}H NMR. The quantification is done directly in the ^{1}H NMR spectra by establishing the relationship between the areas of a characteristic signal of each fatty acid and the signal of the glycerol moiety.

2.6. Analysis of Antioxidant Compounds

Antioxidants are classified as either enzymatic or nonenzymatic based on their catalytic activity (Table 2.2). Enzymatic antioxidants are dependent on specific cofactors and exhibit a high level of specificity toward reactive species of their respective substrates. However, non-enzymatic antioxidants have ability to neutralize all types of free radicals [125]. Therefore, it is recommended that at least two different types of assays should be used for antioxidant analyses [126].

The antioxidant capacity of food samples is generally determined using several assays, such as Trolox Equivalent Antioxidant Capacity (TEAC), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH). In contrast to the water-soluble ABTS•+ radical, DPPH• is characterized by its hydrophobic nature, requiring the use of organic solvents for conducting its reactions. DPPH assay is based on hydrogen atom transfer. However, in the presence of strong hydrogen-bonding solvents like methanol, the release of hydrogen atoms is restricted, leading to a major increase in transfer of single electron [127].

Table 2.2. Classification of antioxidant assays.

Enzymatic antioxidant assay	**Non enzymatic antioxidant assay**	
	Lipid peroxidation-based assays	***Electron & radical based assays***
Superoxide dismutase assay	LPO scavenging assay	DPPH scavenging assay
Catalase assay	TBA scavenging assay	ABTS scavenging assay
Peroxidase assay	β-carotene bleaching assay	FRAP scavenging assay
Ascorbate peroxidase assay	Conjugated diene assay	FOX scavenging assay
Ascorbate oxidase assay	MA/HPLC scavenging assay	FTC scavenging assay
Guaiacol peroxidase assay	MA/GC scavenging assay	ACA scavenging assay
Glutathione reductase assay		MTT scavenging assay
		CUPRAC scavenging assay
		Hydrogen peroxide scavenging assay
		Nitric oxide scavenging assay
		Superoxide radical scavenging assay
		Hydroxyl radical scavenging assay
		Phosphomolybdate scavenging assay

* Used citation: Haida and Hakiman [125], Moon and Shibamoto [126] and Liu and Nair [128].

As an essential source of natural antioxidants, whole wheat grains are suitable to produce various functional food products. Several studies have demonstrated the antioxidant properties of whole wheat. The antioxidant activity of the whole wheat kernel varied from 905.5 to 1031.6 mg Trolox/kg DM in ABTS analysis, while the DPPH assay showed antioxidant activity in between 89.0 and 120.2 mg Trolox/kg DM. For both antioxidant assays, whole Turkish einkorn wheat had the highest values, while whole Armenian emmer wheat had the lowest values [74]. However, Akman, Yeşildağ and Zengin [68] reported ABTS and DPPH values for wheat extracts in the range of 11.6 to 21.8 mg TE/g and 12.4 to 53.4 mg TE/g, respectively, in their study of modern bread and durum wheat cultivars alongside ancient, landrace, and hybrid wheat genotypes. In another study, Swiss red wheat grain was found to have ABTS amount of 14.67 TE µmol/g of wheat, however its DPPH result was determined to be 20 mg/mL ED50 (effective dose for 50% of radical inhibition) value [129]. Same study also revealed the correlation ($r=0.94$, $p=0.02$) between the scavenging activities of DPPH and ABTS radicals for ethanolic extracts. Heo, Lee, Park, Kim, Jeong and Lee [130] have reported the ABTS and DPPH radical-scavenging activities as 45.04 and 3.57 mg TE/g in methanolic extracts of 41 Korean whole wheat cultivars, respectively. In Indian bread wheat from six different agro-

climatic zones, the average antioxidant activity of whole wheat was found to be 4.4 µM Trolox Eq./g and 12.3 % discoloration by ABTS and DPPH assays, respectively [131]. Martini, Taddei, Ciccoritti, Pasquini, Nicoletti, Corradini and D'Egidio [132] studied 10 Italian durum genotypes and found the total antioxidant activity values in the range of 40.48 to 51.10 mmol TEAC/kg DM by using ABTS assay. When ABTS and DPPH methods were used to study the antioxidant activity of colored durum wheat landraces from Ethiopia, both radicals showed highest values for purple wheat varieties than red colored ones [133].

OBJECTIVES OF THE RESEARCH

The aim of the study was to evaluate certain wheat genotypes that are cultivated by local farmers under on-farm conservation program and are anticipated to have high bioactive contents responsible for numerous nutritional and health benefits. For this, the total phenolic contents (soluble-free, soluble-conjugated, insoluble-bound) with their individual composition, total flavonoids, and total antioxidant capacity using the free radical scavenging activity of ABTS and DPPH assays were determined in whole wheat samples. Additionally, the variations of lipophilic bioactive compounds such as phytosterols, steryl ferulates and fatty acid composition were investigated in hexane extracts of Turkish wheat genotypes. Subsequently, Sünter was selected as a representative wheat for advanced purification and characterization due to its high bioactive contents in local wheat varieties. Metabolites such as phenolic acids, fatty acids, sterol/stanols, steryl ferulates, α-tocopherol, phospholipids, sugars, sugar alcohols, organic acids etc., were identified with the use of GC-MS, GC-FID, NMR (^{1}H and ^{13}C NMR) and HPLC-DAD.

The easy and reliable quantification of the bioactive compounds might increase the importance of local wheat genotypes and contribute valuable information for the awareness of local farmers. This study also supports the protection of local wheat cultivation and utilization of nutritional and health benefit approach to evaluate and describe these local varieties in ongoing breeding programs, ensuring their availability for future generations. Furthermore, the study model for the analysis of the local wheat genotypes can also be used as a framework for analyzing other indigenous wheat varieties on a global scale.

3. MATERIAL AND METHODS

3.1. Materials and Chemicals

3.1.1. Materials

The research material consists of 18 wheat varieties, including 4 monococcum, 3 dicoccum, 4 durum, and 7 aestivum wheat genotypes, was supplied by the Ministry of Agriculture and Forestry, Field Crops Central Research Institute, TAGEM, Ankara (Table 3.1). These wheat seeds were initially collected from local farmers cultivated under on-farm conservation program, and then grown at the Gölbaşı-İkizce Research and Production Farm in Ankara (latitude 39.57 N and longitude 32.63 E) during the field trials conducted in the years of 2019 and 2020. The altitude is 1200 m above sea level, and the average annual temperature and precipitation are 11 °C and 377 mm, respectively [134]. The images of wheat genotypes (both with and without husk) are included in ANNEX 1. All wheat seeds were re-cultivated in the same field and climatic conditions using standard agronomic practices.

At the beginning of the research, Mirzabey 2000, Eminbey, Bayraktar 2000 and Demir 2000 were selected as commercial controls. During the research, Mergüze, Atasiyez, and Kafkas were also registered as commercial wheat varieties.

The preliminary investigation for local wheat varieties was carried out under the framework of Erasmus+ KA107 International Credit Mobility and BAP-FUK-2019-17752 Project titled “Identification and Characterization of Bioactive Components in Some Local Wheat (*Triticum* spp.) Varieties” in the Department of Horticulture, Michigan State University, USA. Pre-experiment results of hexane-methanol sequential extraction, TLC, MPLC, PTLC, GC-MS, NMR, MTT, and LPO and their optimization trials are included in ANNEX 2.

Table 3.1. List of wheat genotypes selected from on-farm conservation program.

Genotypes	Botanical name	Ploidy	Status	Origin
Mergüze	*Triticum monococcum* L. *ssp. monococcum*	A^mA^m	Commercial**	Kastamonu
Atasiyez	*Triticum monococcum* L. *ssp. monococcum*	A^mA^m	Commercial**	Kastamonu
Siyez 4	*Triticum monococcum* L. *ssp. monococcum*	A^mA^m	Pure line	Kastamonu
Siyez Pop	*Triticum monococcum* L. *ssp. monococcum*	A^mA^m	Local	Kastamonu
Kafkas	*Triticum turgidum* L. *ssp. dicoccum*	A^uA^uBB	Commercial**	Ardahan
Kavılca kırmızı	*Triticum turgidum* L. *ssp. dicoccum*	A^uA^uBB	Pure line	Kars
Gacer	*Triticum turgidum* L. *ssp. dicoccum*	A^uA^uBB	Local	Kayseri
Mirzabey 2000	*Triticum turgidum* L. *ssp. durum*	A^uA^uBB	Commercial*	Central Anatolia
Eminbey	*Triticum turgidum* L. *ssp. durum*	A^uA^uBB	Commercial*	Central Anatolia
Karakılçık	*Triticum turgidum* L. *ssp. durum*	A^uA^uBB	Local	Hatay
Sarı buğday	*Triticum turgidum* L. *ssp. durum*	A^uA^uBB	Local	Eskişehir
Bayraktar 2000	*Triticum aestivum* L. *ssp. aestivum*	A^uA^uBBDD	Commercial*	Central Anatolia
Demir 2000	*Triticum aestivum* L. *ssp. aestivum*	A^uA^uBBDD	Commercial*	Central Anatolia
AK-702	*Triticum aestivum* L. *ssp. aestivum*	A^uA^uBBDD	Local	Eskişehir
Köse 220/33	*Triticum aestivum* L. *ssp. vulgare*	A^uA^uBBDD	Local	Sivas, Erzurum
Sünter	*Triticum aestivum* L. *ssp. vulgare*	A^uA^uBBDD	Local	Eastern Anatolia
Zerun	*Triticum aestivum* L. *ssp. vulgare*	A^uA^uBBDD	Local	Sivas, Erzurum
Spelt S. başak	*Triticum aestivum* L. em Thell spelta	A^uA^uBBDD	Pure line	Central Anatolia

* Initially chosen as commercial controls.
**During research, some local wheat varieties were registered as commercial varieties.

3.1.2. Chemicals and Reagents

High-purity analytical standards such as 4-hydroxybenzoic acid, 2-hydroxycinnamic acid, gallic acid, vanillic acid, ferulic acid, syringic acid, sinapic acid, caffeic acid, protocatechuic acid, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Supelco FAME37 mixture, 5α-cholestane, campesterol, stigmasterol, β-sitosterol, α-tocopherol and γ-oryzanol were purchased from Sigma-Aldrich, Steinheim, Germany. Chlorogenic, ρ-coumaric acid and DPPH (2,2-diphenyl-1-picrylhydrazyl) were supplied by the European Pharmacopia Reference Laboratory (Germany), HWI Pharma Services GmbH (Germany) and Toronto Research Chemicals Inc. (Canada), respectively.

HPLC grade methanol, isopropanol, ethyl acetate, chloroform, toluene, n-hexane, cyclohexane, pyrogallol, potassium persulfate, potassium hydroxide, sodium hydroxide, sodium carbonate, Folin-Ciocalteu reagent, ABTS (2,2′-azinobis (3-ethylbenzothiazoline- 6-sulfonic acid)), methoxyamine hydrochloride, pyridine, and N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), have been obtained from Sigma-Aldrich Steinheim, Germany. Analytical-grade glacial acetic acid, diethyl ether, and ethanol were obtained from ISOLAB (Germany), and hydrochloric acid was bought from Merck, Darmstadt, Germany.

Silica gel 60 F_{254} plates (20×20 cm) for TLC and PTLC were purchased from Merck, Darmstadt, Germany. For MPLC, normal phase silica gel 60 (15-40 µm particle size) and reverse phase LiChroprep RP-18 (15-25 µm particle size) were provided by Merck, Darmstadt, Germany.

3.2. Sampling and pretreatment

Husked wheat genotypes were manually dehulled and cleaned after harvesting to eliminate broken seeds and other foreign objects. A laboratory-scale flour mill was used to grind the seeds into a fine powder (60 mesh size) in accordance with the AACC No:26-50 method [135]. Each sample of wheat flour was lyophilized and vacuum-packed as a single flour fraction in moisture-proof packaging and kept at −20 °C.

3.3. Physiochemical Analysis

3.3.1. Thousand kernel weight and ash content

The thousand kernel weight (TKW) was determined according to the method described by Khodarahmi, Soughi, Shahbazi, Jafarby and Khavarinejad [136], and the total of 1000

cleaned wheat kernels from each wheat variety were counted randomly in duplicates, and their weights were recorded in grams. Moreover, ash content (%) of whole wheat flour was determined according to AACC No: 08-01 method [135].

3.3.2. Extraction of phenolic compounds

Extraction of soluble-free, soluble-conjugated, insoluble-bound, and total phenolic compounds (including flavonoids) were performed according to Moore, Hao, Zhou, Luther, Costa and Yu [99]. At first, soluble-free and soluble-conjugated phenolic compounds were extracted from whole grain flour with the use of acetone/methanol/water (7:7:6, v/v/v) solvent mixture. Then soluble-conjugated and insoluble-bound phenolic compound extracts and whole grain flour were hydrolyzed with 2N NaOH to release free phenolic compounds. After adjusting pH (2) with 6N HCl, all samples were extracted four times with the mixture of ethyl ether and ethyl acetate (1:1, v/v). Then the combined supernatants were concentrated under a gentle stream of nitrogen at 35 °C and reconstituted in 1 mL of 30% methanol in ultrapure water (Milli-Q, 18.2 MΩ cm). All extracts were stored at −20 °C for further analysis (Figure 3.1).

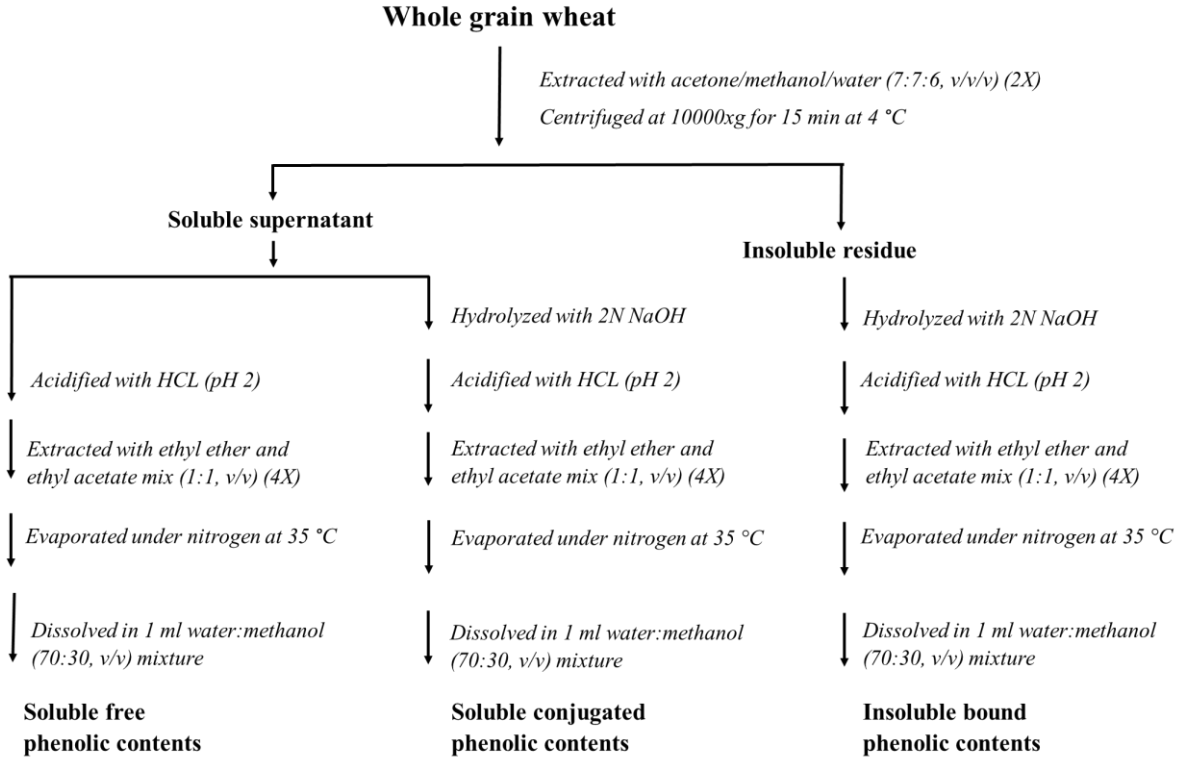


Figure 3.1. Protocol for extraction of phenolic compounds (modified from [99]).

3.3.3. Total phenolic contents

The soluble-free, soluble-conjugated, insoluble-bound, and total phenolic contents in whole grain wheat samples were determined using the Folin-Ciocalteau method [137]. The appropriate dilutions of the extracts (200 µl for soluble and 30 µl for insoluble phenolic contents) were mixed with 800 µL of 0.2 N Folin-Ciocalteau reagent and incubated for 5 minutes. Subsequently, 800 µL of saturated Na_2CO_3 solution was added to the resulting mixture and allowed to react for approximately 60 minutes, until the distinctive blue color became apparent. The mixture was then centrifuged at a rate of 6100×g for 5 minutes and the absorption of the supernatant was measured using a spectrophotometer at a wavelength of 760 nm. Quantifications were carried out as mg of gallic acid equivalent (GAE) per kg of whole grain flour on DM basis, with each trial being replicated three times.

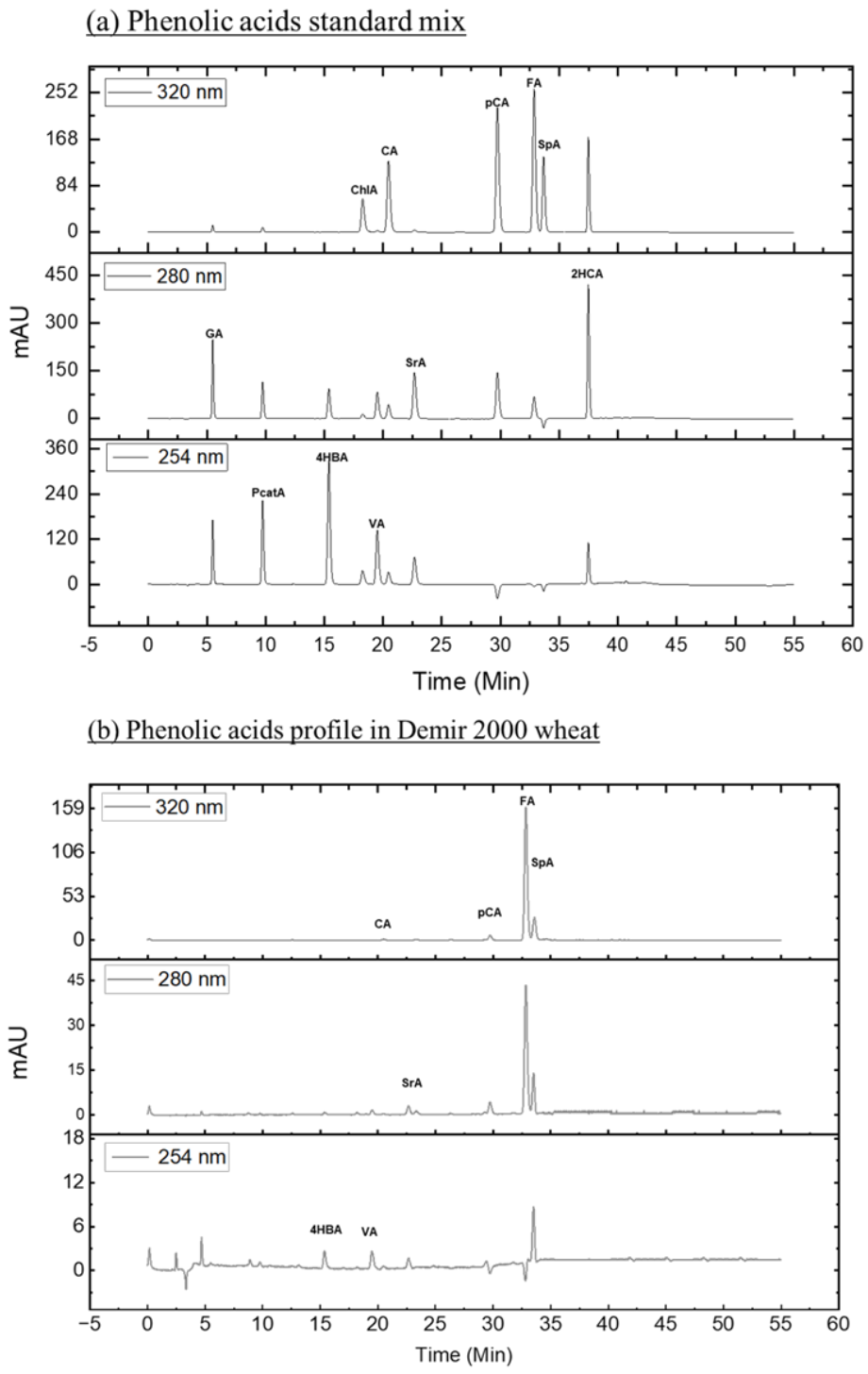
3.3.4. Phenolic acid composition

For the extraction of total phenolic acids, whole wheat flour was directly hydrolyzed with 2N NaOH to release all phenolic acids into their free forms. After the pH was adjusted to 2 with 6N HCl, the hydrolysate was extracted four times with ethyl acetate and ethyl ether (1:1, v/v). The resulting extracts were dried with N_2 gas at 35 °C, reconstituted in 1 mL of 30% methanol, filtered through a 0.22 µm PTFE syringe type filters and stored at −20 °C until HPLC analysis.

Quantitative analyses of individual phenolic acid compositions were performed according to the reported method of Irakli, Samanidou, Biliaderis and Papadoyannis [138]. HPLC system with diode array detector (Agilent Technologies, 1200 series; DAD G1315B, Waldbronn, Germany), and Nucleosil 100-5 C18ec (250 × 4.6 mm I.D. with 5 µm particle size) column (Macherey-Nagel, Düren, Germany) was used for chromatographic separation of phenolic acids (20 µl injection volume) at temperature of 30 °C. Mobile phase consists of pure methanol (A) and 1% acetic acid in Milli-Q water (B), and elution was achieved at the flow rate of 1.3 mL/min with the following gradient program; 90-80% B for 10 min, 80-75% B for 10 min, 75-65% B for 10 min, 65-35% B for 10 min, and column re-equilibration with 90% B for 10 min.

The optimal wavelengths for detecting the peaks of 4-hydroxybenzoic, protocatechuic, and vanillic acids were found to be 254 nm. Similarly, gallic, syringic, and 2-hydroxycinnamic acids showed their peak absorptions at 280 nm. Moreover, chlorogenic,

caffeic, ρ-coumaric, ferulic, and sinapic acids displayed their optimal absorption wavelengths at 320 nm. (Figure 3.2).



GA (Gallic acid); **PcatA** (Protocatechuic acid); **4HBA** (4-hydroxybenzoic acid); **ChlA** (Chlorogenic acid); **CA** (Caffeic acid); **VA** (Vanillic acid); **SrA** (Syringic acid); **pCA** (p-coumaric acid); **FA** (Ferulic acid); **SpA** (Sinapic acid); **2HCA** (2-hydroxycinnamic acid)

Figure 3.2. HPLC chromatograms for phenolic acids in (a) analytical standard mixture and (b) wheat sample.

The analytical standard solution mixture was prepared in different concentrations (1-60 µg/mL) for calibration by using 30% methanol and their retention time (R_t), wavelength (nm), regression values (R^2), LOD and LOQ values were given in Table 3.2. The coefficient of determination (R^2) for gallic, protocatechuic, 4-hydroxybenzoic, chlorogenic, vanillic, caffeic, syringic, ρ-coumaric, ferulic, sinapic, and 2-hydroxycinnamic acids was calculated as ≥ 0.99 (ANNEX 3). The results of individual phenolic acids were calculated as mg/kg DM of whole grain flour.

Table 3.2. Retention time (Rt), wavelength (nm), regression values (R^2), LOD and LOQ values for phenolic acid standards mix by HPLC-DAD.

Phenolic acids	**Retention time (min)**	**λ [a] (nm)**	**Calibration curves [b]**	**R^2**	**LOD [c] (µg/mL)**	**LOQ [d] (µg/mL)**
Gallic acid	5.397	280	y=38.565x-42.452	0.9994	1.952	5.914
Protocatechuic acid	9.698	254	y=45.012x–9.9821	0.9999	0.876	2.654
4-hydroxybenzoic acid	15.407	254	y=85.434x–1.9622	0.9999	0.705	2.137
Chlorogenic acid	18.257	320	y=19.711x–19.912	0.9992	2.148	6.510
Vanillic acid	19.526	254	y=43.100x–3.8192	0.9999	0.665	2.014
Caffeic acid	20.483	320	y=41.384x–22.642	0.9997	1.265	3.833
Syringic acid	22.688	280	y=46.564x–3.0676	0.9999	0.582	1.763
ρ-coumaric acid	29.766	320	y=72.829x–4.0491	0.9999	0.678	2.053
Ferulic acid	32.868	320	y=77.600x–2.7454	0.9999	0.659	1.997
Sinapic acid	33.650	320	y = 34.755x – 21.806	0.9986	2.874	8.710
2-hydroxycinnamic acid	37.503	280	y = 79.895x – 13.245	0.9999	0.881	2.670

[a] Wavelength

[b] y = response area & x = standard concentration,

[c] LOD = Limit of Detection,

[d] LOQ = Limit of Quantification.

3.3.5. Total flavonoid contents

Total flavonoid contents (TFC) in all wheat samples were determined using the colorimetric method of Serpen, Gökmen, Karagöz and Köksel [137], after minor

modifications. In brief, an appropriate dilution of the extracts (200 µl) was mixed with 100 µl of 5% $NaNO_2$ for 5 minutes and a flavonoid-aluminum complex was formed by adding 1 mL of 10% $AlCl_3$. After adding 500 µl of 1N NaOH to the mixture, centrifugation was achieved at 10000×g for 5 minutes at 25 °C. After 15 min of incubation period, the absorbance of the supernatant was measured using a UV/Vis spectrophotometer at a wavelength of 510 nm. The results were expressed as mg catechin equivalents (CE) per kg of whole wheat flour on a DM base.

3.4. Total Antioxidant Capacity

Total antioxidant capacity was determined by the direct QUENCHER-based approach [139]. ABTS and DPPH assays were used for the determination of total antioxidant capacity in wheat genotypes.

3.4.1. Preparation of ABTS•+ and DPPH• radical solutions

A stock solution of ABTS•+ radical was prepared at a final concentration of 7 mmol/L ABTS and 2.45 mmol/L potassium persulfate ($K_2S_2O_8$). To achieve this, 38.41 mg ABTS in 5 mL ultrapure water and 6.615 mg $K_2S_2O_8$ in 5 mL ultrapure water were dissolved separately. Then both solutions were combined and left in the dark for 15 hours at room temperature before being used to prepare a working solution.

Similarly, the stock solution of the DPPH• radical was prepared in a final concentration of 0.5 mmol/L DPPH. For this, 20 mg of the DPPH• radical was first dissolved in 50 mL ethanol then further diluted with 50 mL ultrapure water.

The working solutions of $ABTS^{\bullet+}$ and DPPH• radicals were freshly prepared by diluting the stock solutions with the 50% ethanol till the absorbance reach in between 0.75-0.8 at 734 and 520 nm, respectively.

3.4.2. Calibration with Trolox reference standard

The inhibition capacities of both ABTS•+ and DPPH• radicals were determined and converted to TEAC by using the Trolox reference standard. Standard Trolox solutions were prepared using methanol as the solvent, with concentrations ranging from 0 to 600 µg/mL Trolox. Standard calibration curves were determined by mixing 0.1 mL of a standard solution of each concentration with 10 mL of ABTS·+ and DPPH• radical working solution separately, and their absorbances were measured separately at 734 and 520 nm, respectively. The coefficient of determination (R^2) for ABTS•+ and DPPH•

radicals against the Trolox standard was determined as 0.997 and 0.999, respectively (ANNEX 3).

3.4.3. Measurements of Total antioxidant capacity

Approximately 10 mg of whole wheat flour sample was separately mixed with 10 mL working solution of either ABTS or DPPH radicals, shaken for 27 min at 350 rpm, and centrifuged (Sigma3-18K, Germany) at 6100×g for 2 min. And the absorbance of the supernatants was measured by spectrophotometer at the wavelength of 734 nm for ABTS assay and 510 nm for DPPH assay. Finally, all results were measured as Trolox equivalent antioxidant capacity present in 1 kg DM (mmol TEAC/kg DM).

3.5. Extraction of Bioactive Compounds from Wheat Samples

The extraction of bioactive compounds from whole wheat samples was performed using a sequential extraction method consisting of two different solvent systems (Figure 3.3). The lyophilized whole wheat flour samples were extracted three times overnight with n-hexane (1:10, w/v). The extraction was performed in a sealed container placed in an incubated shaker (SI-300R, Lab Companion, Daejeon, South Korea) at 200 rpm. After extraction, the obtained extract was centrifuged at 10,000×g and 4 °C for 15 minutes, and the supernatant 1 was evaporated to dryness with a rotary vacuum evaporator at 35 °C to obtain hexane extract. Similarly, the remaining residue 1 was again extracted with methanol (1:10, w/v) and dried extract (methanol extracts) was recovered from supernatant 2. All dried extracts were weighed for their mass balance and stored at −20 °C before further analysis [140].

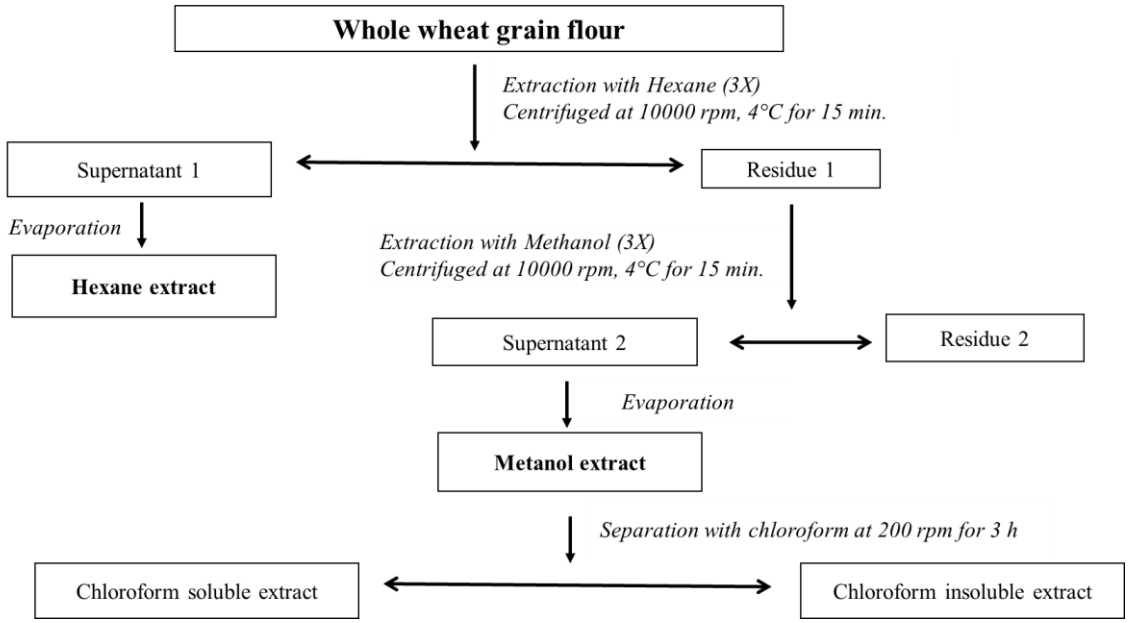


Figure 3.3. Flow chart for sequential extraction using hexane and methanol solvents.

3.5.1. Extraction of Sünter wheat variety

The Sünter wheat variety was selected as a representative of all wheat samples for further purification (TLC, MPLC, PTLC) of bioactive compounds due to its high phenolic content in local wheat varieties. For this, about 250 g of whole grain flour from Sünter wheat variety was extracted with hexane (2.5 L, 3×), yielding 4.57 g of crude hexane extract. The remaining residue 1 was extracted again with methanol (2.5 L, 3×), and 8 g of crude methanol extract was obtained (Figure 3.3). A part of the crude methanol extract (4 g) was mixed with 100 mL chloroform for 3 hours at 200 rpm. The chloroform soluble fraction was subsequently isolated and evaporated to dryness, yielding 1.7 g of chloroform-soluble extract. Similarly remaining insoluble fraction was also dried to yield 2.3 grams of chloroform insoluble extract [141, 142]. All extracts were stored at −20 °C until further analysis.

3.6. Identification of Bioactive Components from Wheat Samples

3.6.1. Fatty acid composition

All hexane extracts (approximately 25 mg) were diluted with 2.5 mL of n-hexane solvent and preparation of fatty acid methyl esters (FAMEs) was carried out with 250 µL of 2N methanolic KOH solution. The saponified portion of the extracts was then precipitated by centrifugation at 5000×g for 15 min, and the supernatant was transferred to a 1.5 mL vial [143].

The methylated fatty acids in the extracts were analyzed by GC-FID system (Thermo Fisher Scientific, Trace GC Ultra, Milan, Italy), with the use of Optimawax column (30 m × 0.25 I.D., 0.25 µm, Macherey-Nagel, Düren, Germany). The oven temperature program was arranged from 140 °C (5 min) to 240 °C (10 min), with an increase of 4 °C/min. The carrier gas was helium (1 mL/min), and the detector temperature was kept at 250 °C. Fatty acids were identified with the help of Supelco FAME-37 standard mixture and their contents were determined as percentage.

3.6.2. Phytosterol profile

The extractions of phytosterols/stanols in hexane extracts were carried out according to the methods of Jekel, Vaessen and Schothorst [144]. For this, approximately 100 mg of hexane extract was mixed in 900 µL toluene solution and 100 µL internal standard (5α-cholestane, 5 mg/mL). The resulting mixture was then gently dried under stream of N_2 gas for 2 min. Then, 8 mL of 3% pyrogallol ethanolic solution and 0.5 mL of saturated KOH solution were added and whole mixture was placed in water bath at 80°C for 30 min to facilitate saponification. After cooling to room temperature, 20 mL of cyclohexane and 12 mL of ultrapure water were added and thoroughly mixed for 5 min. After phase separation, 17 mL of the upper organic extract was carefully removed and dried in a rotary evaporator at 45°C. Subsequently, 1 mL of cyclohexane and a 0.5 mL of 3% pyrogallol aqueous solution were added to the obtained residue and vortexed for 1 minute. After centrifugation at 13000 rpm for 10 minutes at 4°C, the supernatant was transferred to a GC vial after passing through a 0.22 µm syringe filter and stored at −20°C.

Qualitative and quantitative analyses of phytosterols in hexane extracts were performed by GC-MS (Agilent 7890B GC/ 5977A Series MSD Systems, Agilent Technology Inc., Santa Clara, CA, USA) and GC-FID (Thermo Fisher Scientific, Trace GC Ultra, Milan, Italy) devices, with Agilent 19091S-413 HP-5ms (30 m x 0.32 mm I.D. x 0.25 µm, Agilent J&W Scientific, USA) and Alltech EC-1 (30 m × 0.32 mm I.D. × 0.25 µm, Deerfield, IL, USA) columns, respectively.

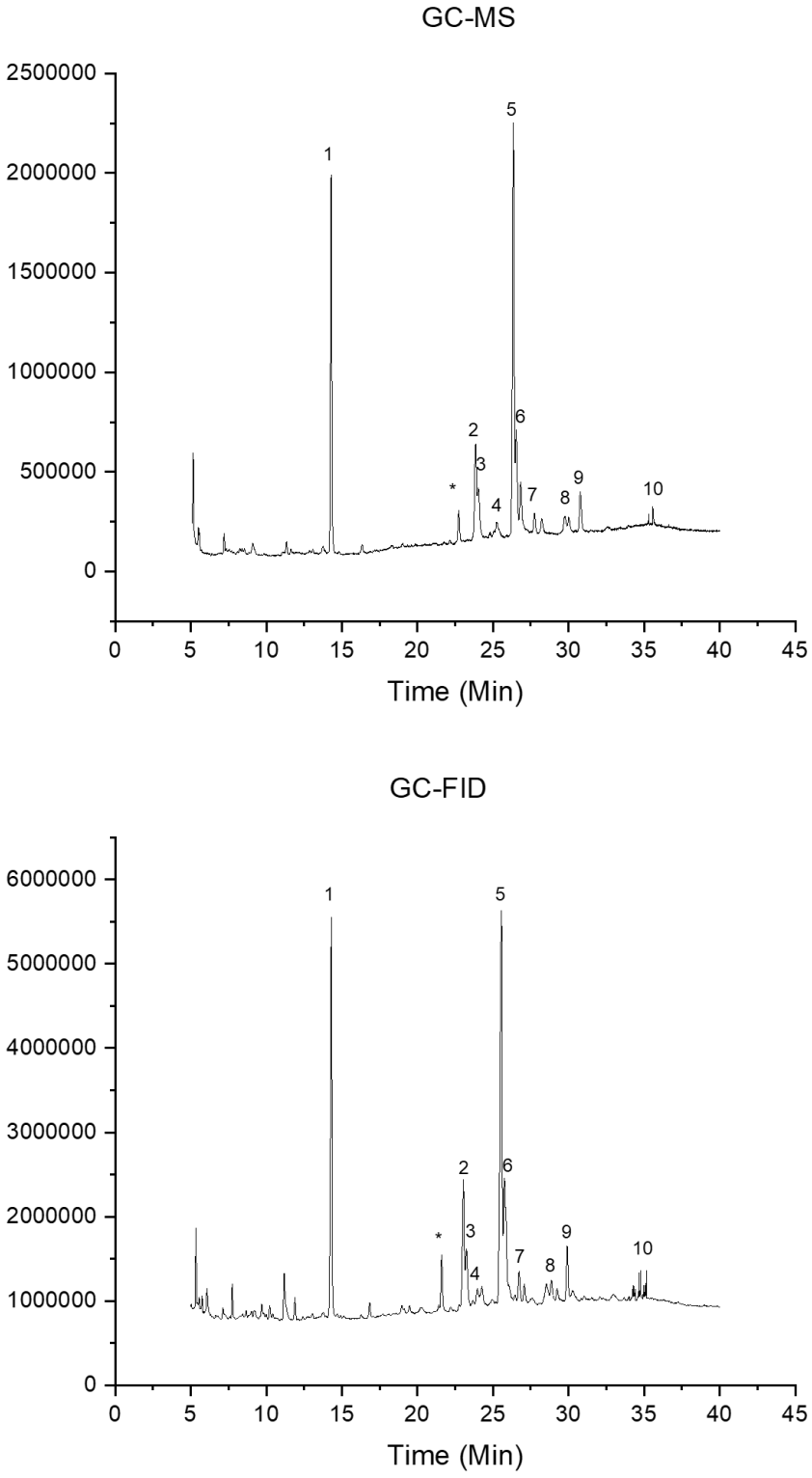
The oven temperature program was arranged from 220 °C (5 min) to 280 °C (10 min), with an increase of 10°C/min. The carrier gas was helium (1 mL/min), and the detector temperature was kept at 280 °C (Sterol/stanol chromatogram in supplementary material). The amounts of phytosterols were calculated according to the following equation using the 5α-cholestane as an internal standard [145].

$$\mathrm{Ps} = \frac{\mathrm{M}is \times \mathrm{A}st \times \mathrm{R}}{\mathrm{A}is \times \mathrm{M}y}$$

*P*s* = Phytosterol amount (mg/g), R = Response factor, M*is* = Internal standard amount (mg), M*y* = Wheat lipids (mg), A*is* =Peak area of the internal standard, A*st* = peak area of sterol.

The response factor in the formula used to determine the amount for each of the compounds in GC chromatograms; was calculated as the ratio of the peak area of the internal standard (5α-cholestane) to the peak area of the external standards (campesterol, stigmasterol, and β-sitosterol) at the same concentration (200 ppm). Due to the lack of standards for campestanol, β-sitostanol and Δ5-avenasterol, their total ion chromatographs (TIC) were identified with an accuracy greater than 90% by comparing the spectra and retention index data of mass spectral libraries like National Institute of Standards and Technology (NIST) MS search version 2.4 and Wiley W10N14 (John Wiley & Sons, Hoboken, NJ, USA) above an accuracy of 90%. This confirmation of MS based peaks was also accomplished by comparing the phytosterol profiles of earlier literature [145-147].

The above-mentioned method was applied to observe the phytosterol profile via GC-FID, and a comparable profile was determined through GC-MS chromatography (Figure 3.4). In addition to the confirmed phytosterols (campesterol, stigmasterol, and β-sitosterol), the mass spectra also indicated the presence of campestanol, β-sitostanol, and Δ5-avenasterol, which have already been discovered in the early [9, 47].



Peak 1. 5α-cholestane, 2. campesterol, 3. campestanol, 4. stigmasterol, 5. β-sitosterol, 6. β-sitostanol, 7. Δ5-avenasterol, 8. unknown, 9. unknown, 10. unknown. * α-tocopherol

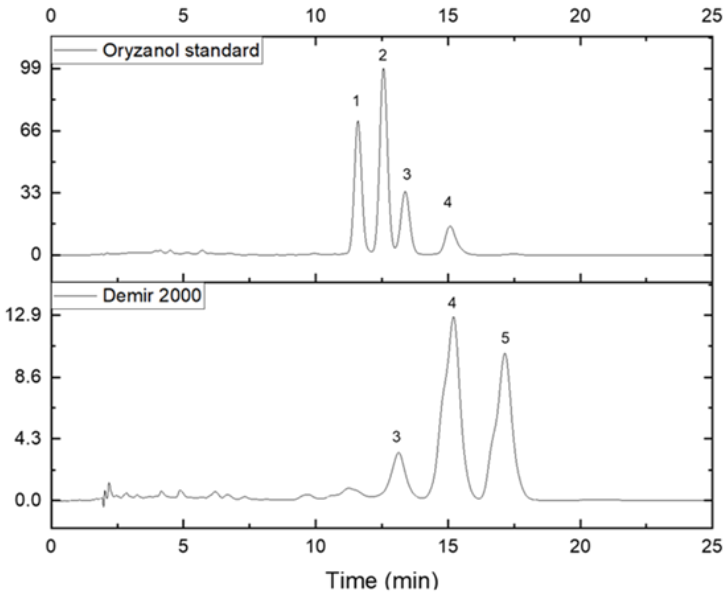
Figure 3.4. GC-MS and GC-FID chromatograms of phytosterols/stanols in wheat.

3.6.3. Steryl ferulate analysis

The analysis of steryl ferulates in hexane extracts was carried out according to the method of Lei, Chen, Liu, Wang, Zhao and Chen [148]. The hexane extracts (5 mg) were mixed with 500 µl of isopropanol and vortexed for 1 min. The resulting extracts were then transferred to the HPLC vial after passing through 0.22 µm syringe type filters, and subsequently stored at −20 °C.

HPLC chromatograms of steryl ferulates (20 µL injection) were obtained using C18 Inertsil ODS3 column (250 mm × 4.6 mm I.D., Hichrom Ltd., Reading, UK) and HPLC-DAD detector (Agilent Technologies, 1200 series; DAD G1315B, Waldbronn, Germany) at 325 nm with acetonitrile and methanol (60:40, v/v) under isocratic conditions (1.5 mL/min). The calibration curve was produced by using the total peak area of oryzanol standard (R^2=0.9997). The amount of steryl ferulates in hexane extracts was determined as "mg γ-oryzanol equivalent" per kg of whole grain flour on DM base.

The HPLC chromatogram of steryl ferulates in the γ-oryzanol standard and wheat is compared in Figure 3.5. The oryzanol standard is composed of cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, campesteryl ferulate, and campestanyl ferulate & β-sitosteryl ferulate [149]. The peaks (3. and 4.) in wheat were confirmed by comparing the retention times of the respective oryzanol peaks. The fifth peak has been identified as sitostanyl ferulate, according to the reported literature [21].



Peak 1. cycloartenyl ferulate, 2. 24-methylene cycloartanyl ferulate, 3. campesteryl ferulate, 4. campestanyl ferulate & β-sitosteryl ferulate and 5. sitostanyl ferulate.

Figure 3.5. Steryl ferulate profiles in γ-oryzanol standard and wheat sample.

In parallel, HPLC-DAD-MS analysis was also conducted to identify the presence of sitostanyl ferulate in the fifth peak. This identification is consistent with the reported literature [110]. Both DAD and MS spectra (positive ion mode) of the fifth peak are given in Figure 3.6.

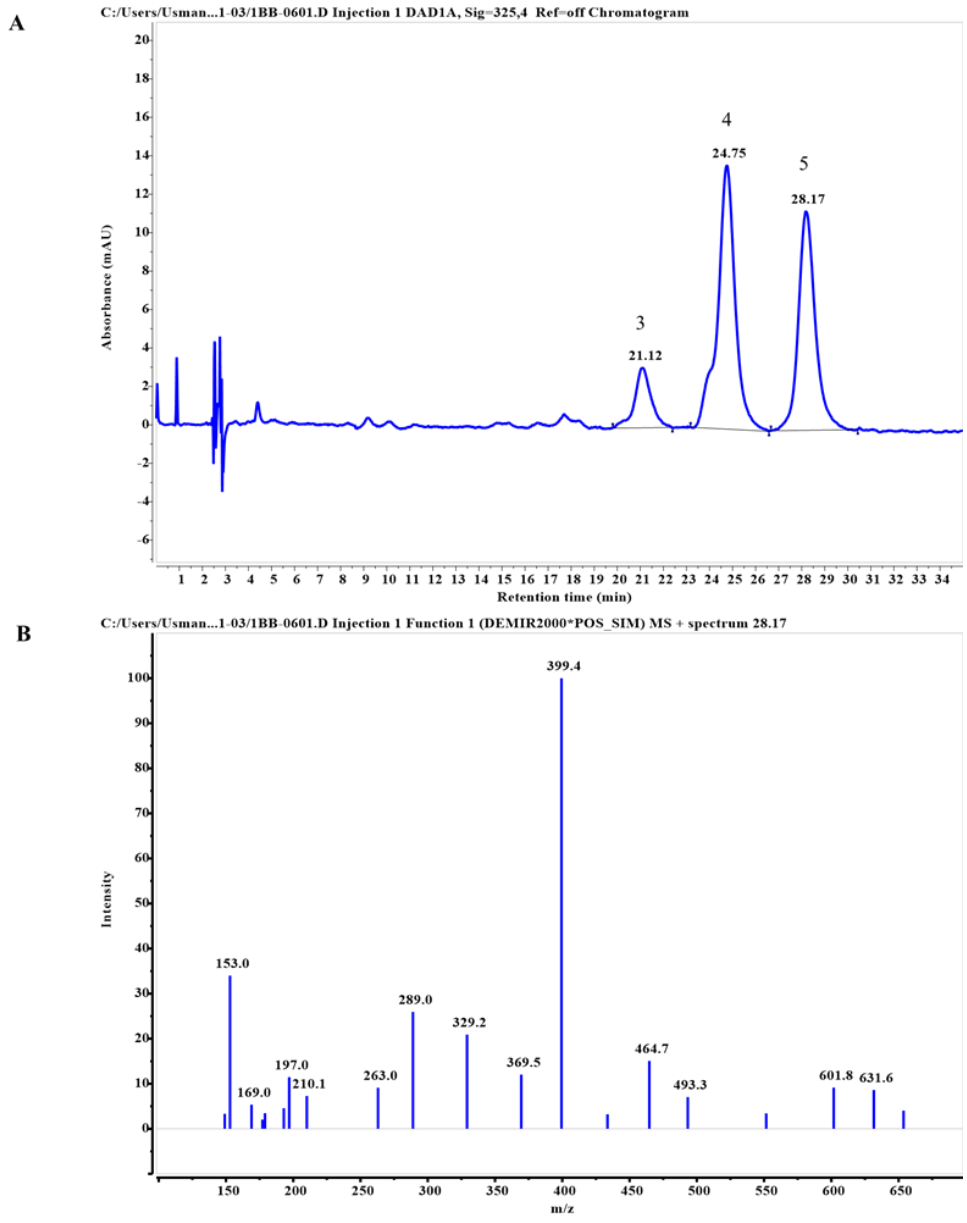


Figure 3.6. Steryl ferulate profiling of wheat after reversed-phase separation (A) DAD spectrum and (B) ES-API MS spectrum (positive ions mode) of 5th peak (identified as sitostenyl ferulate).

Steryl ferulate composition was analyzed using an Agilent 1200 HPLC-DAD linked to an Agilent 6130 (Quadrupole LC/MS) MSD mass spectrometer with an API-ES interface (Agilent Technologies, Palo Alto, CA, USA). The mass spectrometer operating conditions were quadrupole temperature (100 °C), nitrogen flow rate (11 L/min), gas temperature (325 °C), and nebulizer pressure (40 psi). The mass spectrometer was operated in positive mode at capillary voltage of 4000 V.

3.7. Isolation and Purification of Components in Sünter Wheat Extracts

The hexane and methanol extracts of Sünter wheat variety were prepared (see Section 3.5.1) and further purification and identification analyses on these extracts were performed according to flow chart (Figure 3.7).

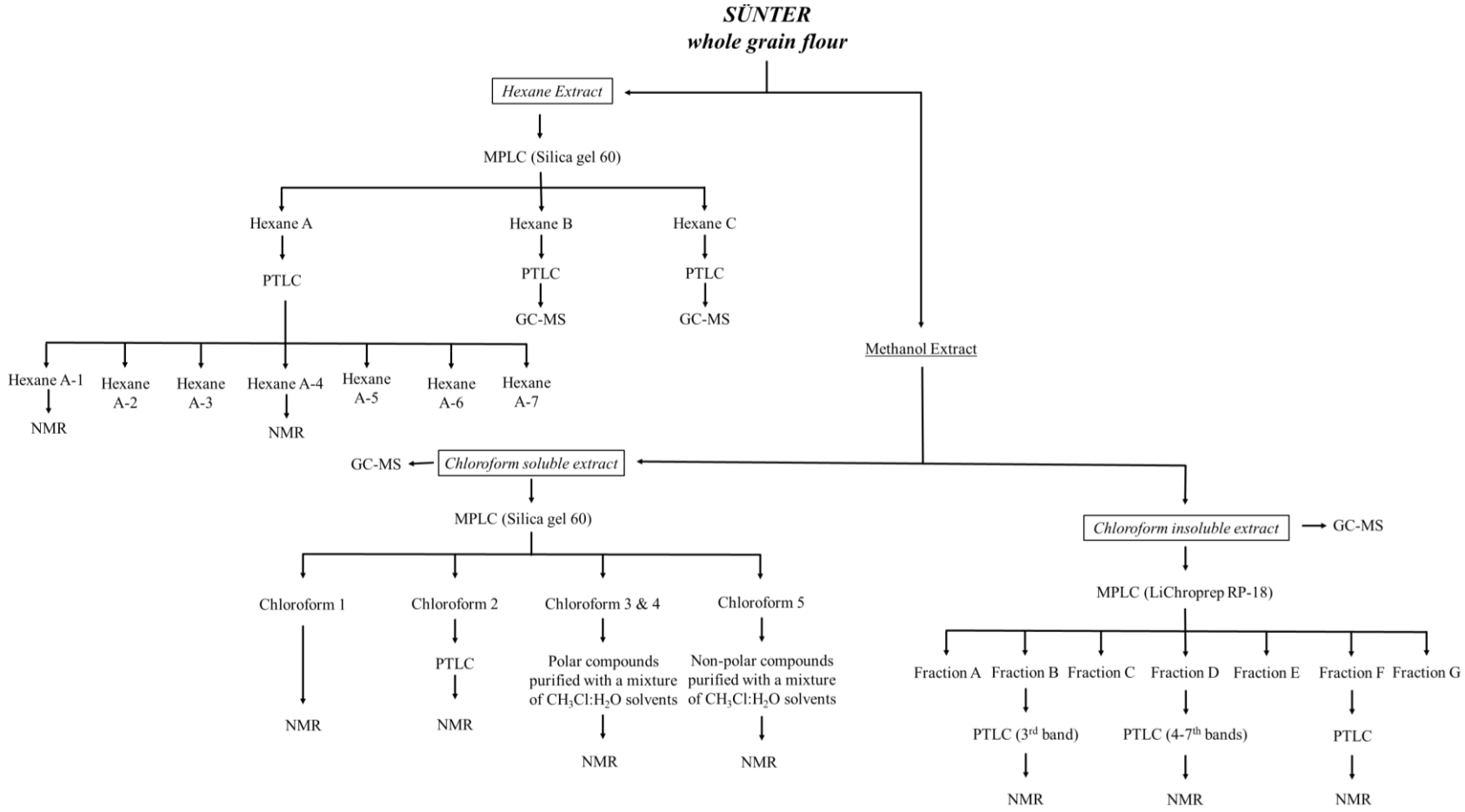


Figure 3.7. Flow chart of purification and identification of Sünter wheat extracts.

3.7.1. Purification of hexane extract

Following the addition of approximately 200 µL of hexane to 20 mg of crude hexane extract, the resulting solution was subsequently applied onto a TLC plate. Various ratios of hexane and acetone (10:1, 4:1, and 2:1, v/v) were employed to separate the polar and non-polar components present in the extract. The aforementioned procedure facilitated the determination of the optimal solvent ratios for achieving effective separation in column chromatography. The TLC plates were subjected to examination using wavelengths of 366 nm and 254 nm. For normal eye visualization, a 10% sulfuric acid in methanol solution was then sprayed onto the plates and heated at 120 ºC for 5 min [150].

The crude hexane extract (1.59 g) was mixed with 1 mL of hexane-acetone (10:1, v/v) to remove insoluble precipitates. The obtained supernatant containing 1.45 g hexane extract was transferred to an MPLC column (ACE, 21 × 300 mm, 100 mL) filled with normal phase silica (silica gel 60, 15-40 µm particle size). Hexane-acetone gradient profiles such as 10:1, 4:1, 2:1, 1:1 v/v and pure acetone were used at the rate of 3 mL/min, and total 20 fractions of 20 mL each were collected from the MPLC column. The TLC analysis was again performed to determine the retention factors (Rf) of the molecules in each fraction. Rf values were obtained by dividing the distance traveled by the molecule divided by the distance traveled by the solvent front on the plate. By combining fractions with similar Rf values, three major fractions were obtained. The first 7 fractions were combined and labeled as A, the second 4 fractions were combined as B, and the third 9 fractions were combined as C major-fractions.

Fractions A, B and C were further purified by PTLC. Fraction A was separated into 7 sub-fractions, which were scraped from PTLC plate and diluted with hexane-acetone (10:1) solution and the mixture was filtered through Gooch filter (70 micron). Subsequently, 1st and 4th sub-fractions were characterized with the help of ^{1}H NMR and ^{13}C NMR for identification [150]. Other subfractions were not pure enough for further characterization.

Similarly, PTLC was applied to fractions B and C, but highly complex sub-fractions (10 for B and 17 for C) were visualized. Therefore, these two fractions were combined and identified with GC-MS after derivatization with MSTFA (see Section 3.9.1). These fractions were not employed for NMR characterization due to their low purity.

3.7.2. Purification of methanol extract

Methanol extracts of Sünter were prepared and re-extracted with chloroform as described in Section 3.5 (Figure 3.3).

3.7.2.1. Purification of chloroform soluble extract

First TLC was performed to determine solvent mixture (chloroform-methanol, 15:1, v/v) for optimum separation of both polar and non-polar components. Then MPLC column (ACE, 21 × 300 mm, 100 mL) filled with silica gel 60 (15-40 µm particle size) was used for isolation of chloroform soluble extract. Chloroform-methanol gradient profiles such as 30:1, 20:1, 10:1, 5:1, 1:1 v/v and pure methanol were used at the rate of 1 mL/min, and total 24 fractions of 15 mL volume each were collected according to increasing polarity. These fractions were confirmed by applying on TLC plate by using solvent mix of chloroform and methanol (15:1, v/v). Fractions with the same Rf distances on TLC plate were assumed to have similar molecule/s. As a result, MPLC fractions having same Rf on TLC were combined and five separate groups were obtained: chloroform 1 (fractions 1-8), chloroform 2 (fractions 9-12), chloroform 3 (fractions 13-16), chloroform 4 (fractions 17-19), and chloroform 5 (fractions 20-24).

- Chloroform 1 (456.6 mg) was directly characterized by NMR.
- Chloroform 2 was first purified on a PTLC plate using a chloroform-methanol solvent system (15:1, v/v) and a pure UV-active band was scraped for NMR analysis.
- Chloroform 3 and 4 with similar TLC profiles, were dissolved in chloroform and polar metabolites (96.1 mg) were extracted with water prior to NMR characterization.
- Similarly, chloroform 5 was further extracted with a chloroform and water solvent mixture (1:1, v/v), to afford chloroform-soluble (13.1 mg) and water-soluble (217.4 mg) extracts. Subsequently chloroform-soluble non extract was selected for NMR characterization (Figure 3.8).

3.7.2.2. Purification of chloroform insoluble extract

MPLC column (ACE, 21 × 300 mm, 100 mL) filled with LiChroprep RP-18 (15-40 µm particle size) was used for the isolation of chloroform insoluble extract.

Water-methanol gradient profiles such as 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90 v/v and pure methanol were used at the rate of 2 mL/min, and total 24 fractions of 15 mL volume each were collected. The fractions with similar Rf distances on TLC plate (chloroform-methanol-water, 3:6:1, v/v/v) were combined into 7 major fractions: A (fractions 1 & 2), B (fractions 3-7), C (fractions 8-9), D (fractions 10 &11), E (fraction 12), F (fractions 13 & 14), and G (fractions 15-24).

- Fraction B, which exhibited a complex profile in TLC plate, was subjected to PTLC (chloroform-methanol-water, 3:6:1, v/v/v) in order to isolate the UV-active band, which was subsequently scraped off and subjected to characterization by NMR analysis.
- Similarly, fraction D was also applied to PTLC plate, and the purified UV-active band was characterized by NMR analysis.
- Finally, fraction F was directly analyzed using nuclear magnetic resonance (NMR).

Fractions A, E and G were not discarded due to absence of any active molecule. On the other hand, Fraction C was thought to have a similar chemical profile to that of Fraction B, therefore not be characterized by NMR.

3.8. Identification and Characterization of Bioactive Compounds

3.8.1. Gas chromatography – Mass spectrometry (GC-MS)

Sünter whole wheat flour was first extracted with methanol-water (80:20, v/v) solution and supernatant was dried in rotary evaporator at 35 ºC. For derivatization, the dried extracts were mixed with 250 µL of methoxyamine hydrochloride (prepared in 20 mg/mL pyridine) and the mixture was placed in a shaking incubator set to 37 °C and 400 rpm for 90 minutes. Subsequently, 250 µL of the derivatizing agent N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was added to the mixture, and another 90 minutes of incubation at 37 °C and 400 rpm was performed [151]. Similarly, the derivatization of other extracts (such as hexane B, hexane C, chloroform soluble and chloroform insoluble extracts of Sünter wheat) was also carried out and all derivatized samples were stored at −20 °C before GC-MS analysis.

GC-MS (Agilent 7890B GC/ 5977A Series MSD Systems, Agilent Technology Inc., Santa Clara, CA, USA) system with Agilent 19091S-413 HP-5ms (30 m × 0.32 mm I.D. × 0.25 µm, Agilent J&W Scientific, USA) column was used. The oven temperature was

programmed to increase at the rate of 4 °C/min from 80 °C (1 min) to 280 °C (9 min). The carrier gas was helium, the velocity was set to 1 mL/min and the detector temperature was kept at 280 °C.

The peak identification of all metabolites was conducted through a comparative analysis of NIST MS search version 2.4 and Wiley W10N14 spectral libraries, ensuring an accuracy threshold of 80%. Additionally, these metabolites were also compared with the composition of wheat in previously reported literature [152].

3.8.2. Nuclear magnetic resonance (NMR)

The characterization of the pure components was conducted by employing two separate NMR spectroscopy techniques: proton (^{1}H NMR, 400 MHz) and carbon (^{13}C NMR, 100 MHz) NMR, as described by Liu, Singh and Nair [153]. The chemical shift range observed in proton NMR (^{1}H NMR) spectrum is generally narrow and exhibits a range of 1-10 ppm, whereas carbon NMR (^{13}C NMR) displays a significantly broader chemical shift range, between 0 to 200 ppm. The chemical environment surrounding the hydrogen atoms within the molecule affects both the number and position of peak signals observed in the proton NMR spectrum. Similarly, the carbon NMR spectrum is obtained but peak signals are affected by the chemical environment surrounding the carbon atoms. However, obtaining strong and readable peak signals in the carbon NMR spectrum typically requires a longer scanning time.

The same protons of a substance give peaks at different frequency values (Hz) depending on the strength of magnetic field in NMR device. NMR spectra were obtained in the form of free induction decay (FID) signals. These values are converted to delta (δ) units by integration and displayed in terms of constant chemical shift, generally expressed in ppm (parts per million) by multiplying the actual values by 10^{6}. The position of each peak signal in the NMR spectrum is typically determined by comparing the signal of reference standard TMS (tetramethylsilane). The signal of TMS was utilized to establish the starting point (0 ppm) for locating the proton or carbon signals in both NMR spectra. Following this, the signal for the deuterated chloroform ($CDCl_3$) solvent was observed at 7.28 ppm in the ^{1}H NMR spectrum and at 77.2 ppm in the ^{13}C NMR spectrum. On the other hand, signal for the deuterated water (D_2O) solvent was detected at 4.8 ppm in the ^{1}H NMR spectrum. The area below the ^{1}H NMR peak is proportional to the number of hydrogen atoms in the molecule.

3.9. Statistics

The obtained data was evaluated using the SPSS Statistics Base 26.0 package program with analysis of variance (ANOVA-1) and Duncan's Post-hoc test was applied to investigate the statistically significant difference between means [154]. The relationship among various bioactive compounds was determined with the help of Heatmap after data normalization using GraphPad Prism version 9.0 (GraphPad Software, MA, USA).

4. RESULTS AND DISCUSSION

Turkish wheat varieties (14 local and 4 commercial) from monococcum, dicoccum, durum, and aestivum genotypes were investigated for their bioactive composition to select best local cultivars for breeding programs. Due to its rich phenolic contents, Sünter local variety has been chosen for further extraction, purification, and identification of metabolites.

4.1. Physiochemical Analyses

4.1.1. Thousand kernel weight and ash contents

The TKW has an important role in evaluation of wheat grain yield. In this study, TKW values were found in the range of 26.20 and 54.02 g for all wheat varieties (Table 4.1). Eminbey, Demir 2000 and Gacer wheat varieties have significantly high TKW values in durum, aestivum and dicoccum genotypes, respectively. However, no significant difference was observed among monococcum wheat varieties in terms of THW values ($p>0.05$). In all wheat genotypes, TKW values decreased in the order of durum > aestivum > dicoccum > monococcum. Similarly, durum genotype exhibited high TKW values due to the presence of relatively large sized wheat grains, as reported by Wang and Fu [155].

Ash contents of wheat varieties were determined in between 1.19 to 1.80 % as presented in Table 4.1. In all wheat genotypes, average ash contents reduced in the order of durum ≥ monococcum > dicoccum > aestivum. Wheat varieties such as Mirzabey 2000, Bayraktar 2000, Atasiyez and Gacer have significantly high ash content values in durum, aestivum, monococcum and dicoccum genotypes, respectively. Similar to our study, the ash content of five different Canadian wheat varieties has been reported in between 1.18% and 1.75% [156]. Whereas European wheat varieties cultivated in the years 1992-1994, have been reported with high ash contents of 1.76 – 1.86% [157].

The ash content of the food serves as a quantitative indicator of the overall mineral composition. As a significant indicator of chemical quality, it has a direct effect on technological properties, extraction yield, mineral content, and nutritional labeling of wheat flour [158].

The relationship between TKW values and ash content % was found to be weak. It is assumed that monococcum, dicoccum and aestivum wheat grains have smaller kernel sizes (higher bran-to-endosperm ratio), which may lead to higher ash contents [159].

Table 4.1. Thousand kernel weight (g) and ash content (%) of wheat genotypes.

Wheat genotypes	Thousand kernel weight	Ash contents
Monococcum		
Mergüze	26.78 ± 1.35 [a]	1.41 ± 0.00 [c]
Atasiyez	26.69 ± 0.20 [a]	1.50 ± 0.01 [a]
Siyez-4	25.93 ± 1.01 [a]	1.45 ± 0.00 [b]
Siyez Pop	26.20 ± 0.89 [a]	1.37 ± 0.01 [d]
average	**26.40 ± 0.93**	**1.43 ± 0.05**
Dicoccum		
Kafkas	31.54 ± 0.59 [c]	1.25 ± 0.01 [c]
Kavılca kırmızı	32.94 ± 0.67 [b]	1.33 ± 0.01 [b]
Gacer	36.11 ± 0.56 [a]	1.62 ± 0.03 [a]
average	**33.53 ± 2.07**	**1.40 ± 0.18**
Durum		
Mirzabey 2000	50.29 ± 1.99 [b]	1.80 ± 0.01 [a]
Eminbey	54.02 ± 0.90 [a]	1.47 ± 0.01 [b]
Karakılçık	43.14 ± 1.28 [c]	1.35 ± 0.01 [c]
Sarı buğday	50.51 ± 0.33 [b]	1.19 ± 0.01 [d]
average	**49.49 ± 4.24**	**1.45 ± 0.24**
Aestivum		
Bayraktar 2000	43.89 ± 2.83[b]	1.80 ± 0.02 [a]
Demir 2000	48.38 ± 1.60 [a]	1.34 ± 0.03 [cd]
AK-702	35.21 ± 0.69 [e]	1.46 ± 0.04 [b]
Köse 220/33	40.52 ± 0.34 [c]	1.35 ± 0.02 [c]
Sünter	29.48 ± 0.19 [f]	1.25 ± 0.01 [de]
Zerun	38.00 ± 0.57 [d]	1.22 ± 0.01 [e]
Spelt S. başak	39.68 ± 0.31 [cd]	1.30 ± 0.08 [cde]
average	**39.31 ± 5.82**	**1.39 ± 0.19**

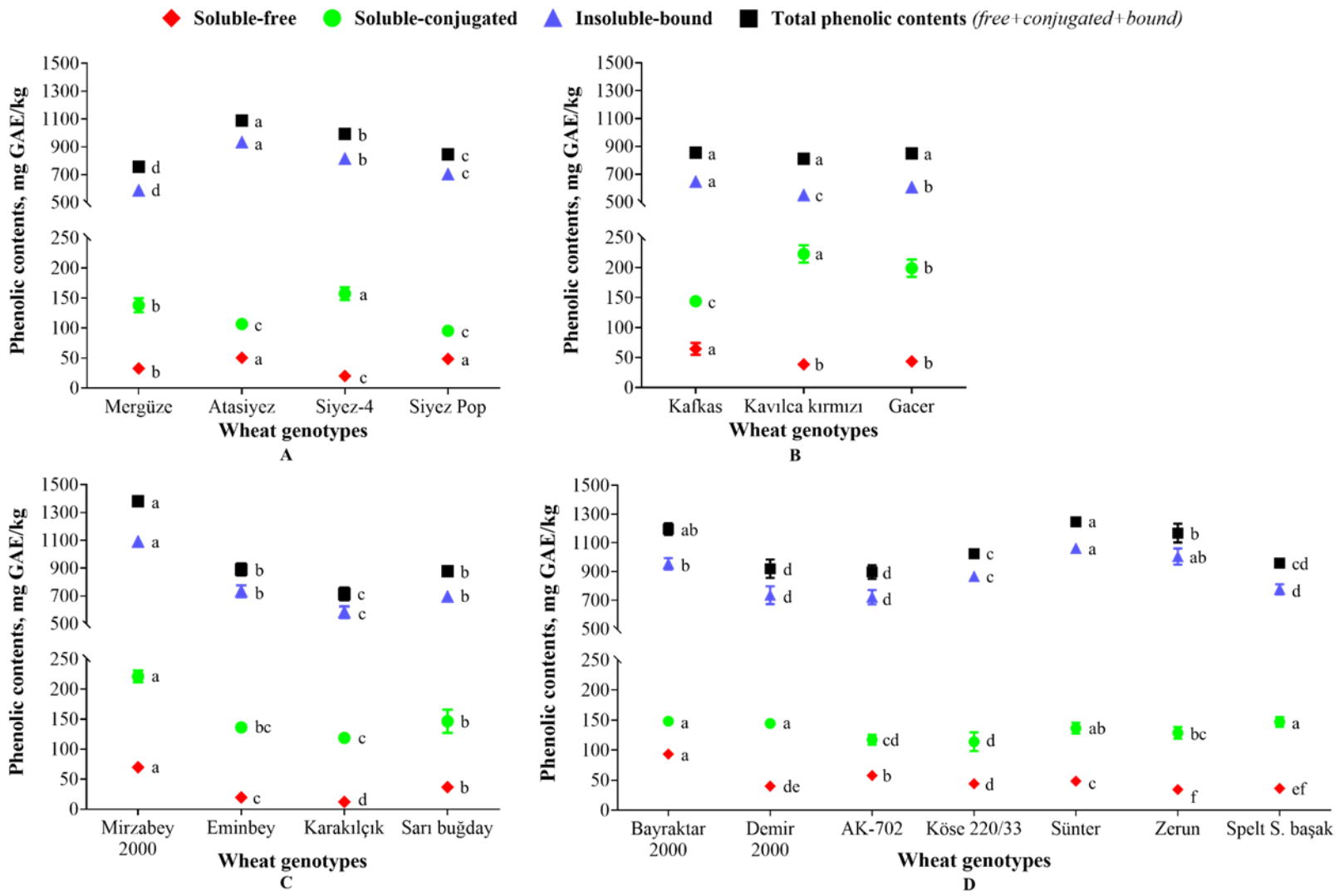
Different lowercase letters in the same results indicate significant values ($p < 0.05$), (Mean ± SD, n = 2).

4.1.2. Total phenolic contents

The results of soluble-free, soluble-conjugated, insoluble-bound, and total phenolic contents (TPC) of monococcum, dicoccum, durum, and aestivum wheat genotypes are presented in Figure 4.1. In monococcum genotype (Figure 4.1A), Atasiyez variety (recently commercialized), has substantial amounts of soluble-free, insoluble-bound and TPC (50.43, 932.37, 1,089.38 mg GAE /kg, respectively) except for the soluble-conjugate phenolics. On the other hand, the maximum soluble-conjugated phenolics for Siyez-4 was around 157.28 mg GAE /kg. Additionally, Mergüze wheat has the lowest TPC (757.49 mg GAE /kg), primarily consisting of soluble-conjugated phenolic contents. Although, TPC values of all varieties in dicoccum genotype were found similar (Figure 4.1B). Kafkas wheat (recently commercialized) has the highest contents of soluble-free and

insoluble-bound phenolics (64.54 and 646.24 mg GAE /kg, respectively). The soluble-conjugated phenolic contents were statistically high in Kavılca kırmızı (222.65 mg/kg) wheat as compared to other wheat varieties of the dicoccum genotype ($p<0.05$).

Mirzabey 2000 has been observed with the highest soluble-free (69.74 mg/kg) soluble-conjugated (221.25 mg/kg), insoluble-bound (1090.39 mg/kg), and total phenolic contents (1381.38 mg/kg) among durum genotype and all wheat varieties. (Figure 4.1C). Similarly, high soluble-free phenolic contents were found in Bayraktar 2000 (93.42 mg/kg) wheat in aestivum wheat genotypes (Figure 4.1D). Bayraktar 2000, Demir 2000, Spelt S. başak and Zerun have high contents of soluble-conjugated phenolic compounds, as compared to AK-702, and Köse 220/33 wheat varieties. Also, significant amounts of insoluble-bound phenolics were obtained in Sünter (1061.28 mg/kg) and Zerun (1004.28 mg/kg) varieties. Nevertheless, local Sünter wheat variety has the highest total phenolic contents (1246.30 mg GAE/kg DM) among aestivum genotype and all local wheat varieties.



Values with different lowercase letters in the same results are statistically different (p <0.05), (Mean ± SD, n = 4).

Figure 4.1. Phenolic contents in (a) monococcum, (b) dicoccum, (c) durum, and (d) aestivum wheat genotypes.

Overall, TPC varied from 757.49 to 1,089.38 mg for monococcum, 811.39 to 854.77 mg for dicoccum, 713.92 to 1381.38 mg for the durum, and 897.05 to 1,246.30 mg GAE/kg DM for the aestivum wheat genotypes. Similarly, comparable results of the total phenolic contents were reported in various European wheat landraces [160]. In addition, the bound phenolic contents in the durum and aestivum genotypes are agreeable with the previously reported literature [107, 138, 161]. However, Serpen, Gökmen, Karagöz and Köksel [137] have reported higher total phenolic contents in the emmer (*T. dicoccum* ssp. dicoccum) compared to einkorn (*T. monococcum* ssp. monococcum) wheat, which were in the range of 5.38 to 8.58 and 2.55 to 4.73 µmol GAE/g of whole wheat, respectively. This contradiction is due to the possible variations in the wheat varieties.

4.1.3. Phenolic acid composition

Seven phenolic acids such as ferulic, sinapic, ρ-coumaric, vanillic, syringic, caffeic, and 4-hydroxybenzoic acids in all wheat genotypes were determined by comparison to the mixture of 11 different analytical standards, as shown in Figure 3.2 (a, b). The phenolic acids composition of all wheat samples was found to be similar except caffeic acid, which is primarily found in trace levels in the monococcum group (Table 4.2). It has been explained by literature that caffeic acid is very low in whole wheat grains and has no significant effect on the sum of individual phenolic acids composition [97]. Ferulic and sinapic acids were observed as major phenolic acids, while other phenolic acids such as ρ-coumaric, vanillic, syringic, 4-hydroxybenzoic, and caffeic acids, were determined in trace amounts.

The amount of total phenolic acids varied from 672.51 – 720.77 mg for monococcum, 588.18 – 677.92 mg for dicoccum, 681.83 – 815.08 mg for durum, and 624.10 – 878.97 mg/kg DM for aestivum wheat genotypes. In monococcum genotypes, Mergüze and Siyez-4 wheat varieties have been observed with high ρ-coumaric and vanillic acids. Also, Mergüze was the only wheat in the monococcum family where caffeic acid was not detected. The levels of ferulic, sinapic, and 4-hydroxybenzoic acids are high in Atasiyez followed by the Siyez Pop wheat variety. Kafkas and Kavılca kırmızı varieties of the dicoccum genotype exhibit significant levels of ferulic, ρ-coumaric, and syringic acids, whereas Kavılca kırmızı wheat was also rich in sinapic acid. Similarly, Gacer wheat has been found to contain high amounts of 4-hydroxybenzoic and vanillic acids.

Table 4.2. Phenolic acid profile of wheat genotypes (mg/kg DM of whole grain flour).

Wheat genotypes	4-hydroxybenzoic acid	Vanillic acid	Caffeic acid	Syringic acid	ρ-coumaric acid	Ferulic acid	Sinapic acid	Total phenolic acids
Monococcum								
Mergüze	4.96 ± 0.19 ^{b}	12.62 ± 0.83 ^{a}	n.d.	6.91 ± 0.58 ^{a}	52.87 ± 3.51 ^{a}	412.22 ± 13.43 ^{b}	182.93 ± 8.13 ^{b}	672.51 ± 26.36 ^{b}
Atasiyez	5.38 ± 0.07 ^{a}	11.62 ± 0.16 ^{b}	15.32 ± 0.43 ^{b}	6.35 ± 0.16 ^{b}	45.00 ± 2.36 ^{b}	440.45 ± 5.70 ^{a}	196.66 ± 5.46 ^{a}	720.77 ± 9.92 ^{a}
Siyez-4	4.37 ± 0.16 ^{c}	12.25 ± 0.08 ^{ab}	17.56 ± 1.19 ^{a}	7.36 ± 0.16 ^{a}	51.15 ± 2.71 ^{a}	421.92 ± 9.19 ^{b}	173.96 ± 2.35 ^{b}	688.56 ± 10.60 ^{b}
Siyez Pop	5.10 ± 0.23 ^{b}	11.76 ± 0.32 ^{b}	15.50 ± 0.32 ^{b}	6.39 ± 0.20 ^{b}	40.45 ± 4.26 ^{b}	424.56 ± 10.67 ^{b}	177.99 ± 10.15	681.73 ± 25.92 ^{b}
average	**4.95 ± 0.41**	**12.06 ± 0.58**		**6.75 ± 0.52**	**47.37 ± 5.90**	**424.79 ± 13.85**	**182.88 ± 10.92**	**690.89 ± 25.84**
Dicoccum								
Kafkas	5.29 ± 0.18 ^{b}	15.03 ± 0.54 ^{b}	14.07 ± 0.15 ^{a}	7.93 ± 0.26 ^{b}	33.60 ± 0.28 ^{a}	421.39 ± 4.16 ^{a}	180.60 ± 3.96 ^{b}	677.92 ± 4.75 ^{a}
Kavılca kırmızı	4.37 ± 0.10 ^{c}	14.15 ± 0.25 ^{c}	n.d.	8.77 ± 0.50 ^{a}	33.07 ± 1.38 ^{a}	409.98 ± 3.99 ^{a}	195.63 ± 2.83 ^{a}	665.98 ± 7.53 ^{a}
Gacer	7.04 ± 0.44 ^{a}	17.06 ± 0.71 ^{a}	n.d.	6.65 ± 0.22 ^{c}	23.85 ± 1.46 ^{b}	351.63 ± 25.86 ^{b}	181.96 ± 11.66	588.18 ± 40.20 ^{b}
average	**5.57 ± 1.18**	**15.41 ± 1.36**		**7.79 ± 0.96**	**30.17 ± 4.80**	**394.33 ± 34.78**	**186.06 ± 9.69**	**644.03 ± 46.79**
Durum								
Mirzabey 2000	12.44 ± 0.73 ^{a}	16.06 ± 0.83 ^{a}	n.d.	5.81 ± 0.27 ^{d}	28.99 ± 1.48 ^{a}	519.72 ± 25.86 ^{a}	194.79 ± 8.45 ^{c}	777.81 ± 36.36 ^{a}
Eminbey	8.99 ± 0.74 ^{a}	13.03 ± 0.42 ^{b}	n.d.	7.46 ± 0.27 ^{b}	23.28 ± 1.33 ^{a}	553.53 ± 37.63 ^{a}	208.80 ± 7.44 ^{b}	815.08 ± 46.60 ^{a}
Karakılçık	6.26 ± 0.17 ^{c}	15.68 ± 0.37 ^{a}	n.d.	8.40 ± 0.17 ^{a}	23.49 ± 0.18 ^{a}	461.35 ± 7.73 ^{b}	166.65 ± 4.46 ^{c}	681.83 ± 11.73 ^{b}
Sarı buğday	8.13 ± 0.21 ^{b}	15.51 ± 0.33 ^{a}	n.d.	6.66 ± 0.09 ^{c}	22.57 ± 0.79 ^{a}	536.39 ± 14.17 ^{a}	224.93 ± 6.85 ^{a}	814.20 ± 21.56 ^{a}
average	**8.96 ± 2.36**	**15.07 ± 1.32**		**7.08 ± 1.01**	**24.58 ± 2.82**	**517.75 ± 41.86**	**198.79 ± 22.97**	**772.23 ± 62.98**
Aestivum								
Bayraktar 2000	7.50 ± 0.07 ^{c}	16.59 ± 0.18 ^{cd}	n.d.	10.68 ± 0.25 ^{d}	15.04 ± 0.35 ^{f}	406.66 ± 3.78 ^{d}	167.64 ± 15.53	624.10 ± 19.91 ^{d}
Demir 2000	6.59 ± 0.09 ^{d}	15.53 ± 0.50 ^{d}	14.45 ± 0.37 ^{a}	18.19 ± 0.32 ^{a}	20.59 ± 0.46 ^{de}	510.95 ± 7.75 ^{b}	237.86 ± 1.62 ^{b}	824.17 ± 7.28 ^{b}
AK-702	7.48 ± 0.28 ^{c}	16.61 ± 0.65 ^{cd}	n.d.	8.64 ± 0.46 ^{e}	23.28 ± 0.52 ^{c}	449.86 ± 14.67 ^{c}	211.46 ± 13.93	717.33 ± 30.31 ^{c}
Köse 220/33	7.81 ± 0.33 ^{b}	18.17 ± 1.49 ^{b}	n.d.	12.56 ± 1.01 ^{bc}	21.63 ± 1.40 ^{d}	514.10 ± 33.68 ^{b}	238.60 ± 22.70	812.88 ± 60.40 ^{b}
Sünter	6.28 ± 0.03 ^{d}	17.13 ± 0.57 ^{bc}	n.d.	12.02 ± 1.22 ^{c}	25.77 ± 0.60 ^{b}	550.65 ± 13.91 ^{a}	267.12 ± 9.16 ^{a}	878.97 ± 24.79 ^{a}
Zerun	8.33 ± 0.17 ^{a}	25.36 ± 0.57 ^{a}	n.d.	13.30 ± 0.20 ^{b}	20.01 ± 1.61 ^{e}	430.69 ± 6.50 ^{c}	237.64 ± 5.67 ^{b}	735.33 ± 1.19 ^{c}
Spelt S. başak	6.51 ± 0.04 ^{d}	13.63 ± 0.59 ^{e}	n.d.	9.96 ± 0.26 ^{d}	27.25 ± 0.24 ^{a}	496.58 ± 9.25 ^{b}	176.70 ± 14.86	730.64 ± 9.95 ^{c}
average	**7.21 ± 0.74**	**17.57 ± 3.56**		**12.19 ± 2.97**	**21.94 ± 3.89**	**479.93 ± 50.79**	**219.58 ± 36.22**	**760.49 ± 83.55**

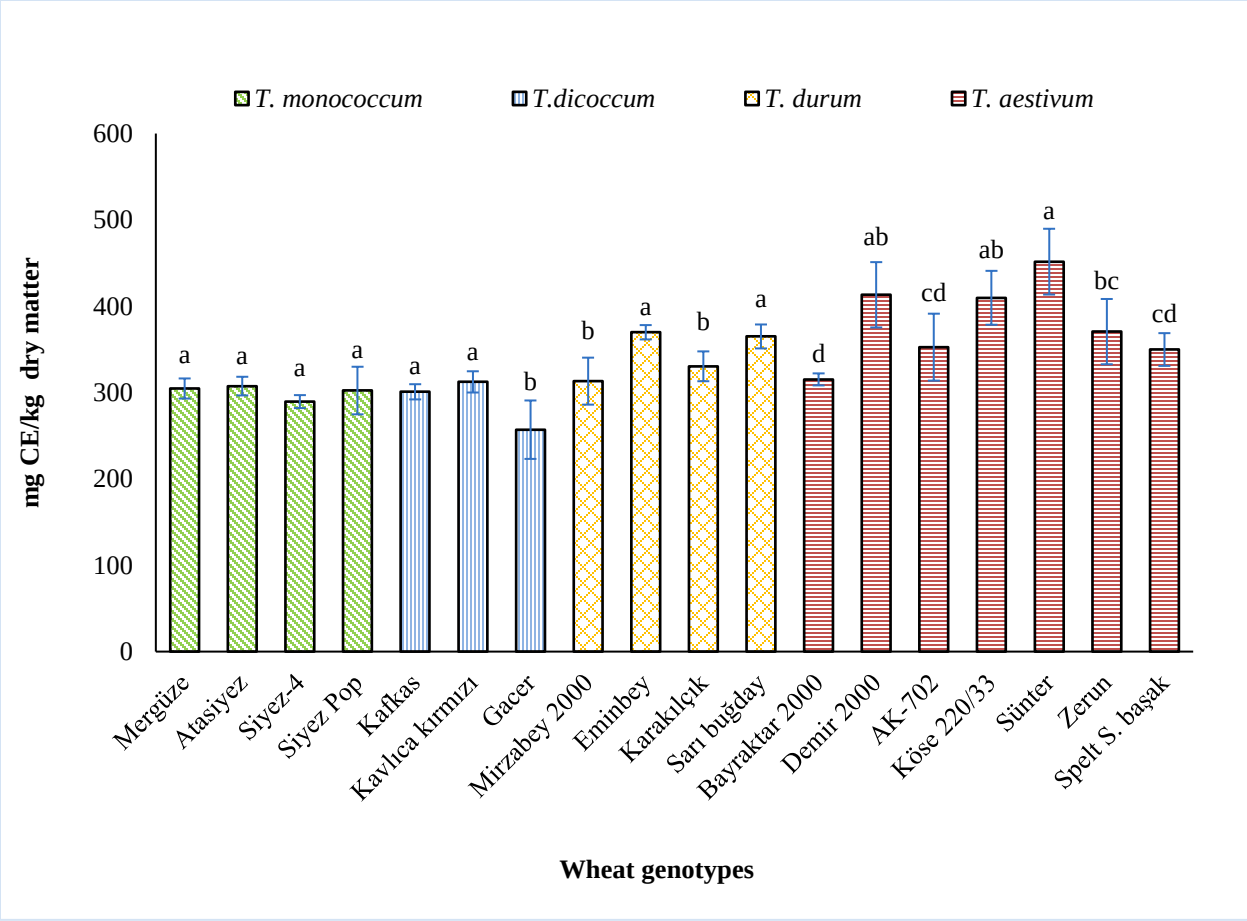
Values with different lowercase letters in the same results and individual group are statistically different ($p < 0.05$); Mean ± SD, n = 4); n.d..: not detected.

The maximum levels of ferulic acid have been identified in Eminbey and Mirzabey 2000 of durum genotypes. On the other hand, Sarı buğday and Karakılçık wheat has a high content of sinapic and syringic acids, respectively. In aestivum genotypes, significant amounts of ferulic and sinapic acids have been identified in Sünter, whereas caffeic acid was only detected in Demir 2000 wheat. Similarly, vanillic, and 4-hydroxybenzoic acids are prominent in Zerun, but ρ-coumaric acid is quite abundant in Spelt S. başak wheat. These phenolic acid compositions and quantities in Turkish wheat were found to be comparable to those reported in the literature for durum and aestivum wheat genotypes [138, 162, 163].

4.1.4. Total flavonoid contents

The results of the total flavonoid contents (TFC) in monococcum, dicoccum, durum, and aestivum wheat genotypes are presented in Figure 4.2. The variation in TFC values ranged from 289.54 to 307.52 mg for monococcum, 257.02 to 312.38 mg for dicoccum, 313.25 to 369.86 mg for durum, and 315.27 to 451.63 mg CE/kg DM for aestivum wheat genotypes. TFC contents decreased in order; from aestivum (mean, 380.43 mg CE/kg DM) and durum (mean, 344.64 mg CE/kg DM) to monococcum (mean, 301.03 mg CE/kg DM) and dicoccum (mean, 290.10 mg CE/kg DM) genotypes.

In the monococcum, the flavonoid contents did not exhibit significant changes ($p>0.05$). On the other hand, the difference in TFC was statistically significant in the dicoccum, durum, and aestivum genotypes ($p<0.05$). The wheat varieties such as Kavılca kırmızı (312.38 mg CE/kg DM), and Eminbey (369.86 mg CE/kg DM) exhibit maximum TFC values in the dicoccum and durum genotypes, respectively. Among the aestivum genotypes, Sünter (451.63 mg CE/kg DM) wheat exhibited a higher TFC value than the commercially available Demir 2000 (413.26 mg CE/kg DM) variety. In comparison to our results, Dinelli, Carretero, Di Silvestro, Marotti, Fu, Benedettelli, Ghiselli and Gutiérrez [107] observed reduced flavonoid contents in modern and old varieties of durum wheat, a discrepancy that could potentially be attributed to the elevated temperatures experienced in their study.



Values with different lowercase letters in the same results and individual group are statistically different (p <0.05), (Mean ± SD, n = 4).

Figure 4.2. Total flavonoid contents of wheat genotypes.

4.2. Total Antioxidant Capacity

The ABTS results showed that the Trolox equivalent antioxidant capacity values were determined to be between 20.24 – 21.01 mmol for monococcum, 20.37 – 20.61 mmol for dicoccum, 17.10 – 30.11 mmol for durum, and 18.23 – 22.00 mmol TEAC/kg DM for aestivum wheat genotypes (Table 4.3). On the other hand, Trolox equivalent antioxidant capacity values measured by DPPH radical assay ranged from 4.34 to 4.76 mmol for monococcum, 5.02 to 5.81 mmol for dicoccum, 4.11 to 4.44 mmol for durum, and 3.37 to 4.24 mmol TEAC/kg DM for aestivum wheat genotypes (Table 4.3). When the antioxidant capacity values from two different methods were compared, no major difference in variation of total antioxidant capacity was observed among the wheat genotypes for the results of ABTS and DPPH assays, but TEAC values were significantly low for

DPPH• radical. This can be explained by the different scavenging abilities of the ABTS•+ and DPPH• radicals to various antioxidant groups. ABTS assay is useful in the evaluation of electron-donating potential while DPPH determines the hydrogen-transfer capability of antioxidants.

ABTS and DPPH assays use different scavenging radicals and, therefore, their results cannot be directly compared with each other. In our study, we found that the total antioxidant capacity values were not significantly different among the wheat varieties in each antioxidant assay. Among all wheat varieties, Mirzabey 2000 (durum) and Gacer (dicoccum) have the highest antioxidant capacity for ABTS and DPPH radicals, respectively. The observed differences in antioxidant values between the ABTS and DPPH assays can be attributed to the interaction of the radicals with both soluble and bound phenolics. The ABTS assay predominantly measures the interaction of radicals with bound phenolics, which explains why Mirzabey 2000, with high levels of bound phenolics, exhibits high TEAC values. On the other hand, DPPH assay primarily interacts with soluble phenolics, and therefore, Gacer rich in soluble phenolics, exhibits high antioxidant values. Similarly, Bayraktar 2000 and Demir 2000 varieties in aestivum genotype have been observed with the highest total antioxidant capacity in ABTS and DPPH assays, respectively.

The DPPH-based antioxidant activity values of Turkish bread and durum wheat varieties were reported to range from 3.59 to 4.64 µmol Trolox Equivalent/g flour [164], which is consistent with the DPPH results obtained in the wheat varieties investigated in this thesis. In the same study, ABTS-based results were revealed in between 10.53 and 12.23 µmol TE/g flour, which are lower than the findings of our study. YİĞİT and Erekul [165] also reported slightly higher antioxidant values (ranging from 11.89 to 26.33% of DPPH inhibition) for various Turkish bread wheat varieties. Similar to our findings, Serpen, Gökmen, Karagöz and Köksel [137] have reported the ABTS based total antioxidant capacity in between 16.92 to 20.64 mmol and 19.00 to 23.84 mmol TEAC/kg for Turkish monococcum and dicoccum genotypes, respectively.

DPPH (% inhibition) values of Indian bread wheats from different agro-climatic zones were determined in between 6.0 – 25.0% but ABTS assay results (2.0 to 10.0 µmol TE/g) were much lower than our findings [131]. On the other hand, several studies have also reported significantly higher antioxidant activities in pigmented wheat varieties compared to conventional ones [166-168].

Table 4.3. Total antioxidant capacity (mmol TEAC/kg DM) of wheat genotypes.

Wheat genotypes		ABTS	DPPH
Monococcum			
Mergüze		20.40 ± 0.35 [a]	4.34 ± 0.07 [a]
Atasiyez		20.73 ± 1.43 [a]	4.64 ± 0.18 [a]
Siyez-4		21.01 ± 1.01 [a]	4.76 ± 0.37 [a]
Siyez Pop		20.24 ± 1.54 [a]	4.43 ± 0.23 [a]
	average	**20.60 ± 1.05**	**4.54 ± 0.27**
Dicoccum			
Kafkas		20.37 ± 0.50 [a]	5.02 ± 0.36 [b]
Kavılca kırmızı		20.50 ± 0.63 [a]	5.22 ± 0.27 [b]
Gacer		20.61 ± 0.40 [a]	5.81 ± 0.19 [a]
	average	**20.49 ± 0.46**	**5.35 ± 0.43**
Durum			
Mirzabey 2000		30.11 ± 044 [a]	4.44 ± 0.26 [a]
Eminbey		17.15 ± 0.61 [b]	4.12 ± 0.32 [a]
Karakılçık		19.17 ± 0.16 [a]	4.11 ± 0.40 [a]
Sarı buğday		17.10 ± 0.53 [b]	4.26 ± 0.20 [a]
	average	**20.95 ± 5.60**	**4.16 ± 0.33**
Aestivum			
Bayraktar 2000		22.00 ± 0.22 [a]	3.74 ± 0.06 [bc]
Demir 2000		18.46 ± 0.16 [cd]	4.24 ± 0.22 [a]
AK-702		19.12 ± 0.64 [bcd]	4.20 ± 0.23 [a]
Köse 220/33		19.33 ± 0.52 [bc]	3.37 ± 0.37 [c]
Sünter		19.48 ± 0.78 [b]	4.20 ± 0.29 [a]
Zerun		18.23 ± 0.28 [d]	3.66 ± 0.29 [bc]
Spelt S. başak		20.37 ± 0.28 [a]	4.14 ± 0.23 [ab]
	average	**19.57 ± 1.27**	**3.94 ± 0.39**

Values with different lowercase letters in the same results and individual group are statistically different ($p < 0.05$), Mean ± SD, n = 4.

4.3. Bioactive profile in wheat

4.3.1. Extraction yield

The yield of hexane and methanol extraction for all wheat genotypes ranged from 1.61 to 3.41 and 3.36 to 5.46 g/100 g of whole grain flour on a DM basis, respectively (Table 4.4). The average extract quantity for both hexane and methanol decreased in the following order among wheat genotypes: monococcum > dicoccum > durum > aestivum.

Atasiyez wheat has the highest yield of extraction for both hexane and methanol extracts in all wheat genotypes. Furthermore, it was observed that the Kavlıca kırmızı and Gacer wheat varieties

(*T. dicoccum*) exhibited elevated yields of hexane and methanol extracts. Similarly, the Spelt S. başak and Bayraktar 2000 varieties (*T. aestivum*) demonstrated substantial yields for hexane and methanol extracts, respectively.

Table 4.4. Hexane and methanol extraction yields (g/100 g DM) of wheat genotypes.

Wheat genotypes		Hexane extraction yield	Methanol extraction yield
Monococcum			
Mergüze		2.49 ± 0.00 ^{b}	4.74 ± 0.05 ^{b}
Atasiyez		3.41 ± 0.29 ^{a}	5.39 ± 0.23 ^{ab}
Siyez-4		2.43 ± 0.10 ^{b}	5.46 ± 0.10 ^{a}
Siyez Pop		2.70 ± 0.07 ^{b}	5.30 ± 0.40 ^{ab}
	average	**2.76 ± 0.43**	**5.22 ± 0.35**
Dicoccum			
Kafkas		2.60 ± 0.15 ^{ab}	4.50 ± 0.14 ^{a}
Kavılca kırmızı		2.79 ± 0.02 ^{a}	4.61 ± 0.10 ^{a}
Gacer		2.37 ± 0.07 ^{b}	4.92 ± 0.45 ^{a}
	average	**2.59 ± 0.20**	**4.68 ± 0.29**
Durum			
Mirzabey 2000		2.25 ± 0.10 ^{a}	4.67 ± 0.01 ^{a}
Eminbey		2.13 ± 0.03 ^{a}	4.25 ± 0.12 ^{b}
Karakılçık		2.00 ± 0.37 ^{a}	3.97 ± 0.00 ^{c}
Sarı buğday		1.91 ± 0.07 ^{a}	3.82 ± 0.01 ^{c}
	average	**2.07 ± 0.20**	**4.18 ± 0.35**
Aestivum			
Bayraktar 2000		1.64 ± 0.01^{b}	4.37 ± 0.08 ^{a}
Demir 2000		1.66 ± 0.04 ^{b}	3.60 ± 0.00 ^{c}
AK-702		1.99 ± 0.34 ^{b}	3.52 ± 0.08 ^{cd}
Köse 220/33		1.61 ± 0.02 ^{b}	3.36 ± 0.00 ^{d}
Sünter		1.96 ± 0.24 ^{b}	3.61 ± 0.22 ^{c}
Zerun		1.69 ± 0.02 ^{b}	3.60 ± 0.02 ^{c}
Spelt S. başak		2.40 ± 0.03 ^{a}	3.92 ± 0.02 ^{b}
	average	**1.85 ± 0.30**	**3.71 ± 0.33**

Different lowercase letters in the same results indicate significant values ($p < 0.05$), (Mean ± SD, n = 2).

Giambanelli, Ferioli, Koçaoglu, Jorjadze, Alexieva, Darbinyan and D'Antuono [169] have reported the lipid contents in the range of 18 to 28.5 g/kg DM for various ancient and modern wheat varieties from Georgia, Italy, Turkey, Armenia, and Bulgaria, which are comparable to hexane extract yield (1.61 – 3.41%) in the thesis. Similarly, the fat contents of monococcum and dicoccum wheat varieties have been obtained in between 23.7 and 31.5 g/kg on a DM basis [170]and in another study lipid contents of wheat samples were determined in between 1.92 and

2.39% [113]. In contrast, the total lipid contents of 10 durum wheat cultivars in central and southern Italy were reported between 2.97% and 3.54%, with an average value of 3.22% on dry basis [10], which was higher than durum wheats studied in this thesis.

An inverse relationship was observed between thousand kernel weight values and extraction yields of both hexane and methanol extracts. The monococcum and dicoccum genotypes, which have low TKW, exhibited higher extraction yields compared to the aestivum and durum genotypes with high TKW values. This phenomenon can be justified by the fact that the bran and germ fractions of the wheat grain are usually rich in bioactive components and lipids. Therefore, small-sized wheat grains have a larger bran-to-endosperm ratio and a higher extraction yield than large-sized grains of the same weight [171].

4.3.2. Fatty acid composition

The fatty acids in all wheat varieties were observed in the following decreasing order: linoleic acid > oleic acid > palmitic acid > α-linolenic acid > stearic acid > eicosenoic acid > palmitoleic acid (Table 4.5). For all wheat samples, linoleic, oleic, and palmitic acids were observed as the major fatty acids, whereas α-linolenic, stearic, eicosenoic, and palmitoleic acids were detected in trace amounts. The GC-FID method was utilized to identify eight essential fatty acids present in the hexane extracts of all wheat samples. Figure 4.3 presents the fatty acid profile of wheat samples.

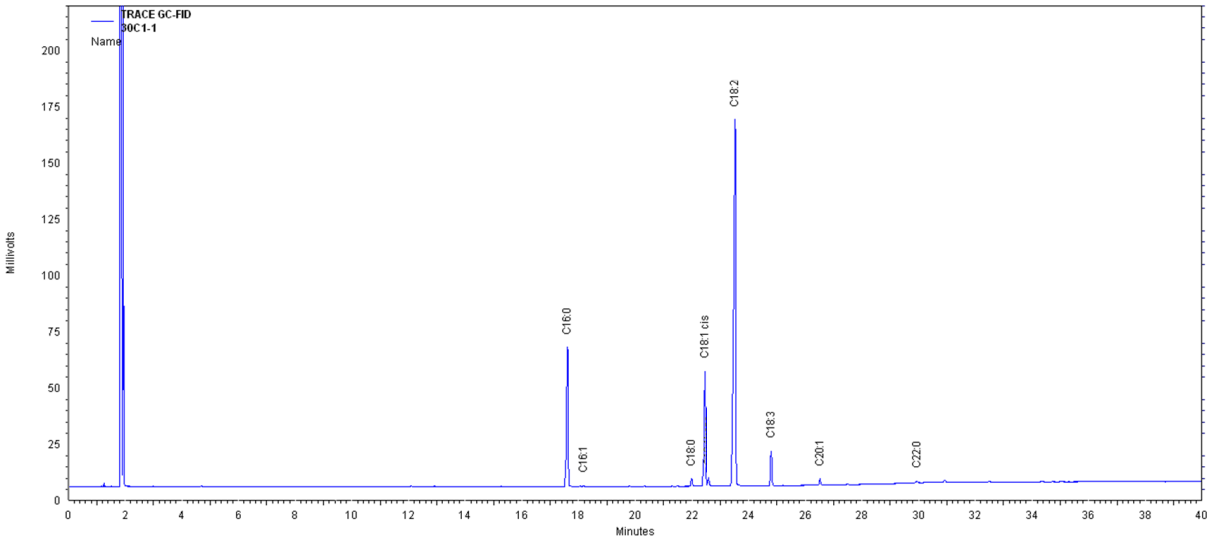


Figure 4.3. Fatty acid profile of the Sunter wheat sample.

In monococcum genotypes, Siyez-4 wheat was found to be rich in terms of linoleic, stearic, eicosenoic, and other fatty acids, whereas Atasiyez wheat has significant levels of oleic and palmitic acids. Furthermore, monococcum genotypes were found to have higher levels of eicosenoic acid than steric acid. Gacer and Kafkas (commercial) wheat varieties of the dicoccum genotype have statistically important amounts of linoleic and α-linolenic acids, respectively. Moreover, substantial quantities of oleic, palmitic, and steric acids were observed in Kavılca kırmızı wheat. The linoleic and α-linolenic acids were high in Karakılçık wheat of durum genotype, whereas Sarı buğday was found rich in terms of palmitic, palmitoleic, eicosenoic, and other fatty acids. The Köse 220/33 wheat has statistically high linoleic, palmitic, and palmitoleic acids in aestivum genotypes. Similarly, α-linolenic and stearic acids were abundant in commercial Bayraktar 2000 wheat, along with high linoleic acid. On the other hand, Spelt S. başak wheat was found to contain higher levels of oleic and eicosenoic acids.

Overall, the monococcum and dicoccum genotypes were rich in oleic and eicosenoic acids, while palmitic and linoleic acids were considerably higher in durum and aestivum wheat genotypes. In addition, commercial Bayraktar 2000 (aestivum) was observed to be rich in terms of both linoleic and α-linolenic fatty acids, followed by some local wheat varieties such as Köse 220/33, Sünter, and AK-702. However, α-linolenic acid level was found to be consistent among all wheat genotypes.

Matea and Bele [172] conducted a study on three different Romanian wheat varieties using GC-FID to determine the fatty acid profile. They reported that linoleic acid had the highest concentration, ranging from 59% to 62%, which aligns with the results of the present study (49.14% - 61.15%). Similarly, Suchowilska, Wiwart, Borejszo, Packa, Kandler and Krska [173] observed a comparable fatty acid profile among various wheat varieties, including monococcum, dicoccum, spelta, and aestivum. Another study by Kan [6] analyzed the fatty acids of Turkish durum and bread wheat varieties using GC-MS, and it revealed a relatively similar fatty acid profile. Recently, quantity of linolenic acid in Italian durum wheats were studied by Narducci, Finotti, Galli and Carcea [10] and linolenic acid amount was determined in the range of 0.5 and 1.14 g/100 g of dry samples. However, the findings of this thesis indicated that Turkish durum wheat has slightly higher amounts of linolenic acid, ranging from 1.06 to 1.20 g/100g (after converting percent fatty acid values into g/100g).

Table 4.5. Fatty acid composition (%) of wheat genotypes.

Wheat genotypes	Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	Linoleic acid	α-linolenic acid	Eicosenoic acid	Other
	C16:0	C16:1	C18:0	C18:1	C18:2, n-6	C18:3, n-3	C20:1	FAs
Monococcum								
Mergüze	13.28 ± 0.02 ^{d}	0.23 ± 0.00 ^{b}	1.10 ± 0.05 ^{a}	28.44 ± 0.02 ^{b}	51.74 ± 0.09 ^{b}	3.15 ± 0.01 ^{b}	1.31 ± 0.01 ^{c}	0.75 ± 0.01
Atasiyez	14.33 ± 0.04 ^{a}	0.23 ± 0.01 ^{a}	1.07 ± 0.03 ^{a}	28.87 ± 0.09 ^{a}	50.39 ± 0.05 ^{d}	3.04 ± 0.01 ^{c}	1.35 ± 0.01 ^{b}	0.70 ± 0.11 ^{b}
Siyez-4	14.16 ± 0.04 ^{b}	0.23 ± 0.00 ^{a}	1.11 ± 0.06 ^{a}	26.70 ± 0.05 ^{d}	52.03 ± 0.05 ^{a}	3.54 ± 0.01 ^{a}	1.38 ± 0.01 ^{a}	0.83 ± 0.02 ^{a}
Siyez Pop	13.68 ± 0.07 ^{c}	0.24 ± 0.00 ^{a}	0.94 ± 0.04 ^{b}	28.33 ± 0.05 ^{c}	51.17 ± 0.05 ^{c}	3.56 ± 0.02 ^{a}	1.34 ± 0.01 ^{b}	0.74 ± 0.02
average	**13.86 ± 0.43**	**0.24 ± 0.01**	**1.06 ± 0.08**	**28.09 ± 0.85**	**51.33 ± 0.65**	**3.32 ± 0.24**	**1.34 ± 0.03**	**0.75 ± 0.07**
Dicoccum								
Kafkas	16.43 ± 0.08 ^{a}	0.21 ± 0.00 ^{a}	1.57 ± 0.12 ^{b}	24.51 ± 0.07 ^{c}	51.30 ± 0.13 ^{b}	3.75 ± 0.01 ^{a}	1.15 ± 0.01 ^{b}	1.07 ± 0.02 ^{a}
Kavılca kırmızı	16.41 ± 0.03 ^{a}	0.23 ± 0.02 ^{a}	1.95 ± 0.03 ^{a}	26.61 ± 0.04 ^{a}	49.14 ± 0.01 ^{c}	3.54 ± 0.01 ^{b}	1.16 ± 0.01 ^{b}	0.97 ± 0.02 ^{b}
Gacer	15.89 ± 0.12 ^{b}	0.23 ± 0.06 ^{a}	1.75 ± 0.22	24.85 ± 0.13 ^{b}	52.03 ± 0.18 ^{a}	3.14 ± 0.02 ^{c}	1.20 ± 0.01 ^{a}	0.91 ± 0.05 ^{b}
average	**16.24 ± 0.27**	**0.23 ± 0.03**	**1.76 ± 0.21**	**25.32 ± 0.96**	**50.82 ± 1.29**	**3.48 ± 0.27**	**1.17 ± 0.03**	**0.98 ± 0.07**
Durum								
Mirzabey 2000	17.46 ± 0.07 ^{b}	0.25 ± 0.01 ^{a}	1.68 ± 0.09 ^{a}	22.10 ± 0.06 ^{a}	53.43 ± 0.04 ^{d}	3.62 ± 0.01 ^{a}	0.73 ± 0.01 ^{c}	0.72 ± 0.13 ^{a}
Eminbey	16.73 ± 0.02 ^{c}	0.22 ± 0.01 ^{b}	1.19 ± 0.01 ^{b}	21.58 ± 0.03 ^{b}	55.70 ± 0.12 ^{b}	3.51 ± 0.00 ^{b}	0.62 ± 0.00 ^{d}	0.44 ± 0.12 ^{b}
Karakılçık	16.37 ± 0.05 ^{d}	0.24 ± 0.01 ^{a}	1.16 ± 0.10 ^{b}	19.43 ± 0.04 ^{d}	57.61 ± 0.09 ^{a}	3.62 ± 0.02 ^{a}	0.76 ± 0.02 ^{b}	0.82 ± 0.01 ^{a}
Sarı buğday	18.21 ± 0.06 ^{a}	0.25 ± 0.00 ^{a}	1.21 ± 0.05 ^{b}	19.86 ± 0.04 ^{c}	55.44 ± 0.06 ^{c}	3.39 ± 0.01 ^{c}	0.80 ± 0.01 ^{a}	0.84 ± 0.03 ^{a}
average	**17.19 ± 0.73**	**0.24 ± 0.01**	**1.31 ± 0.23**	**20.74 ± 1.16**	**55.55 ± 1.53**	**3.54 ± 0.10**	**0.73 ± 0.07**	**0.71 ± 0.18**
Aestivum								
Bayraktar 2000	16.87 ± 0.14 ^{c}	0.16 ± 0.04 ^{b}	1.85 ± 0.04 ^{a}	14.10 ± 0.08 ^{f}	61.15 ± 0.06 ^{a}	4.29 ± 0.02 ^{a}	0.69 ± 0.01 ^{c}	0.91 ± 0.02 ^{b}
Demir 2000	17.00 ± 0.28 ^{c}	0.15 ± 0.06 ^{b}	1.05 ± 0.04 ^{e}	16.32 ± 0.23 ^{c}	59.92 ± 0.21 ^{c}	4.04 ± 0.01 ^{c}	0.70 ± 0.01 ^{c}	0.82 ± 0.18 ^{b}
AK-702	17.59 ± 0.06 ^{b}	0.17 ± 0.07 ^{b}	1.44 ± 0.08 ^{b}	15.34 ± 0.07 ^{d}	60.76 ± 0.11 ^{b}	3.49 ± 0.01 ^{e}	0.64 ± 0.03 ^{d}	0.57 ± 0.04 ^{c}
Köse 220/33	18.62 ± 0.04 ^{a}	0.26 ± 0.01 ^{a}	1.44 ± 0.07 ^{b}	13.24 ± 0.01 ^{g}	61.07 ± 0.10 ^{a}	4.09 ± 0.02 ^{b}	0.66 ± 0.03 ^{d}	0.61 ± 0.03 ^{c}
Sünter	17.58 ± 0.05 ^{b}	0.23 ± 0.00 ^{a}	0.90 ± 0.04 ^{f}	14.96 ± 0.08 ^{e}	60.78 ± 0.07 ^{b}	3.94 ± 0.00 ^{d}	0.77 ± 0.01 ^{b}	0.83 ± 0.03 ^{b}
Zerun	17.04 ± 0.04 ^{c}	0.21 ± 0.00 ^{ab}	1.34 ± 0.02 ^{c}	18.10 ± 0.04 ^{b}	58.26 ± 0.06 ^{d}	3.17 ± 0.01 ^{f}	0.76 ± 0.01 ^{b}	1.12 ± 0.02 ^{a}
Spelt S. başak	16.64 ± 0.04 ^{d}	0.24 ± 0.01 ^{a}	1.24 ± 0.06 ^{d}	24.09 ± 0.05 ^{a}	53.14 ± 0.04 ^{e}	2.71 ± 0.01 ^{g}	1.05 ± 0.01 ^{a}	0.89 ± 0.03 ^{b}
average	**17.34 ± 0.64**	**0.20 ± 0.05**	**1.32 ± 0.29**	**16.59 ± 3.45**	**59.30 ± 2.73**	**3.68 ± 0.54**	**0.75 ± 0.13**	**0.82 ± 0.19**

Values with different lowercase letters in the same result and individual groups are statistically different (p <0.05), (Mean ± SD, n = 4).

In addition, the classification of fatty acids (saturated, monounsaturated, polyunsaturated, n-6, and n-3) in wheat was reported in Table 4.6, according to their prospective health advantages. On an average basis, the monococcum genotype exhibited a statistically significant level of unsaturated fatty acids, whereas aestivum has a high concentration of saturated fatty acids. Similar to the findings of Balli, Cecchi, Pieraccini, Innocenti, Benedettelli and Mulinacci [174], the monococcum genotypes in the present study were also observed to have a comparatively high ratio of unsaturated to saturated fatty acids than dicoccum, durum, and aestivum genotypes. The presence of polyunsaturated fatty acids (PUFAs) increases the level of unsaturation in all wheat varieties, but monounsaturated fatty acids (MUFAs) are responsible for this in monococcum genotypes.

Table 4.6. Fatty acid classification (%).

Wheat genotypes	Saturated FAs	MUFAs	PUFAs	Unsaturated FAs	Unsaturated / Saturated FAs	n-6 / n-3 FAs
Monococcum						
Average	15.43 ^{c}	29.91 ^{a}	54.66 ^{c}	84.57 ^{a}	5.49 ^{a}	15.51 ^{ab}
SD	0.44	0.83	0.81	0.44	0.19	1.02
CV (%)	2.9	2.8	1.5	0.5	3.4	6.6
Max	15.98	30.84	55.62	85.18	5.75	16.68
Min	14.82	28.56	53.38	84.02	5.26	14.34
Dicoccum						
Average	18.69 ^{b}	27.02 ^{b}	54.30 ^{c}	81.31 ^{b}	4.35 ^{b}	14.72 ^{b}
SD	0.36	0.95	1.21	0.36	0.10	1.38
CV (%)	1.9	3.5	2.2	0.4	2.4	9.4
Max	19.12	28.33	55.39	81.87	4.52	16.67
Min	18.13	26.13	52.67	80.88	4.23	13.65
Durum						
Average	18.97 ^{ab}	21.92 ^{c}	59.11 ^{b}	81.03 ^{bc}	4.28 ^{bc}	15.72 ^{ab}
SD	0.86	1.07	1.53	0.86	0.24	0.62
CV (%)	4.5	4.9	2.6	1.1	5.6	3.9
Max	20.00	23.28	61.33	81.99	4.55	16.42
Min	18.01	20.64	57.05	80.00	4.00	14.70
Aestivum						
Average	19.21 ^{a}	17.75 ^{d}	63.04 ^{a}	80.79 ^{c}	4.21 ^{c}	16.39 ^{a}
SD	0.64	3.61	3.24	0.64	0.17	1.93
CV (%)	3.3	20.3	5.1	0.8	4.0	11.8
Max	20.60	25.71	65.59	81.84	4.51	19.62
Min	18.16	14.22	55.81	79.40	3.85	14.19

Values with different lowercase letters in individual group are statistically different (p <0.05).

The linoleic acid predominantly found in wheat samples plays a crucial role in the ratio of unsaturated fatty acids to saturated fatty acids. The evaluation of a food item based on the Healthy Eating Index (HEI-2015) includes the assessment of the ratio of unsaturated to saturated fatty acids, which falls under one of the thirteen categories [175]. This ratio should exceed 2.5 in nutritionally acceptable food. Thus, significantly high values of this ratio suggest that all wheat varieties are valuable in terms of their unsaturation ratio.

On the other hand, no significant difference was observed between the ratio of omega 6 to 3 (n-6/n-3) fatty acids in monococcum, dicoccum, durum, and aestivum genotypes. Due to their high unsaturation, the monococcum wheat varieties might be considered a healthier alternative than other wheat genotypes. Our research has demonstrated that the proportion of unsaturated to saturated fatty acids in local wheat genotypes is comparable to that in commercial wheat varieties, supporting the findings of Suchowilska, Wiwart, Borejszo, Packa, Kandler and Krska [173], who reported UFA/SFA in the range of 3.82 – 5.02 for monococcum, dicoccum, spelt and aestivum wheat varieties from all over the world. The overall results of our study indicate that fatty acid composition of local wheat varieties is comparable with commercially available wheat varieties worldwide. This increases their potential to be registered as commercial varieties in the near future.

4.3.3. Phytosterol composition

Phytosterol/stanol composition was measured in both flour and lipid (hexane extract) of whole wheat samples, and β-sitosterol and β-sitostanol were observed as major phytosterol and phytostanol, respectively. Tables 4.7, 4.8. 4.9, and 4.10 show the distribution of phytosterol and stanol contents in wheat varieties of monococcum, dicoccum, durum and aestivum genotypes, respectively.

The average total phytosterol contents in wheat flour were found in the following order; monococcum > dicoccum ≥ durum > aestivum as compared to that of in wheat lipids; durum ≥ aestivum > monococcum ≥ dicoccum. The results indicated that the high phytosterol contents in monococcum genotypes were due to their high yield of hexane extracts. The concentration of stanols was found to be lower than that of sterols in both flour and lipid of whole wheat samples. The ratio of sterol to stanol was calculated to be about 4.3 in aestivum and dicoccum, whereas it was around 3.2 for durum and monococcum genotypes, respectively.

Wheat varieties such as Atasiyez, Kafkas, Eminbey, and Spelt S. başak were found to have high concentrations of major phytosterols/stanols in their respective genotypes for whole grain samples. However, hexane extract of Siyez-4 was found to have statistically high levels of campesterol and β-sitosterol, whereas Atasiyez was significantly rich in stigmasterol content in monococcum genotypes. Among the aestivum genotypes, Demir 2000 (commercial) wheat lipid exhibited statistically high levels of stigmasterol and β-sitosterol, while campesterol was specifically abundant in the lipid extract of Köse 220/33.

Table 4.7. Phytosterol/stanol profile of wheat flour (mg/kg) and wheat lipid (mg/g) of monococcum genotypes.

Wheat genotypes	Campesterol	Campestanol *	Stigmasterol	β-sitosterol	β-sitostanol *	Δ5-avenasterol *	Others **	Total Phytosterol
flour								
Mergüze	69.49 ± 2.24 ^{c}	35.67 ± 1.62 ^{b}	20.46 ± 2.62 ^{b}	362.47 ± 5.17 ^{d}	148.38 ± 2.58 ^{b}	12.73 ± 1.43 ^{b}	70.86 ± 5.44 ^{b}	719.70 ± 9.95 ^{d}
Atasiyez	100.16 ± 3.10 ^{a}	51.15 ± 1.98 ^{a}	41.66 ± 4.80 ^{a}	432.00 ± 2.76 ^{a}	188.73 ± 4.10 ^{a}	6.19 ± 1.86 ^{c}	86.55 ± 12.65 ^{a}	906.45 ± 6.33 ^{a}
Siyez-4	85.51 ± 1.73 ^{b}	33.44 ± 1.39 ^{b}	14.38 ± 1.07 ^{c}	421.73 ± 2.65 ^{b}	143.47 ± 3.59 ^{bc}	12.38 ± 0.45 ^{b}	59.29 ± 2.23 ^{b}	770.19 ± 5.17 ^{c}
Siyez Pop	87.39 ± 1.34 ^{b}	33.98 ± 0.67 ^{b}	13.63 ± 2.03 ^{c}	411.18 ± 5.53 ^{c}	140.51 ± 1.38 ^{c}	25.03 ± 2.27 ^{a}	90.00 ± 4.37 ^{a}	801.72 ± 7.81 ^{b}
average	**85.64 ± 11.53**	**37.42 ± 6.95**	**22.53 ± 12.13**	**406.84 ± 28.08**	**155.27 ± 20.56**	**13.09 ± 6.68**	**77.20 ± 14.81**	**806.77 ± 70.30**
lipid extract.								
Mergüze	2.79 ± 0.09 ^{c}	1.43 ± 0.07 ^{ab}	0.82 ± 0.10 ^{b}	14.57 ± 0.21 ^{c}	5.96 ± 0.10 ^{a}	0.57 ± 0.06 ^{b}	2.85 ± 0.22 ^{ab}	28.92 ± 0.40 ^{c}
Atasiyez	2.94 ± 0.09 ^{c}	1.50 ± 0.06 ^{ab}	1.22 ± 0.14 ^{a}	12.68 ± 0.08 ^{d}	5.54 ± 0.12 ^{b}	0.18 ± 0.05 ^{c}	2.54 ± 0.37 ^{bc}	26.60 ± 0.19 ^{d}
Siyez-4	3.51 ± 0.07 ^{a}	1.37 ± 0.06 ^{b}	0.59 ± 0.04 ^{c}	17.32 ± 0.11 ^{a}	5.89 ± 0.15 ^{a}	0.51 ± 0.02 ^{b}	2.43 ± 0.09 ^{c}	31.63 ± 0.21 ^{a}
Siyez Pop	3.23 ± 0.05 ^{b}	1.26 ± 0.02 ^{c}	0.50 ± 0.07 ^{c}	15.21 ± 0.20 ^{b}	5.20 ± 0.05 ^{c}	0.93 ± 0.08 ^{a}	3.33 ± 0.16 ^{a}	29.66 ± 0.29 ^{b}
average	**3.12 ± 0.30**	**1.38 ± 0.10**	**0.78 ± 0.30**	**14.94 ± 1.74**	**5.65 ± 0.33**	**0.50 ± 0.26**	**2.78 ± 0.43**	**29.23 ± 1.98**

The values with different lowercase letters in same results are statistically different ($p < 0.05$), (Mean ± SD, n = 3).

* Identified by Mass Spectral libraries.

** May include more than one phytosterols.

Table 4.8. Phytosterol/stanol profile of wheat flour (mg/kg) and wheat lipid (mg/g) of dicoccum genotypes.

Wheat genotypes	Campesterol	Campestanol *	Stigmasterol	β-sitosterol	β-sitostanol *	Δ5-avenasterol *	Others **	Total Phytosterol
flour								
Kafkas	105.55 ± 1.70 [b]	40.48 ± 2.16 [a]	28.65 ± 0.75 [a]	433.21 ± 1.19 [a]	142.56 ± 4.85 [a]	7.99 ± 1.07 [b]	64.59 ± 4.02 [a]	823.03 ± 1.62 [a]
Kavılca kırmızı	111.49 ± 1.28 [a]	32.30 ± 0.28 [c]	7.42 ± 1.47 [c]	440.81 ± 3.82 [a]	60.62 ± 2.92 [c]	12.31 ± 2.06 [a]	39.16 ± 0.61 [b]	704.10 ± 2.79 [b]
Gacer	62.06 ± 0.92 [c]	37.71 ± 0.43 [b]	14.78 ± 0.86 [b]	342.66 ± 12.23 [b]	104.02 ± 5.77 [b]	12.56 ± 1.45 [a]	59.35 ± 2.99 [a]	633.14 ± 14.61 [c]
average	**93.03 ± 23.40**	**36.83 ± 3.77**	**16.95 ± 9.38**	**405.56 ± 47.73**	**96.43 ± 37.18**	**10.96 ± 2.61**	**54.36 ± 11.90**	**720.09 ± 83.43**
lipid extract								
Kafkas	4.06 ± 0.06 [a]	1.56 ± 0.08 [a]	1.10 ± 0.03 [a]	16.67 ± 0.05 [a]	5.48 ± 0.19 [a]	0.31 ± 0.04 [b]	2.48 ± 0.15 [a]	31.66 ± 0.06 [a]
Kavılca kırmızı	4.00 ± 0.05 [a]	1.16 ± 0.01 [b]	0.27 ± 0.05 [c]	15.81 ± 0.14 [b]	2.17 ± 0.10 [c]	0.44 ± 0.07 [a]	1.40 ± 0.02 [b]	25.26 ± 0.10 [c]
Gacer	2.62 ± 0.04 [b]	1.59 ± 0.02 [a]	0.62 ± 0.04 [b]	14.44 ± 0.52 [c]	4.38 ± 0.24 [b]	0.53 ± 0.06 [a]	2.50 ± 0.13 [a]	26.69 ± 0.62 [b]
average	**3.56 ± 0.71**	**1.44 ± 0.21**	**0.66 ± 0.36**	**15.64 ± 1.01**	**3.75 ± 1.55**	**0.43 ± 0.11**	**2.13 ± 0.55**	**27.87 ± 2.93**

The values with different lowercase letters in same results are statistically different ($p < 0.05$), (Mean ± SD, n = 3).

* Identified by Mass Spectral libraries.

** May include more than one phytosterols.

Table 4.9. Phytosterol/stanol profile of wheat flour (mg/kg) and wheat lipid (mg/g) of durum genotypes.

Wheat genotypes	Campesterol	Campestanol *	Stigmasterol	β-sitosterol	β-sitostanol *	Δ5-avenasterol *	Others **	Total Phytosterol
flour								
Mirzabey 2000	92.42 ± 2.18 ^{b}	56.47 ± 0.81 ^{b}	25.63 ± 2.46 ^{b}	374.36 ± 3.34 ^{b}	123.33 ± 5.25 ^{b}	10.21 ± 2.22 ^{c}	63.01 ± 0.94 ^{c}	744.79 ± 11.09 ^{b}
Eminbey	99.18 ± 1.47 ^{a}	72.95 ± 1.56 ^{a}	37.54 ± 2.20 ^{a}	398.47 ± 3.81 ^{a}	148.55 ± 1.50 ^{a}	21.24 ± 3.29 ^{a}	69.42 ± 4.09 ^{b}	847.36 ± 4.71 ^{a}
Karakılçık	78.00 ± 0.89 ^{c}	36.10 ± 1.79 ^{d}	20.34 ± 2.67 ^{c}	373.01 ± 2.77 ^{b}	110.77 ± 3.07 ^{c}	16.53 ± 1.40 ^{b}	48.64 ± 2.15 ^{d}	683.38 ± 9.01 ^{c}
Sarı buğday	58.32 ± 1.76 ^{d}	48.89 ± 0.25 ^{c}	22.96 ± 1.48 ^{bc}	313.83 ± 3.08 ^{c}	103.49 ± 1.09 ^{d}	10.16 ± 0.53 ^{c}	74.35 ± 1.35 ^{a}	632.00 ± 7.56 ^{d}
average	**81.98 ± 16.41**	**53.34 ± 14.62**	**26.62 ± 7.14**	**364.92 ± 32.69**	**121.53 ± 18.10**	**14.53 ± 5.19**	**63.93 ± 10.80**	**725.25 ± 87.91**
lipid extract								
Mirzabey 2000	4.11 ± 0.10 ^{b}	2.51 ± 0.04 ^{b}	1.14 ± 0.11 ^{b}	16.64 ± 0.15 ^{b}	5.48 ± 0.23 ^{b}	0.45 ± 0.10 ^{b}	2.80 ± 0.04 ^{c}	33.10 ± 0.49 ^{c}
Eminbey	4.65 ± 0.07 ^{a}	3.42 ± 0.07 ^{a}	1.76 ± 0.10 ^{a}	18.66 ± 0.18 ^{a}	6.96 ± 0.07 ^{a}	0.99 ± 0.15 ^{a}	3.25 ± 0.19 ^{b}	39.69 ± 0.22 ^{a}
Karakılçık	3.91 ± 0.04 ^{c}	1.81 ± 0.09 ^{c}	1.02 ± 0.13 ^{b}	18.69 ± 0.14 ^{a}	5.55 ± 0.15 ^{b}	0.83 ± 0.07 ^{a}	2.44 ± 0.11 ^{d}	34.25 ± 0.45 ^{b}
Sarı buğday	3.05 ± 0.09 ^{d}	2.56 ± 0.01 ^{b}	1.20 ± 0.08 ^{b}	16.41 ± 0.16 ^{b}	5.41 ± 0.06 ^{b}	0.53 ± 0.03 ^{b}	3.89 ± 0.07 ^{a}	33.04 ± 0.40 ^{c}
average	**3.93 ± 0.60**	**2.58 ± 0.63**	**1.28 ± 0.31**	**17.60 ± 1.14**	**5.85 ± 0.68**	**0.70 ± 0.24**	**3.12 ± 0.59**	**35.19 ± 2.95**

The values with different lowercase letters in same results are statistically different ($p < 0.05$), (Mean ± SD, n = 3).

* Identified by Mass Spectral libraries.

** May include more than one phytosterols.

Table 4.10. Phytosterol/stanol profile of wheat flour (mg/kg) and wheat lipid (mg/g) of aestivum genotypes.

Wheat genotypes	Campesterol	Campestanol *	Stigmasterol	β-sitosterol	β-sitostanol *	Δ5-avenasterol *	Others **	Total Phytosterol
flour								
Bayraktar 2000	55.37 ± 0.99 ^{d}	20.92 ± 0.28 ^{de}	10.22 ± 0.09 ^{d}	308.01 ± 0.83 ^{d}	48.87 ± 1.18 ^{e}	36.95 ± 4.45 ^{c}	41.02 ± 0.55 ^{b}	521.38 ± 4.42 ^{d}
Demir 2000	54.86 ± 1.39 ^{d}	24.55 ± 0.40 ^{c}	14.50 ± 0.49 ^{ab}	339.49 ± 4.81 ^{b}	132.37 ± 2.71 ^{b}	16.13 ± 1.58 ^{e}	55.39 ± 2.24 ^{b}	637.29 ± 6.02 ^{b}
AK-702	55.00 ± 1.78 ^{d}	27.36 ± 1.05 ^{b}	13.91 ± 1.94 ^{bc}	314.46 ± 0.16 ^{c}	84.29 ± 2.87 ^{d}	53.07 ± 0.70 ^{a}	48.00 ± 1.56 ^{b}	596.09 ± 7.99 ^{c}
Köse 220/33	60.16 ± 1.69 ^{c}	22.42 ± 1.25 ^{d}	11.72 ± 0.28 ^{cd}	289.99 ± 4.57 ^{e}	49.24 ± 3.41 ^{e}	43.01 ± 0.28 ^{b}	48.34 ± 0.52 ^{b}	524.88 ± 3.59 ^{d}
Sünter	65.15 ± 1.49 ^{b}	20.25 ± 0.85 ^{e}	12.73 ± 2.09 ^{bc}	336.87 ± 1.85 ^{b}	91.88 ± 2.31 ^{c}	23.30 ± 3.06 ^{d}	52.22 ± 3.42 ^{b}	602.40 ± 2.52 ^{c}
Zerun	58.66 ± 0.19 ^{c}	25.73 ± 0.96 ^{c}	13.27 ± 0.58 ^{bc}	315.13 ± 0.77 ^{c}	49.49 ± 1.10 ^{e}	45.69 ± 2.34 ^{b}	37.75 ± 0.81 ^{b}	545.71 ± 2.89 ^{d}
Spelt S. başak	77.08 ± 0.40 ^{a}	34.34 ± 1.05 ^{a}	16.55 ± 1.53 ^{a}	395.14 ± 2.88 ^{a}	159.98 ± 2.37 ^{a}	14.95 ± 0.45 ^{e}	101.81 ± 2.28 ^{a}	800.58 ± 6.17 ^{a}
average	**60.90 ± 7.70**	**25.08 ± 4.63**	**13.27 ± 2.19**	**328.44 ± 32.28**	**90.27 ± 43.16**	**32.14 ± 14.81**	**53.11 ± 18.86**	**597.66 ± 85.39**
lipid extract								
Bayraktar 2000	3.38 ± 0.06 ^{bc}	1.28 ± 0.02 ^{d}	0.62 ± 0.01 ^{c}	18.82 ± 0.05 ^{b}	2.99 ± 0.07 ^{d}	2.26 ± 0.27 ^{b}	251 ± 0.03 ^{de}	31.85 ± 0.27 ^{d}
Demir 2000	3.30 ± 0.08 ^{cd}	1.48 ± 0.02 ^{ab}	0.87 ± 0.03 ^{a}	20.43 ± 0.29 ^{a}	7.97 ± 0.16 ^{a}	0.97 ± 0.10 ^{c}	3.33 ± 0.13 ^{b}	38.35 ± 0.36 ^{a}
AK-702	2.76 ± 0.09 ^{e}	1.37 ± 0.05 ^{c}	0.70 ± 0.10 ^{bc}	15.79 ± 0.01 ^{f}	4.23 ± 0.14 ^{d}	2.66 ± 0.04 ^{a}	2.41 ± 0.08 ^{e}	29.93 ± 0.40 ^{f}
Köse 220/33	3.73 ± 0.10 ^{a}	1.39 ± 0.08 ^{c}	0.73 ± 0.02 ^{bc}	17.98 ± 0.28 ^{c}	3.05 ± 0.21 ^{d}	2.67 ± 0.02 ^{a}	3.00 ± 0.03 ^{c}	32.54 ± 0.22 ^{c}
Sünter	3.32 ± 0.08 ^{cd}	1.03 ± 0.04 ^{e}	0.65 ± 0.11 ^{c}	17.15 ± 0.09 ^{d}	4.68 ± 0.12 ^{c}	1.19 ± 0.16 ^{c}	2.66 ± 0.17 ^{d}	30.66 ± 0.13 ^{e}
Zerun	3.46 ± 0.01 ^{b}	1.52 ± 0.06 ^{a}	0.78 ± 0.03 ^{ab}	18.61 ± 0.04 ^{b}	2.92 ± 0.06 ^{d}	2.70 ± 0.14 ^{a}	2.23 ± 0.05 ^{f}	32.24 ± 0.17 ^{cd}
Spelt S. başak	3.21 ± 0.02 ^{d}	1.43 ± 0.04 ^{bc}	0.69 ± 0.06 ^{bc}	16.46 ± 0.12 ^{e}	6.66 ± 0.10 ^{b}	0.62 ± 0.02 ^{d}	4.24 ± 0.09 ^{a}	33.34 ± 0.26 ^{b}
average	**3.31 ± 0.28**	**1.36 ± 0.16**	**0.72 ± 0.10**	**17.89 ± 1.50**	**4.75 ± 1.94**	**1.78 ± 0.87**	**2.86 ± 0.63**	**32.81 ± 2.75**

The values with different lowercase letters in same results are statistically different ($p < 0.05$), (Mean ± SD, n = 3).

* Identified by Mass Spectral libraries.

** May include more than one phytosterols.

A recent study conducted by Balli, Cecchi, Pieraccini, Innocenti, Benedettelli and Mulinacci [174], compared the phytosterol contents of ancient and modern wheat varieties. The study revealed that certain ancient varieties exhibited significantly higher phytosterol contents compared to modern wheats. Merah and Mouloungui [9] observed higher total phytosterol contents in durum wheat (average 105.7 mg/100g DM) compared to 79.3 mg/100g DM in dicoccum wheat varieties. However, the findings of our study do not align with those of Merah and Mouloungui [9], since the phytosterol contents of dicoccum and durum wheat varieties were more or less same. Furthermore, our research revealed that the amounts of campesterol (avg. 3.93 mg/g), stigmasterol (avg. 1.28 mg/g), sitosterol (avg. 17.60 mg/g), sitostanol (avg. 5.85 mg/g) and total phytosterols (avg. 35.19 mg/g) in lipid extracts of durum wheat varieties were greater than those of previously reported values of durum wheat samples (2.18, 0.35, 4.71, 2.1, and 15.07 mg/g, respectively) [176].

However, some sterols such as brassicasterol, Δ7-avenasterol, and stigmasta-5,24(25)-dienol, reported by Nurmi, Nystrom, Edelmann, Lampi and Piironen [56] in different wheat studies were not detected in the thesis, but three unknown peaks (8, 9, and 10) were observed, and they could be stigma-7-en-3-ol, γ-sitostenone, and stigmasta-7,24(28)-dien-3-ol, respectively, as named by MS spectral libraries (Figure 3.5).

Consequently, it can be observed that both the flour and lipids of local wheat varieties have higher phytosterol levels compared to commercial ones. This indicates a better quality of wheat grains in terms of bioactive phytosterols. This finding may have significant implications for the selection of local wheat varieties in future breeding programs.

4.3.4. Steryl ferulate contents

The steryl ferulate contents of whole grain flour and lipid extracts are shown in Table 4.11. The average quantities of total steryl ferulates in both wheat flour and lipids were observed in the following order: monococcum ≥ aestivum > durum > dicoccum. As a local wheat variety, Siyez-4 contains a significant amount of total steryl ferulates among all wheat genotypes, including commercial varieties. In the case of aestivum, local wheat varieties were found to possess higher levels of steryl ferulate contents compared to the commercial variety Bayraktar 2000. However, the steryl ferulate contents of the local Spelt S. başak were relatively similar to those of the variety Demir 2000. .

Table 4.11. Steryl ferulate contents of wheat flour (mg/kg) and wheat lipid (mg/g).

Wheat genotypes	Campesteryl ferulate		Campestanyl & sitosteryl ferulates		Sitostanyl ferulate		Total steryl ferulates	
	flour	*lipid extract*	*flour*	*lipid extract*	*flour*	*lipid extract*	*flour*	*lipid extract*
Monococcum								
Mergüze	19.12 ± 0.66 ^{b}	0.77 ± 0.03 ^{b}	72.55 ± 2.33 ^{b}	2.92 ± 0.09 ^{b}	63.44 ± 2.25 ^{a}	2.55 ± 0.09 ^{a}	155.12 ± 5.08 ^{b}	6.23 ± 0.20 ^{b}
Atasiyez	14.76 ± 0.79 ^{c}	0.43 ± 0.02 ^{d}	62.95 ± 3.70 ^{c}	1.85 ± 0.11 ^{d}	41.78 ± 2.61 ^{b}	1.23 ± 0.08 ^{c}	119.48 ± 7.09 ^{c}	3.51 ± 0.21 ^{d}
Siyez-4	24.44 ± 1.95 ^{a}	1.00 ± 0.08 ^{a}	84.88 ± 6.61 ^{a}	3.49 ± 0.27 ^{a}	63.19 ± 5.03 ^{a}	2.60 ± 0.21 ^{a}	172.52 ± 13.59 ^{a}	7.09 ± 0.56 ^{a}
Siyez Pop	18.07 ± 0.92 ^{b}	0.67 ± 0.03 ^{c}	63.86 ± 2.44 ^{c}	2.36 ± 0.09 ^{c}	45.01 ± 1.40 ^{b}	1.66 ± 0.05 ^{b}	126.94 ± 4.77 ^{c}	4.70 ± 0.18 ^{c}
average	**19.10 ± 3.75**	**0.72 ± 0.22**	**71.06 ± 9.83**	**2.65 ± 0.65**	**53.35 ± 10.73**	**2.01 ± 0.61**	**143.51 ± 23.33**	**5.38 ± 1.46**
Dicoccum								
Kafkas	11.04 ± 0.30 ^{b}	0.42 ± 0.01 ^{a}	33.70 ± 1.00 ^{a}	1.30 ± 0.04 ^{a}	18.73 ± 0.55 ^{b}	0.72 ± 0.02 ^{b}	63.46 ± 1.85 ^{a}	2.44 ± 0.07 ^{a}
Kavılca kırmızı	12.04 ± 0.19 ^{a}	0.43 ± 0.01 ^{a}	24.57 ± 0.45 ^{c}	0.88 ± 0.02 ^{b}	12.48 ± 0.26 ^{c}	0.45 ± 0.01 ^{c}	49.09 ± 0.90 ^{c}	1.76 ± 0.03 ^{b}
Gacer	4.88 ± 0.26 ^{c}	0.21 ± 0.01 ^{b}	29.89 ± 1.51 ^{b}	1.26 ± 0.06 ^{a}	22.70 ± 1.14 ^{a}	0.96 ± 0.05 ^{a}	57.48 ± 2.89 ^{b}	2.42 ± 0.12 ^{a}
average	**9.32 ± 3.31**	**0.35 ± 0.11**	**29.39 ± 4.03**	**1.15 ± 0.20**	**17.97 ± 4.45**	**0.71 ± 0.22**	**56.68 ± 6.43**	**2.21 ± 0.34**
Durum								
Mirzabey 2000	9.81 ± 0.56 ^{b}	0.44 ± 0.02 ^{b}	53.33 ± 3.07 ^{a}	2.37 ± 0.14 ^{a}	19.95 ± 1.12 ^{b}	0.89 ± 0.05 ^{c}	83.09 ± 4.74 ^{a}	3.69 ± 0.21 ^{a}
Eminbey	10.01 ± 1.68 ^{b}	0.47 ± 0.08 ^{b}	50.25 ± 6.22 ^{a}	2.35 ± 0.29 ^{a}	21.44 ± 2.68 ^{ab}	1.00 ± 0.13 ^{b}	81.70 ± 10.55 ^{a}	3.83 ± 0.49 ^{a}
Karakılçık	12.32 ± 0.57 ^{a}	0.62 ± 0.03 ^{a}	43.03 ± 1.45 ^{b}	2.16 ± 0.07 ^{a}	23.22 ± 0.73 ^{a}	1.16 ± 0.04 ^{a}	78.57 ± 2.74 ^{a}	3.94 ± 0.14 ^{a}
Sarı buğday	5.39 ± 0.29 ^{c}	0.28 ± 0.01 ^{c}	28.67 ± 1.53 ^{c}	1.50 ± 0.08 ^{b}	14.46 ± 0.84 ^{c}	0.76 ± 0.04 ^{d}	48.53 ± 2.65 ^{b}	2.54 ± 0.14 ^{b}
average	**9.38 ± 2.72**	**0.45 ± 0.13**	**43.82 ± 10.34**	**2.09 ± 0.40**	**19.77 ± 3.66**	**0.95 ± 0.17**	**72.97 ± 15.65**	**3.50 ± 0.63**
Aestivum								
Bayraktar 2000	9.10 ± 0.35 ^{e}	0.56 ± 0.02 ^{d}	39.21 ± 0.71 ^{b}	2.40 ± 0.04 ^{e}	21.14 ± 0.37 ^{e}	1.29 ± 0.02 ^{d}	69.45 ± 1.24 ^{f}	4.24 ± 0.08 ^{e}
Demir 2000	11.53 ± 0.50 ^{d}	0.69 ± 0.03 ^{c}	55.01 ± 1.82 ^{c}	3.31 ± 0.11 ^{a}	43.45 ± 1.31 ^{b}	2.61 ± 0.08 ^{a}	109.99 ± 3.63 ^{b}	6.62 ± 0.22 ^{a}
AK-702	9.19 ± 0.71 ^{e}	0.46 ± 0.04 ^{e}	49.12 ± 3.07 ^{c}	2.47 ± 0.15 ^{de}	30.83 ± 1.95 ^{c}	1.55 ± 0.10 ^{c}	89.14 ± 5.47 ^{d}	4.48 ± 0.27 ^{de}
Köse 220/33	12.46 ± 0.42 ^{cd}	0.77 ± 0.03 ^{a}	41.82 ± 0.61 ^{d}	2.59 ± 0.04 ^{d}	21.75 ± 0.33 ^{e}	1.35 ± 0.02 ^{d}	76.03 ± 1.00 ^{e}	4.71 ± 0.06 ^{d}
Sünter	15.31 ± 0.87 ^{b}	0.78 ± 0.04 ^{a}	55.03 ± 2.02 ^{b}	2.80 ± 0.10 ^{c}	31.04 ± 1.13 ^{c}	1.58 ± 0.06 ^{c}	101.38 ± 3.95 ^{c}	5.16 ± 0.20 ^{c}
Zerun	12.72 ± 0.70 ^{c}	0.75 ± 0.04 ^{ab}	49.37 ± 1.20^{c}	2.92 ± 0.07 ^{bc}	25.68 ± 0.61 ^{d}	1.52 ± 0.04 ^{c}	87.77 ± 2.49 ^{d}	5.18 ± 0.15 ^{c}
Spelt S. başak	16.82 ± 1.10 ^{a}	0.70 ± 0.05 ^{bc}	71.60 ± 3.86 ^{a}	2.98 ± 0.16 ^{b}	58.87 ± 2.93 ^{a}	2.45 ± 0.12 ^{b}	147.28 ± 7.87 ^{a}	6.13 ± 0.33 ^{b}
average	**12.45 ± 2.79**	**0.67 ± 0.12**	**51.59 ± 10.25**	**2.78 ± 0.32**	**33.25 ± 12.88**	**1.76 ± 0.51**	**97.29 ± 24.82**	**5.22 ± 0.84**

Different lowercase letters in same results exhibit statistically different values (p <0.05), (Mean ± SD, n = 4).

The total steryl ferulate content in aestivum wheat flours was measured to be between 67 and 123 µg/g by Nurmi, Lampi, Nystrom and Piironen [48], which is consistent with the values reported in the thesis, ranging from 69 to 147 mg/kg. On the other hand, levels of steryl ferulates in wheat lipids were observed to be higher in comparison to the previous studies conducted by Kumar and Krishna [21] and Nyström, Paasonen, Lampi and Piironen [49] on bran and germ oils. The existing literature on the composition of steryl ferulates in wheat lipids is relatively limited compared to that of rice. Therefore, establishing meaningful comparisons among various wheat genotypes becomes a challenge. On the other hand, considering the significance of steryl ferulates in human metabolism and their potential to reduce cholesterol absorption [20], local wheat genotypes may be a preferable alternative to commercial varieties due to their steryl ferulate contents.

4.4. Purification and Identification of Sünter Extract

The Sünter wheat variety, which exhibits high levels of phenolic and fatty acid content, was chosen for subsequent purification and identification of bioactive components.

4.4.1. Purification of Sünter hexane extract

Hexane extract of Sünter wheat was obtained by method described in Section 3.5.1. The extract consists of nonpolar lipids (triglycerides), phospholipids, and glycolipids. Triglycerides are present in the extract and consist of both saturated and unsaturated fatty acids. The major fatty acid in triglycerides is linolenic acid, followed by palmitic and oleic acids ([177]. These fatty acids in hexane extract were also determined by GC-FID in the present study (Figure 4.4). In addition to triglycerides, it contains minor components such as phytosterols (Section 4.3.3), steryl ferulates (Section 4.3.4), tocopherol, among others.

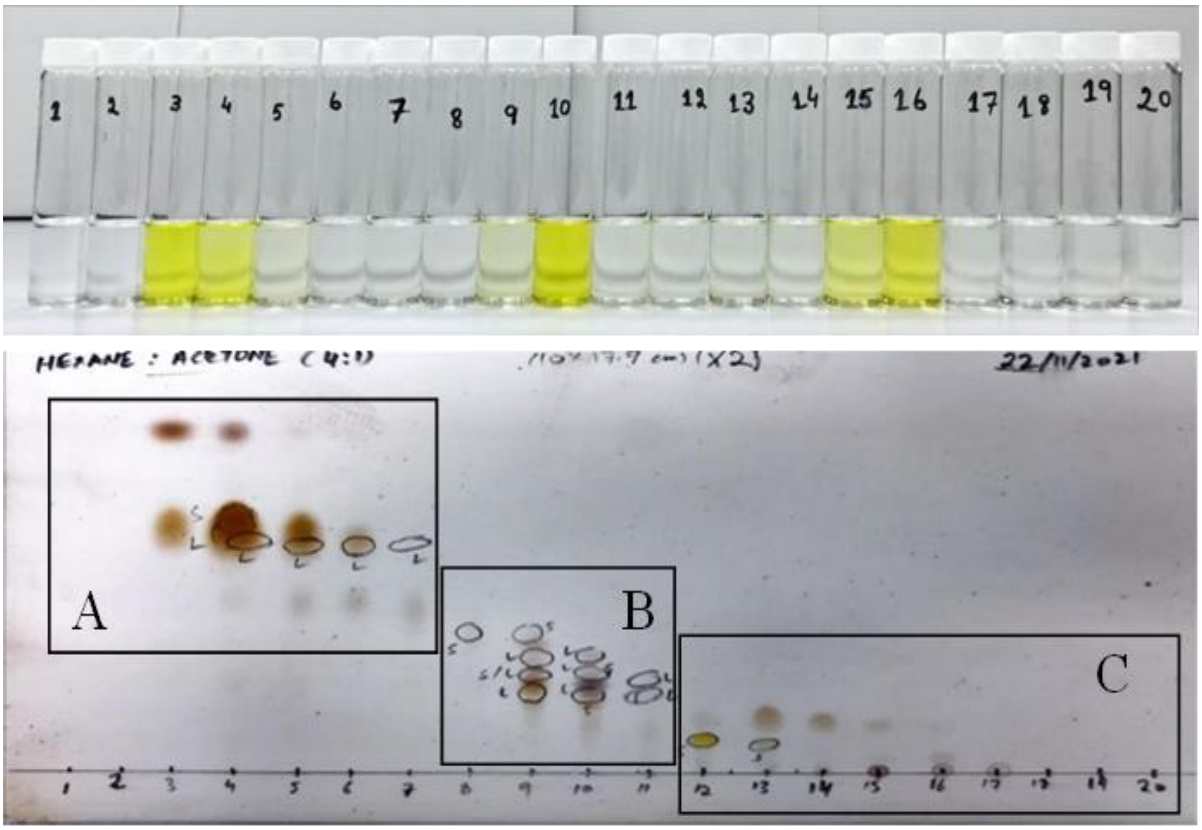


Figure 4.4. Analysis of MPLC fractions using TLC.

The fat-soluble components present in the hexane extract of the Sünter wheat variety were isolated and purified using MPLC, TLC, and PTLC techniques (Section 3.8.1). The twenty fractions (20 ml each) were collected from MPLC silica gel column using various washing profiles. Afterward, these fractions were applied to a TLC plate. According to TLC profiling, these fractions were combined into three primary fractions (A, B, and C) (Figure 4.4). To achieve further purification, the combined fractions were then separately subjected to PTLC. UV fluorescence of metabolites in these major fractions was observed on PTLC under 366 nm and 254 nm wavelengths (Figure 4.5, Figure 4.10, and Figure 4.12). On the other hand, images of UV-inactive metabolites were presented in Figure 4.6, Figure 4.11, and Figure 4.13, after acid charring of PTLC plates.

Purification of Hexane A fraction

In our preliminary studies (ANNEX 3), after MPLC, A fraction was analyzed by NMR and GC-MS. The peaks for glycerol molecules (basic backbone in triglyceride) were detected by both ^{1}H and ^{13}C NMR spectra, and GC-MS analysis also indicated the presence of two main major unsaturated fatty acids (oleic and linolenic acids) and one saturated fatty acid (palmitic acid).

According to this result, in the present study, A fraction was assumed as mixture of triglycerides.

In the PTCL profile of Fraction A, after acid charring, multiple bands (7 bands) were obtained (Figure 4.6B). Only one of the UV-active metabolites (4^{th} band) was detected at 366 nm whereas two of them (2^{nd} and 7^{th} bands) were observed at 254 nm wavelengths (Figure 4.5). After acid charring, 1^{st} band also appeared as UV-active metabolite under UV-366 (Figure 4.6A).

The molecules found in the 4^{th} band displayed a distinct bright blue line at UV 366, indicating a significant presence of double bonds in these compounds. These bands are indicative of the presence of unsaturated fatty acids, as double bonds in the molecules have the property of absorbing light [178].

Therefore, only UV-inactive 1^{st} and UV-active 4^{th} bands were chosen due to high purity for further characterization by NMR analysis. For this, these bands were carefully scraped from the PTLC plate and eluted with appropriate solvent mix.

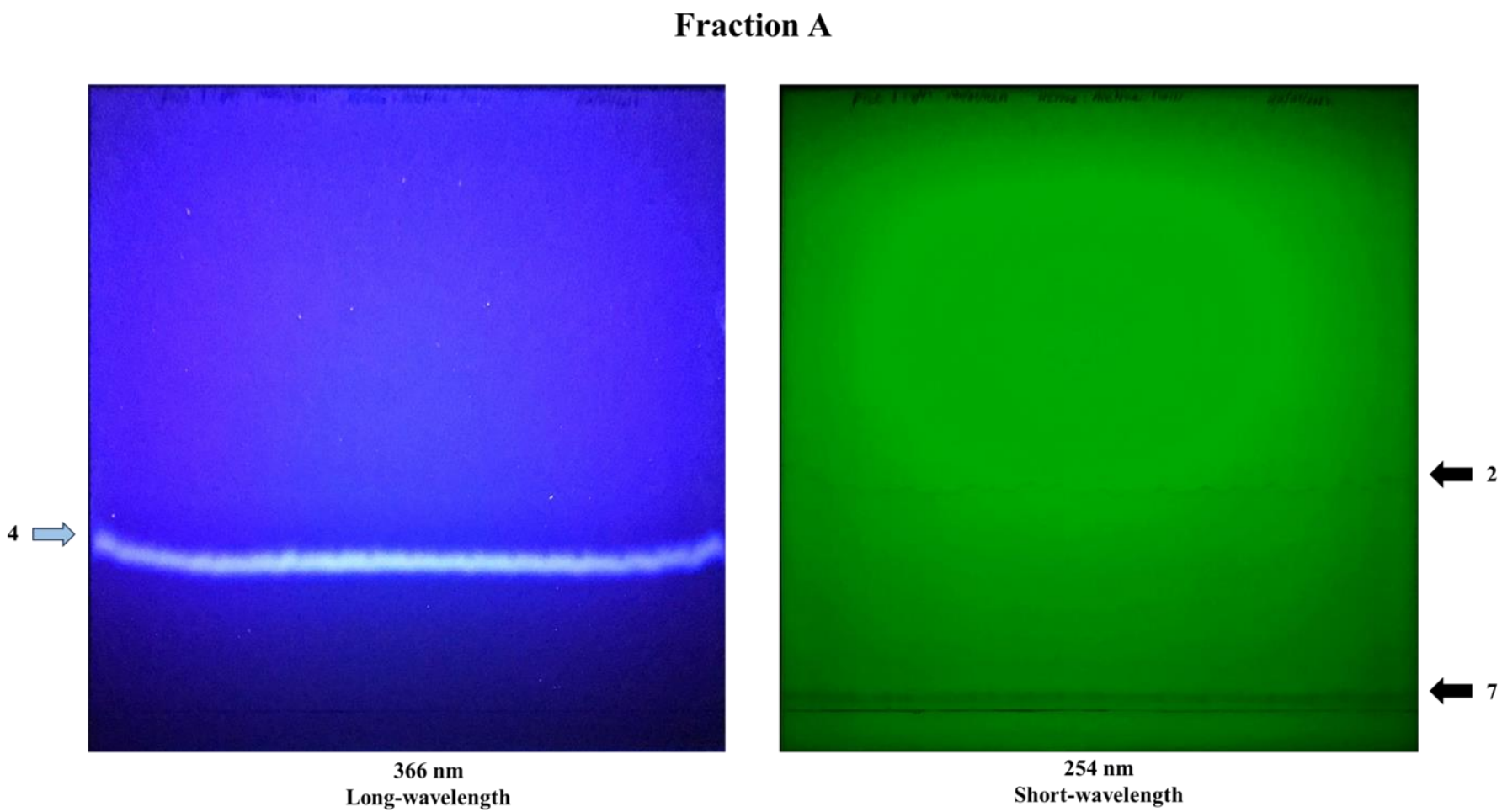


Figure 4.5. PTLC profiling of Fraction A (images captured under UV-366 nm and UV-254 nm)

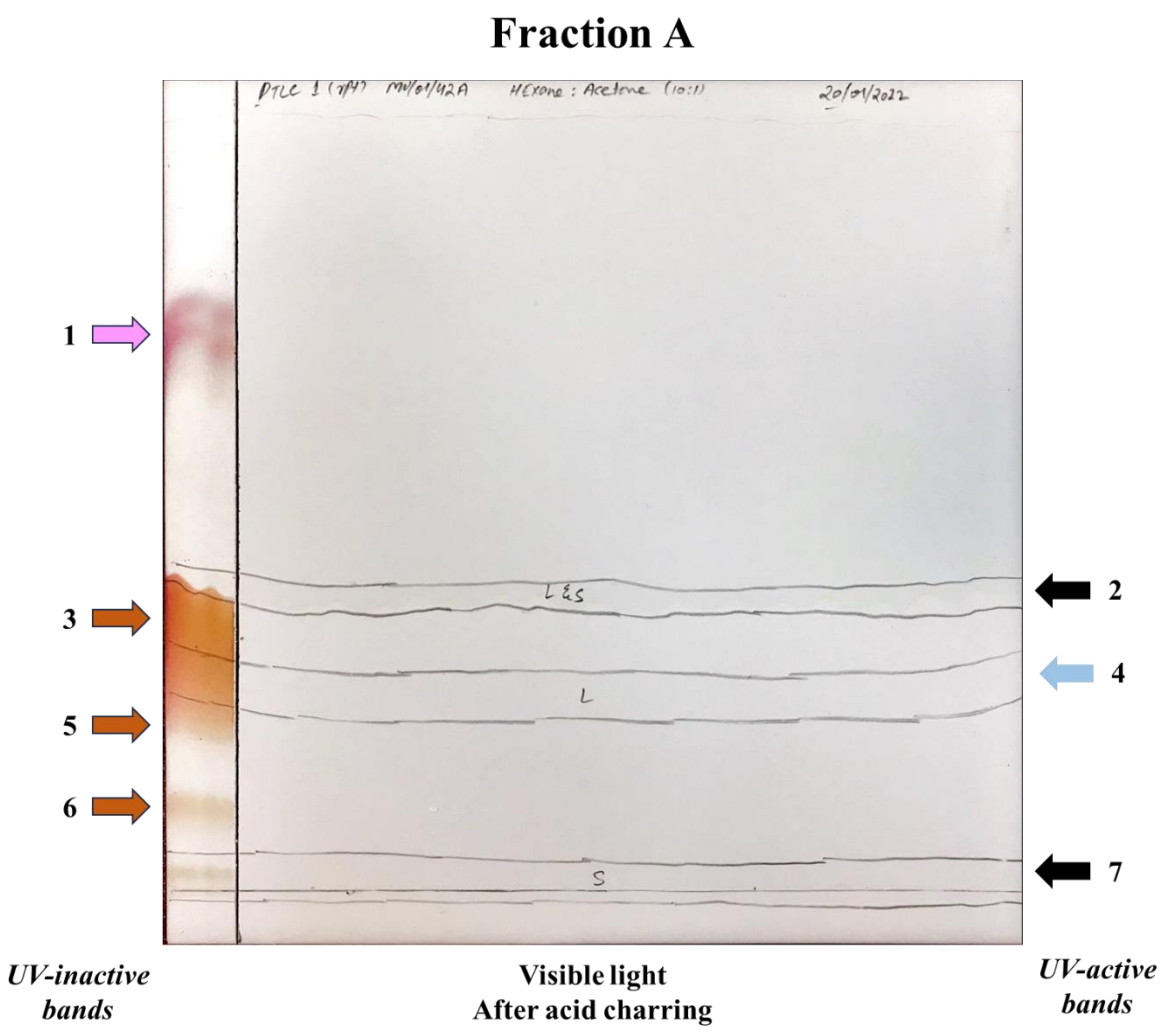


Figure 4.6. PTLC profile of Fraction A (image captured under normal light after acid charring).

NMR characterization of the hexane A 1 band

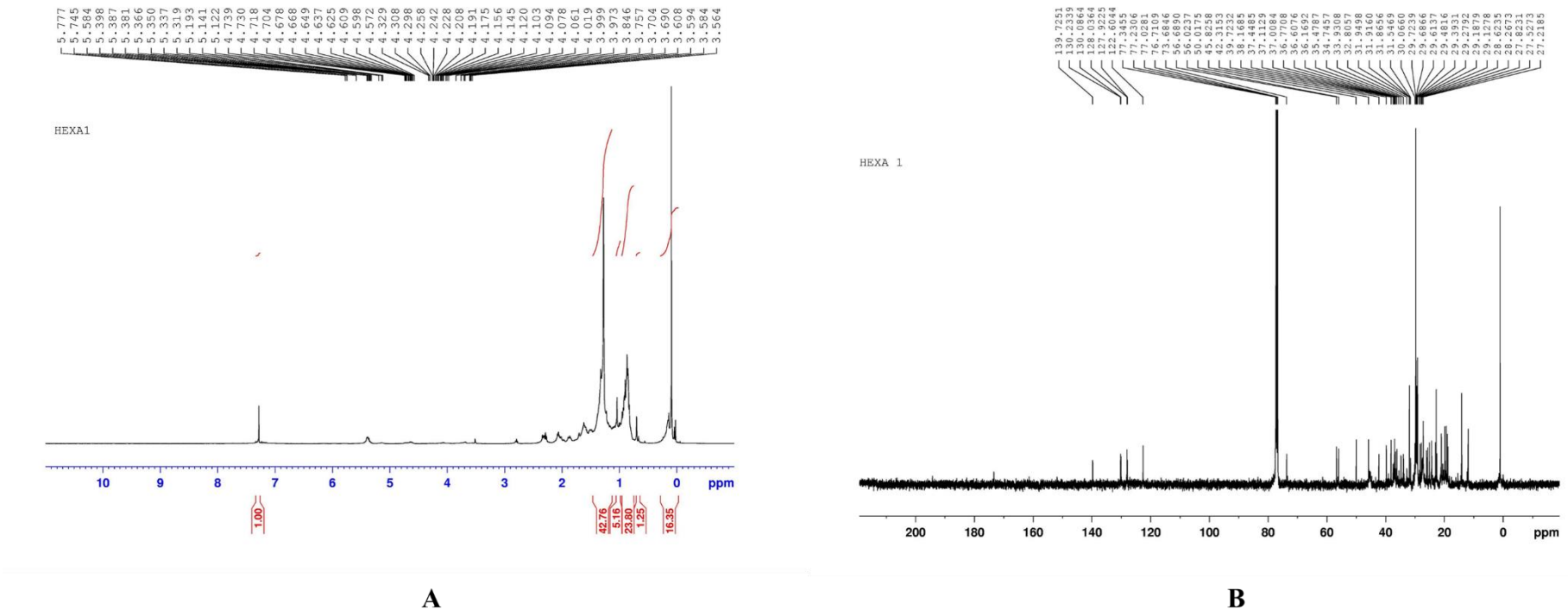
^{1}H NMR Spectrum (400 MHz, $CDCl_3$)

- Peaks falling within the range of 1.2-0.8 ppm (average 1 ppm) in ^{1}H NMR spectra are commonly associated with methane (-CH_3) moiety. Based on the integration results, 24 hydrogens were detected in the range of 0.95-0.79 ppm, which indicates the presence of eight CH_3 moieties (Figure 4.7A).
- Similarly, the signals observed in the range of 1.36-1.20 ppm correspond to 43 hydrogens, indicating the presence of 21 CH_2 moieties.
- Additionally, signals within the range of 6.0-4.5 ppm indicate the presence of double bonds [179]. Consequently, it is reasonable to suggest that molecules with double bonds might be present in the range of 5.42-5.33 ppm.

^{13}C NMR Spectrum (100 MHz, $CDCl_3$)

- In the ^{13}C NMR spectra, signals in the range of 39.7-18.8 ppm further confirm the presence of these hydrocarbons, as moieties such as -CH_3, -CH_2-, and -CH- typically fall within the 0-40 ppm range of the carbon NMR spectrum (Figure 4.7B).
- On the other hand, signals falling within the range of 110-140 ppm correspond to molecules containing -C=C- residues or aromatic hydrocarbons [180].

Although very small peaks indicating double bonds were observed in both ^{1}H and ^{13}C NMR, they did not show any fluorescence under both UV light. But the absorbance appeared after acid charring under UV-366 nm (Figure 4.6). Therefore, 1st band was accepted as primarily composed of **aliphatic hydrocarbons with minor unsaturation**, which could be moieties of fatty acid chains.



A **B**

Figure 4.7. Characterization of Hexane A1 fraction by (A) ^{1}H NMR and (B) ^{13}C NMR.

NMR characterization of the hexane A4 band

^{1}H NMR Spectrum (400 MHz, $CDCl_3$)

- Peaks in the range of 1.05-0.80 ppm indicate 13 hydrogens belonging to 4 CH_3 groups.
- Similarly, 63 hydrogens corresponding to 31 CH_2 groups were observed in the range of 1.50-1.20 ppm (Figure 4.8A).
- The 5 CH_2 residues containing 11 hydrogens were detected in between 1.75-1.57 ppm.
- The 15 hydrogens of 15 CH residues were found in the range of 2.17-2.00 ppm.
- Carboxyl (O=C-CH_3) groups generally present in between 2.6-2.0 ppm, and the integration in the range of 2.90-2.70 ppm indicated 7 hydrogens related to 2 carboxyl moieties.
- Peaks in the range of 6.0-4.5 ppm were characteristic of olefin (R-CH=CH_2) groups, and the integration in the range of 5.54-5.23 ppm indicated 15 hydrogens associated with 2 double bonds. Considering the four peaks related to double bonds, it was confirmed that there are two double bonds present in the linoleic acid structure [124].

However, the presence of two peaks in the 4.36-4.12 ppm range was observed in this ^{1}H NMR spectrum, but these peaks are not characteristic of linoleic acid and are not present in the typical linoleic acid spectra. These peaks belong to the glycerol moiety (as part of triglyceride), as explained by Barison, Pereira da Silva, Campos, Simonelli, Lenz and Ferreira [124].

^{13}C NMR Spectrum (400 MHz, $CDCl_3$)

- Peak signals in the range of 40-0 ppm are attributed to -CH_3, -CH_2-, and -CH- residues, indicating hydrocarbons present in the range of 39.7-18.8 ppm. However, the peaks detected in between 140-110 ppm are characteristic of aromatic hydrocarbons or molecules containing -C=C- residues, suggesting the presence of these groups in this spectrum (Figure 4.8B).

Based on the integration results from both ^{1}H NMR and ^{13}C NMR spectra, it is confirmed that the molecule in question is glycerol ester of linoleic acid ($C_{18}H_{32}O_2$). This conclusion is further supported by comparison with linoleic acid NMR spectra from the literature [181], which show remarkable similarity with the spectra obtained in this study (Figure 4.9).

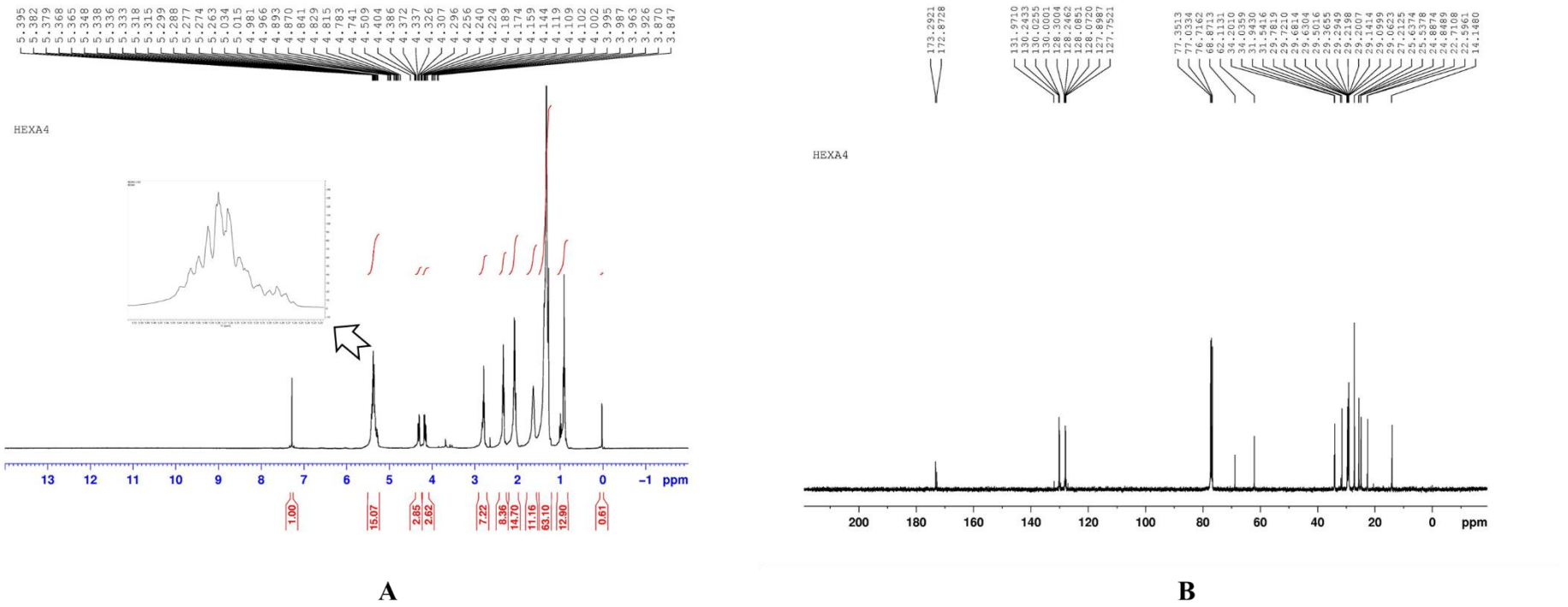


Figure 4.8. Characterization of Hexane A4 fraction by NMR (A) ^{1}H NMR and (B) ^{13}C NMR.

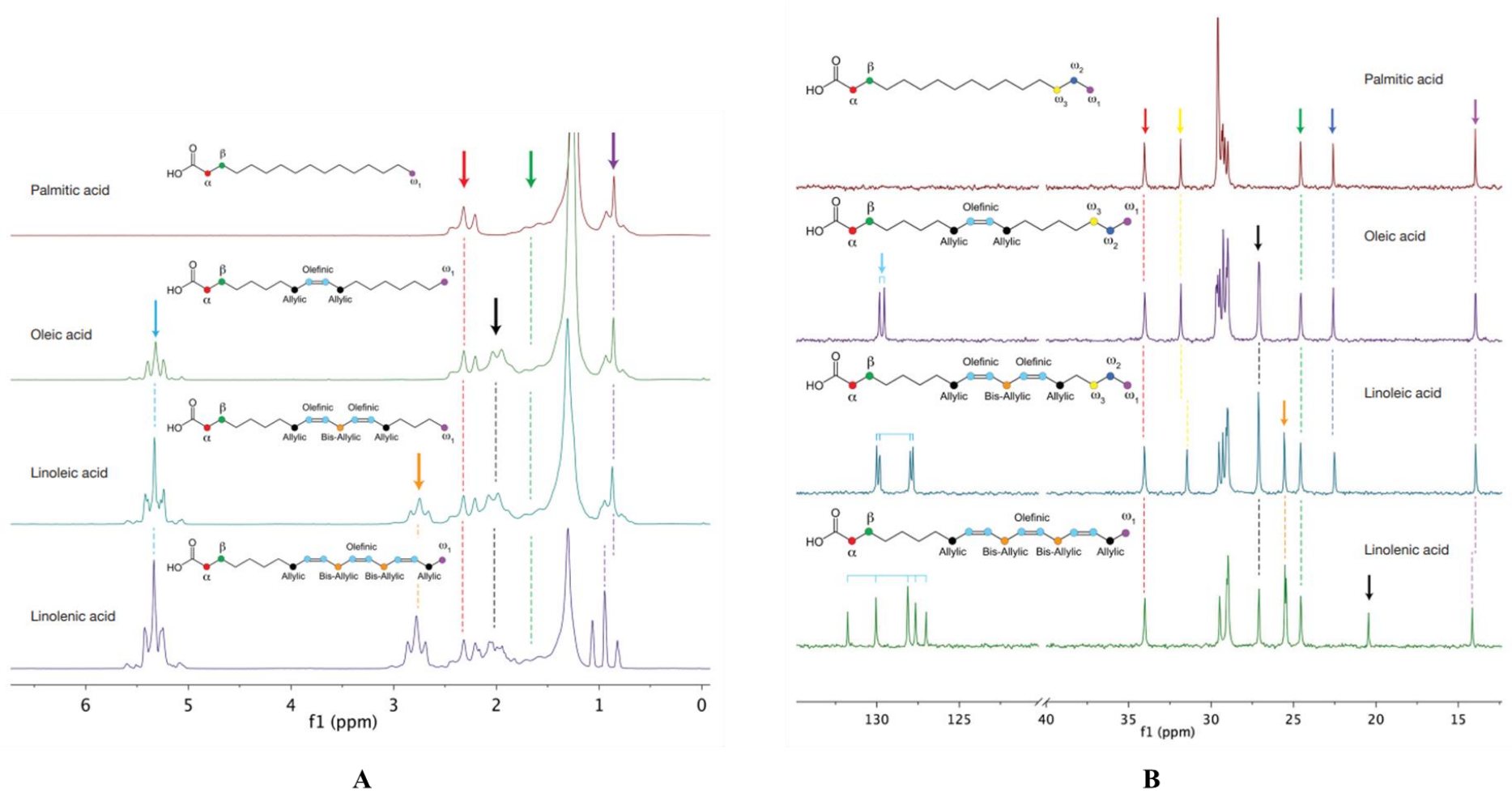


Figure 4.9. (A) ^{1}H NMR and (B) ^{13}C NMR spectra of basic fatty acids (adapted from [181]).

Purification and Identification of Hexane B and C Fractions

Figure 4.10 and Figure 4.12 exhibit the images of the fractions B and C, captured at wavelengths of 366 and 254 nm, respectively. Under UV light, fraction B exhibited 8 bands, while fraction C displayed 13 bands. These UV-active bands are suspected to contain double bonded or aromatic structures.

On the other hand, Figure 4.11 and Figure 4.13 represent fractions B and C after acid charring of PTLC plates. A detailed interpretation of the molecular structures could not be achieved due to the complexity of these bands and their overlapping nature. Therefore, identification of metabolites in these fractions was achieved with the help of GC-MS (Section 4.4.4.2 and Section 4.4.4.3), after derivatization (Figure 4.32 and Figure 4.33).

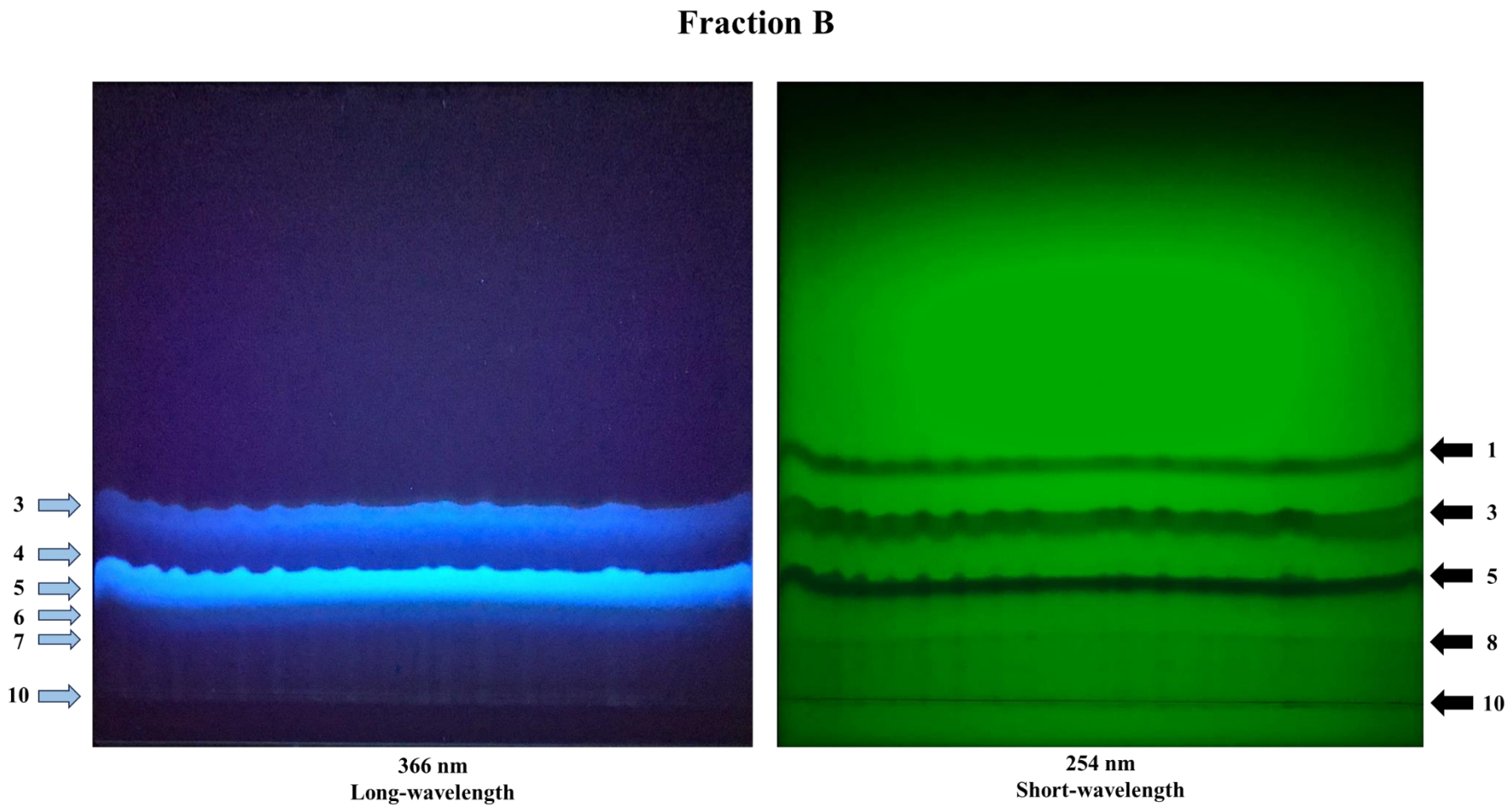


Figure 4.10. PTLC plate of fraction B (images captured under UV-366 nm and UV-266 nm).

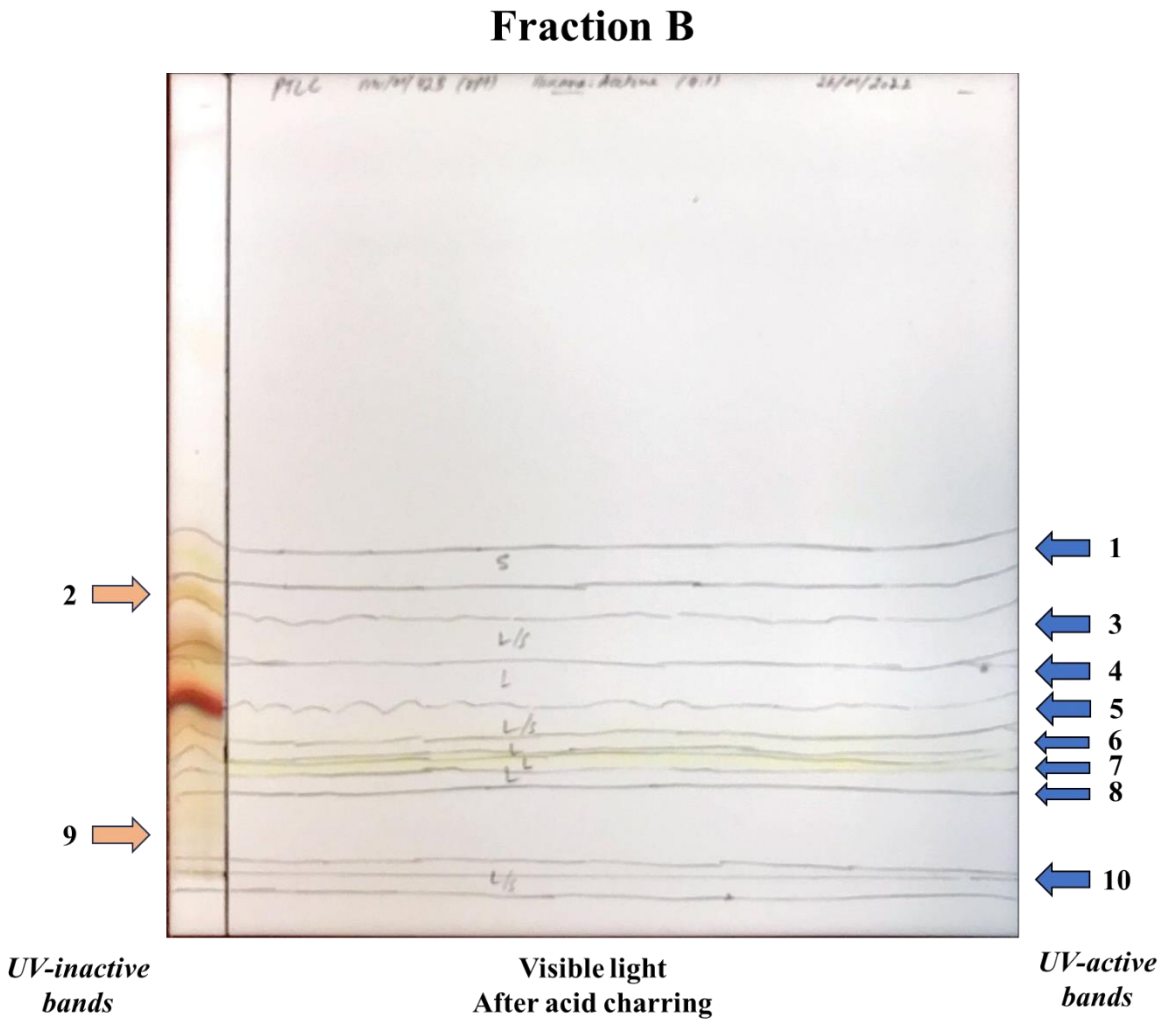


Figure 4.11. PTLC plate of fraction B (image captured under normal light after acid charring).

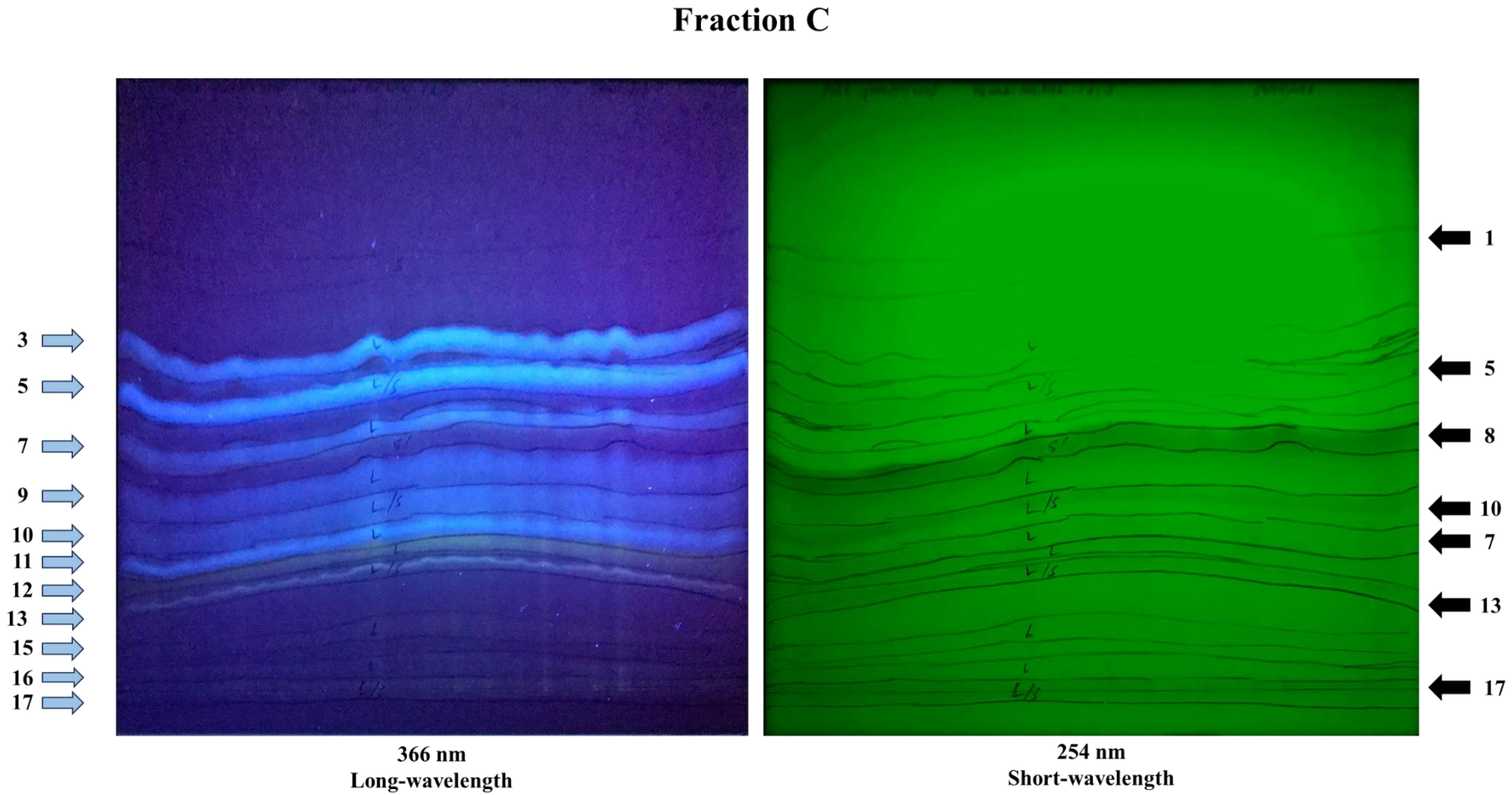


Figure 4.12. PTLC plate of Fraction C (images captured under UV-366 nm and UV-254 nm).

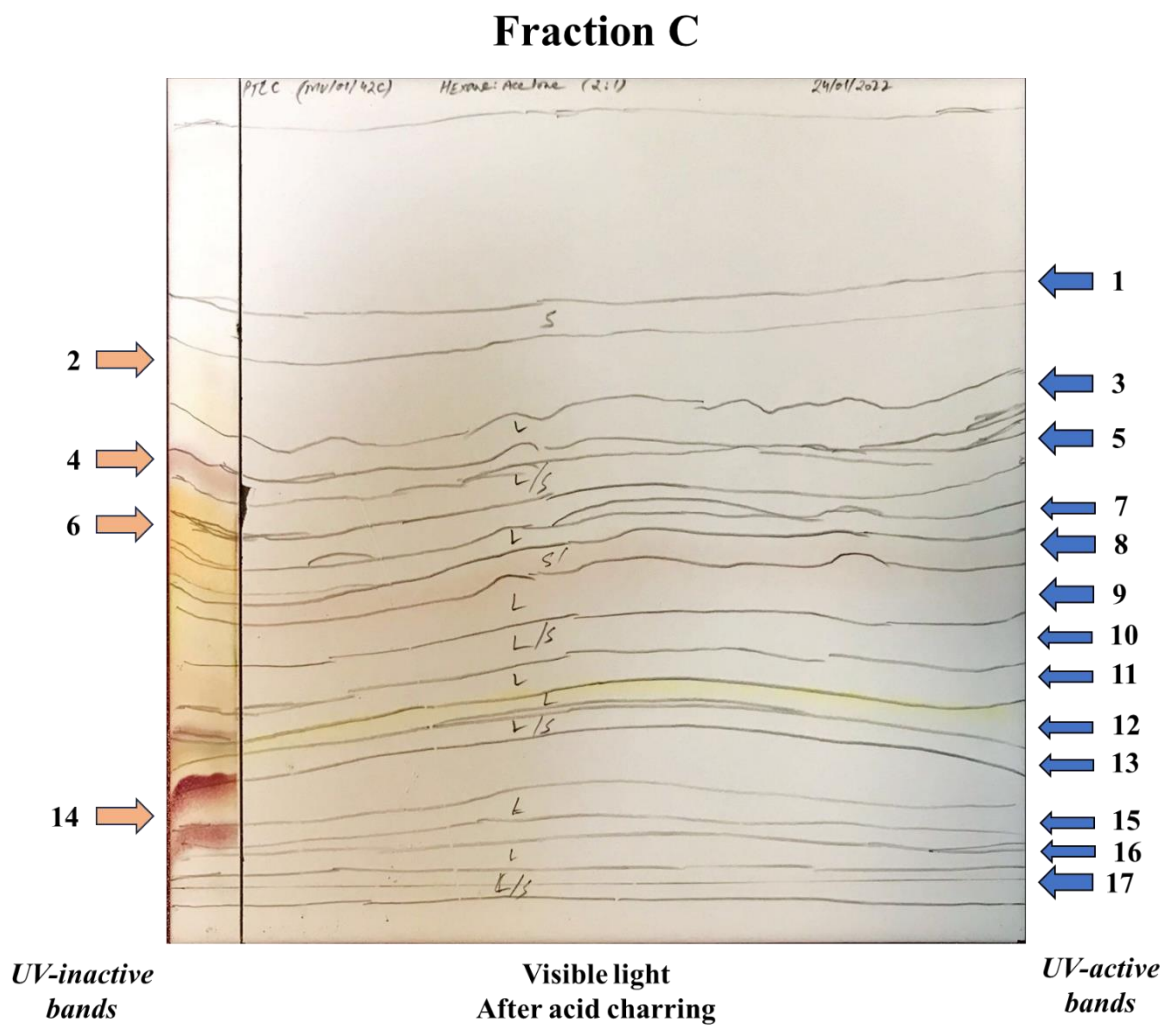


Figure 4.13. PTLC plate of Fraction C (image captured under normal light after acid charring).

4.4.2. Separation of methanol extract with chloroform

The crude methanol extract, along with chloroform-soluble and chloroform-insoluble extracts obtained from Section 3.8.2, underwent TLC using a chloroform-methanol solution (15:1, v/v) as the mobile phase. The fluorescence of mainly nonpolar metabolites was observed under UV-366 nm and UV-254 nm before acid charring (Figure 4.14). Although metabolites in these fractions exhibit some fluorescence under UV-366 nm and UV-254 nm, their emission becomes more prominent in both visible light and UV-366 nm after acid charring. This behavior may be explained by the appearance of UV-active metabolites after acid hydrolysis at high temperature.

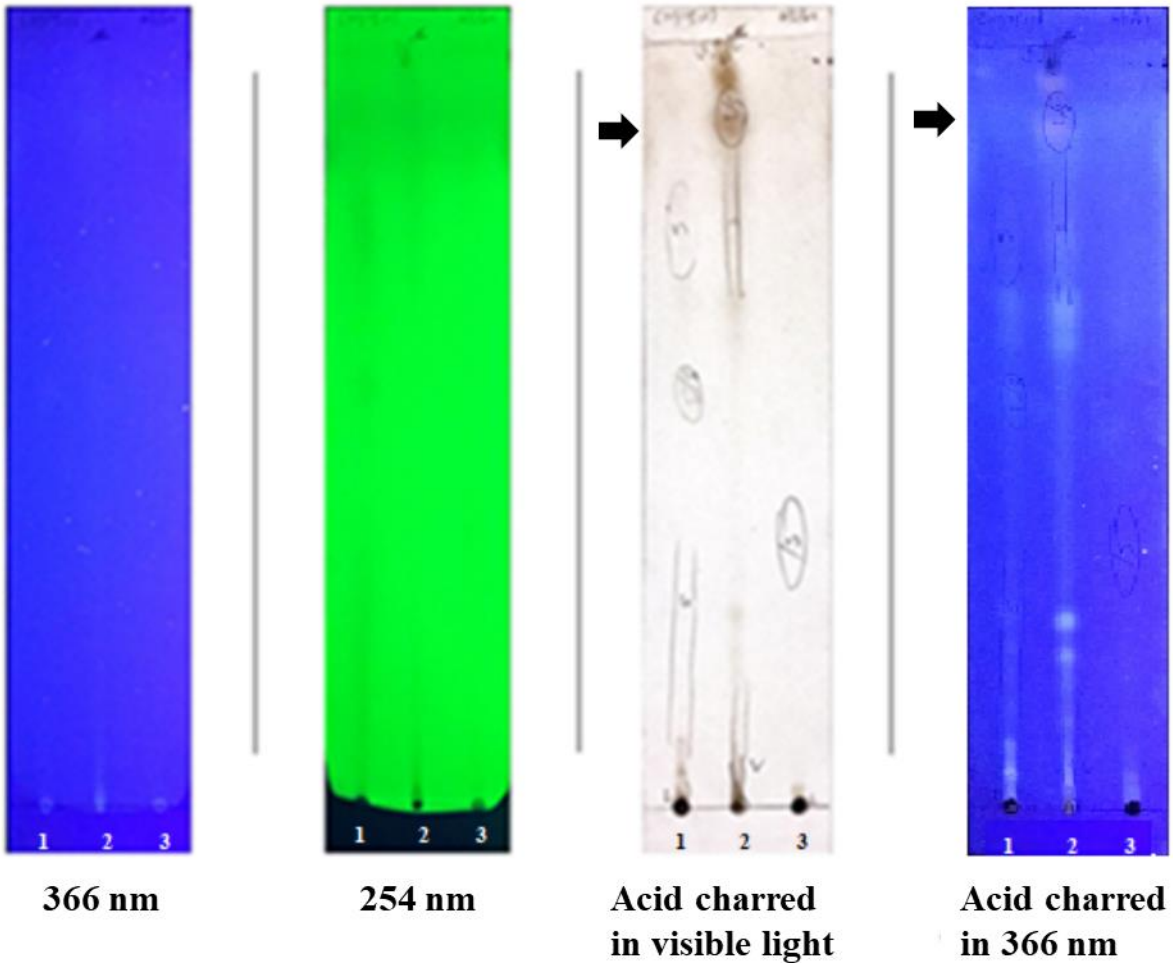


Figure 4.14. Separation of methanol extracts; (1) crude methanol extract, (2) chloroform soluble methanol extract and (3) chloroform insoluble methanol extract.

Crude methanol extract contains all metabolites with poor separation, as it can be seen on TLC plate (Figure 4.14). This separation indicated that major part of crude extract mainly consists of chloroform soluble compounds. As compared to TLC profile of crude methanol extract (at 1st position), it can be observed that the high concentration of chloroform in the mobile phase resulted in rapid separation of the chloroform soluble metabolites on TLC plate (at 2nd position).

However, the polar compounds in the chloroform-insoluble extract (at 3rd position) did not effectively separate, mainly due to the nonpolar nature of the mobile phase.

Based on TLC results, further isolation of chloroform-soluble extracts (non-polar metabolites) and chloroform-insoluble extracts (polar metabolites) was performed individually using MPLC columns filled with silica gel 60 and LiChroprep RP-18, respectively.

4.4.2.1. Purification and identification of chloroform soluble extract

Purification of chloroform soluble extract

In the chloroform-soluble extract of the Sünter variety, all metabolites were isolated and purified with the help of MPLC, TLC, and PTLC Chromatography as previously explained in Section 3.8.2.1. Initially, the extract was subjected to MPLC using a silica gel column, resulting in the separation of the mixture into 24 fractions. Then, collected fractions were individually applied to TLC plates to further analyze and evaluate their composition and purity (Figure 4.15).

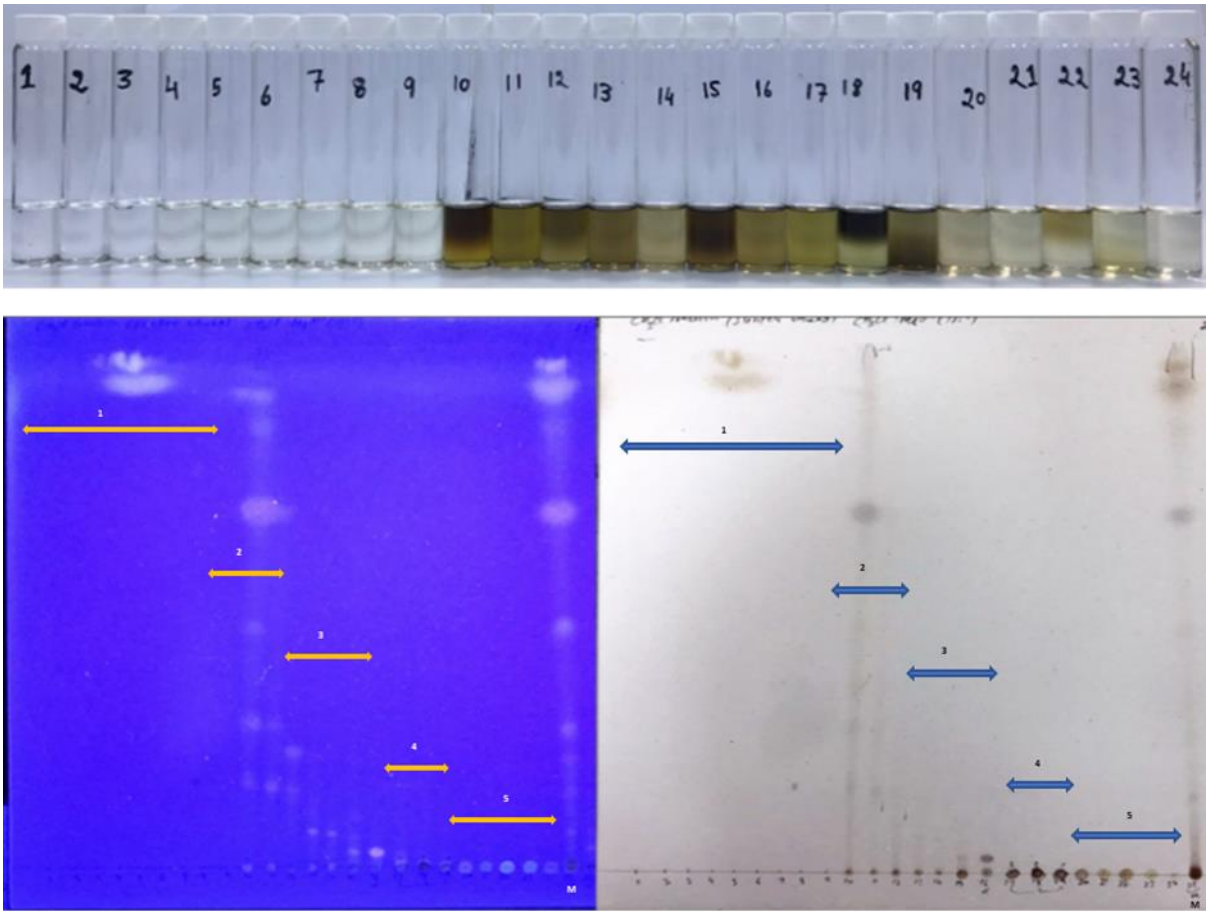


Figure 4.15. (a) Fraction collected from MPLC (b) TLC images of separated fractions (images captured under UV-366 nm and normal light after acid charring).

As a result of TLC, 24 fractions were combined into 5 groups based on their Rf values. The first group exhibits nearly identical Rf values and consists of relatively pure molecules. In contrast, the second group is composed of a highly complex mixture of molecules, mostly indicating non-polar compounds, as they exhibited high Rf values. The third group also contained a mixture of polar molecules with low Rf values. Similarly, the fourth and fifth groups have a few polar molecules. These groups were subsequently combined into 5 major fractions, and their separation on TLC was repeated to get better resolution (Figure 4.15). The TLC plates were examined under UV-366 nm and visible light after acid charring, and the observations for all five fractions mentioned above remain unchanged and were subsequently characterized by NMR.

NMR characterization of chloroform 1 fraction

The Chloroform 1 fraction was considered pure enough according to its TLC profile; thus, it was directly characterized by NMR.

^{1}H NMR Spectrum (400 MHz, $CDCl_3$)

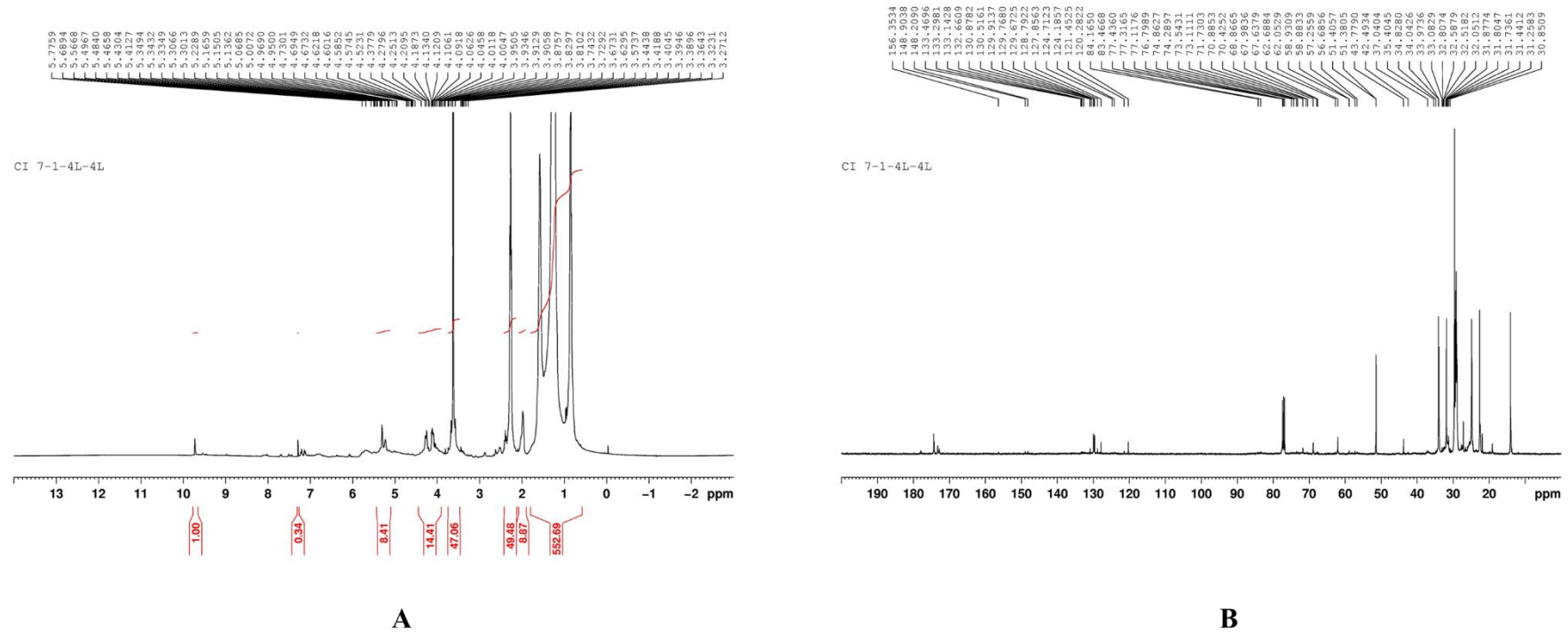
- In the ^{1}H NMR spectrum, two singlet signals were observed: one at δ 9.72 ppm and the other at δ 7.28 ppm. These signals are characteristic of aromatic CH atoms, indicating the presence of an aromatic structure in the compound (Figure 4.16A).
- Additionally, a group of signals was detected in the spectrum at δ 5.40-5.17 ppm. Such signals are typically associated with double bonds, suggesting the presence of an alkene group in the solute compound. This structure may indicate the presence of a fatty acid containing double bonds. Two peaks at 4.30-4.05 ppm were observed, and it is considered that these peaks belong to glycerol backbone [124].
- The singlet peak at 3.63 ppm is attributed to the NH_3 group, which is further confirmed by the value of 3.6 ppm reported in the ^{1}H NMR spectrum of NH_3 peak in the relevant literature [182, 183]. Amiel, Tremblay-Franco, Gautier, Ducheix, Montagner, Polizzi, Debrauwer, Guillou, Bertrand-Michel and Canlet [184] also demonstrated in their ^{1}H NMR study that this peak belongs to **phosphatidylethanolamine**.

^{13}C NMR Spectrum (100 MHz, $CDCl_3$)

1. In the ^{13}C NMR spectrum (Figure 4.16B), it was confirmed by the literature that the signal in the range of δ 174.3-174.1 belongs to the carbonyl (C=O) group [180].
2. Additionally, signals in the 130-129 range were assumed to belong to double bonded structures, likely representing fatty acids [178].

3. The signal at 51.4 was thought to belong to the CH or CH_2O groups.
4. Moreover, signals in the 40-0 range were associated with $-CH_3$, $-CH_2-$ residues, and fatty acids, as they exhibited significant similarity with the ^{13}C NMR spectrum of linoleic acid, which was already confirmed in whole hexane extract.

The presence of **phosphatidylethanolamine diacylglycerols (phospholipids)** in the chloroform 1 fraction was characterized with NMR spectra. This finding was further supported by the GC-MS results of the crude chloroform-soluble extract (Section 4.4.4.4). The GC-MS results indicate the presence of ethanolamine, phosphoric acid esters, and myo-Inositol (Figure 4.34). Gonzalez-Thuillier, Salt, Chope, Penson, Skeggs, Tosi, Powers, Ward, Wilde and Shewry [185] have also reported the presence of polar lipids such as phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylinositol in different milling fractions of wheat.



A **B**

Figure 4.16. Characterization of chloroform 1 fraction by (A) ^{1}H NMR and (B) ^{13}C NMR.

NMR characterization of chloroform 2 fraction

The chloroform 2 fraction was first purified on a PTLC plate using a chloroform-methanol solvent mix. Then the marked area on PTLC (Figure 4.18) was carefully scraped from plate and characterized by NMR.

^{1}H NMR Spectrum (400 MHz, $CDCl_3$)

- In the ^{1}H NMR spectrum of the compound, a single signal was observed at δ 5.38 ppm, which typically corresponds to double bonds, indicating the presence of an alkene group in the compound (Figure 4.17A). However, peak signals between 3.85-3.64 ppm are known to be characteristic of sugars according to the literature [186].
- The signals at 2.45-2.18 ppm represent the R-CO-CH_3 group. Additionally, a set of signals at 2.11-1.94 and 1.92-1.78 ppm corresponds to CH groups, while signals at 1.47-1.17 ppm are attributed to CH_2 groups. The signal at 0.69 ppm is known to belong to the CH_3 group. The spectra obtained in the range of 2.45-0.69 ppm may indicate the presence of **aliphatic fatty acid chains**.

^{13}C NMR Spectrum (100 MHz, $CDCl_3$)

- In the ^{13}C NMR spectrum, signals in the range of δ 80-10 ppm are considered to belong to -CH, -CH_3, and -CH_2- residues, suggesting the presence of **aliphatic fatty acid chains** (Figure 4.17B).

A study by Rech-Cainelli, de Barros, Garcia-Gianni, Sbeghen-Loss, Heinzen, Díaz, Migues, Specht and Cesio [186] demonstrated that the proton and carbon NMRs of the fatty acid disaccharide ester in a natural plant extract (Figure 4.18), which bear close resemblance with the NMRs obtained for the chloroform 2 fraction. Based on this similarity, it is suggested that the molecule in this fraction is a **fatty acid sugar ester**. Additionally, the GC-MS results of chloroform soluble extract (Section 4.4.4), indicated the presence of both octadecanoic acid (stearic acid) and various sugars.

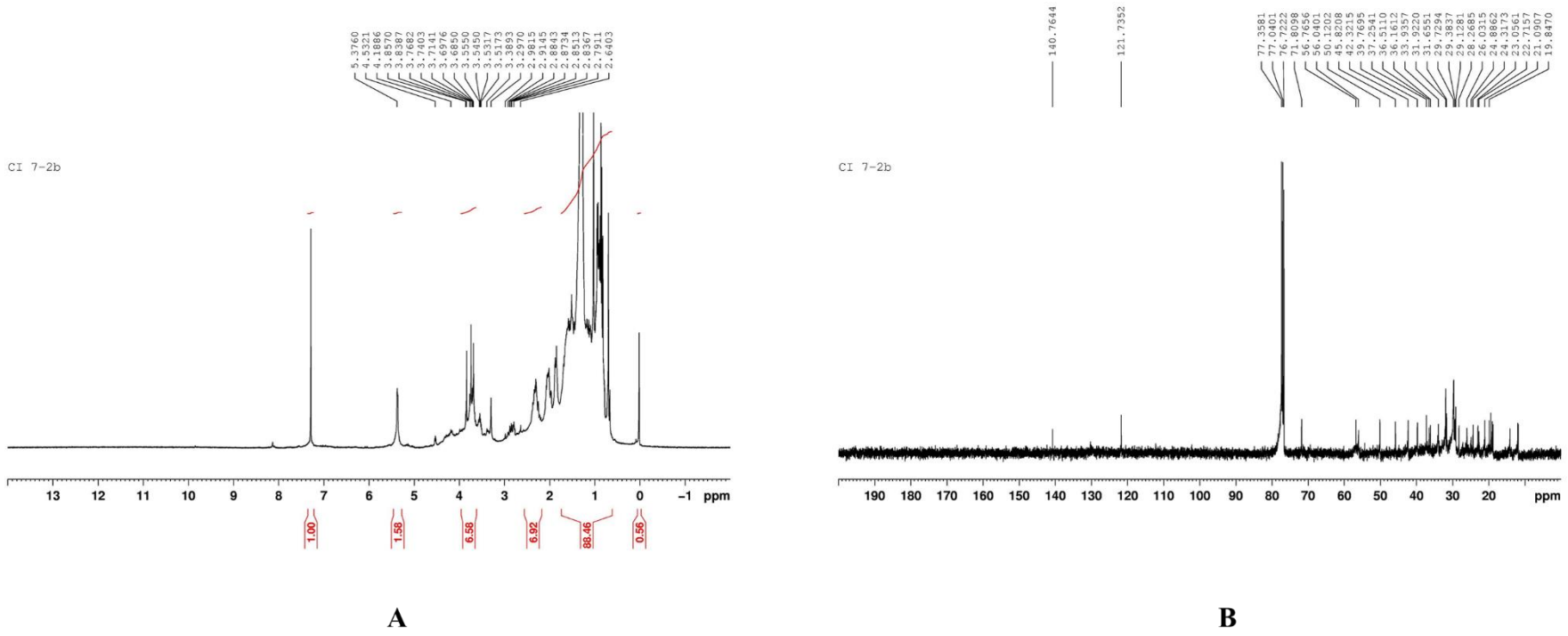
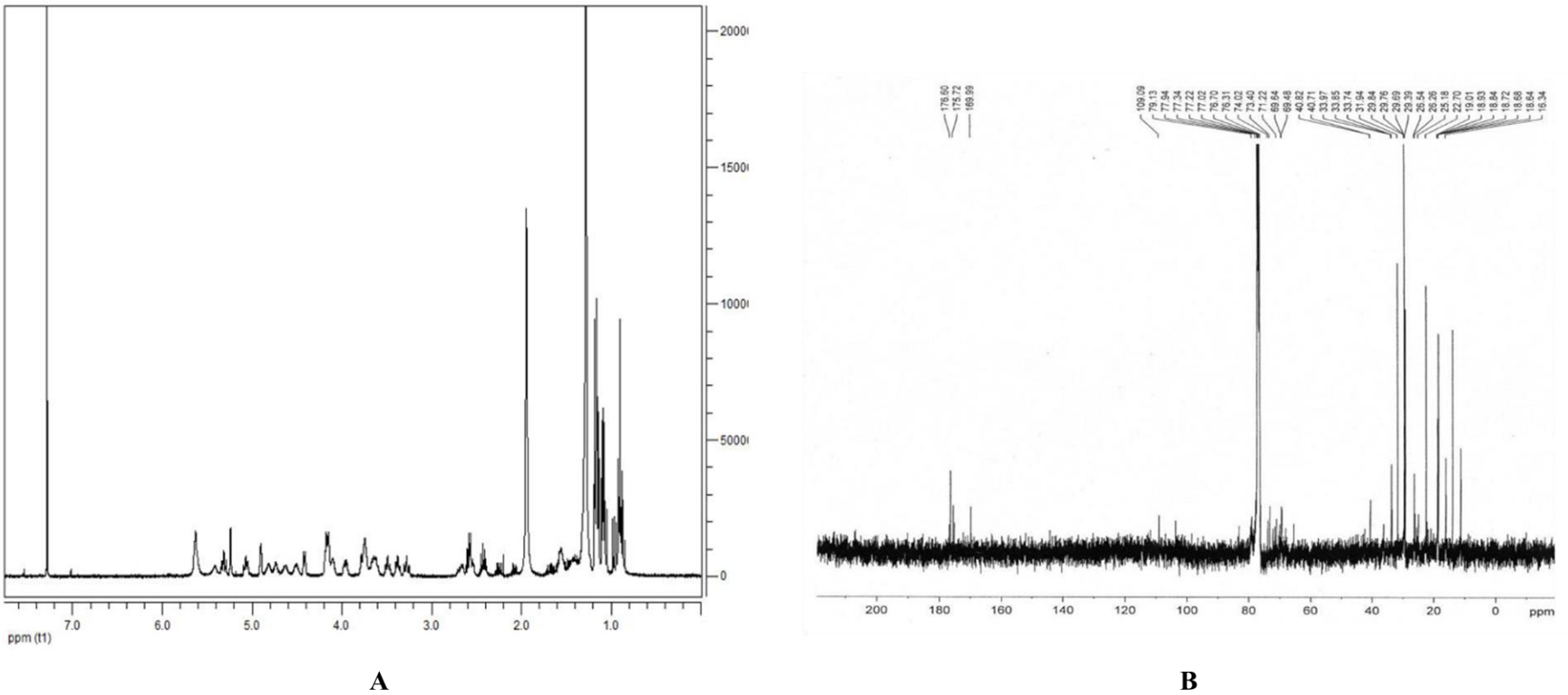


Figure 4.17. Characterization of chloroform 2 fraction by (A) ^{1}H NMR and (B) ^{13}C NMR.



A **B**

Figure 4.18. NMR of fatty acid sugar ester (A) ^{1}H NMR and (B) ^{13}C NMR (adapted from [186]).

NMR characterization of the combined chloroform 3 & 4 fractions

The combined fraction (consisting of 3 & 4 fractions) was subjected to extraction with water to separate the non-polar (NP) and polar (P) metabolites. Separation of these metabolites can be observed on TLC plate (Figure 4.19). NP had at least four different metabolites, but their separation was not successfully achieved. On the other hand, polar metabolites were not isolated by TLC due to their relatively pure nature. Therefore, this polar subfraction was characterized using NMR spectroscopy.

Figure 4.19. TLC analysis of subfractions of combined chloroform 3 and 4 fractions (NP: non-polar and P: polar metabolites).

The **^{1}H NMR Spectrum (400 MHz, D_2O)** and **^{13}C NMR Spectrum (100 MHz, D_2O)** spectra (Figure 4.20A-B) of this fraction exhibit similarities to the NMR spectra obtained for **sugar alcohols (myo-inositol and arabitol)**, as reported in the literature for analytical standards [187]. Additionally, the presence of myo-inositol and arabitol was confirmed in the GC-MS analysis results for entire chloroform soluble fraction (Section 4.4.4.4).

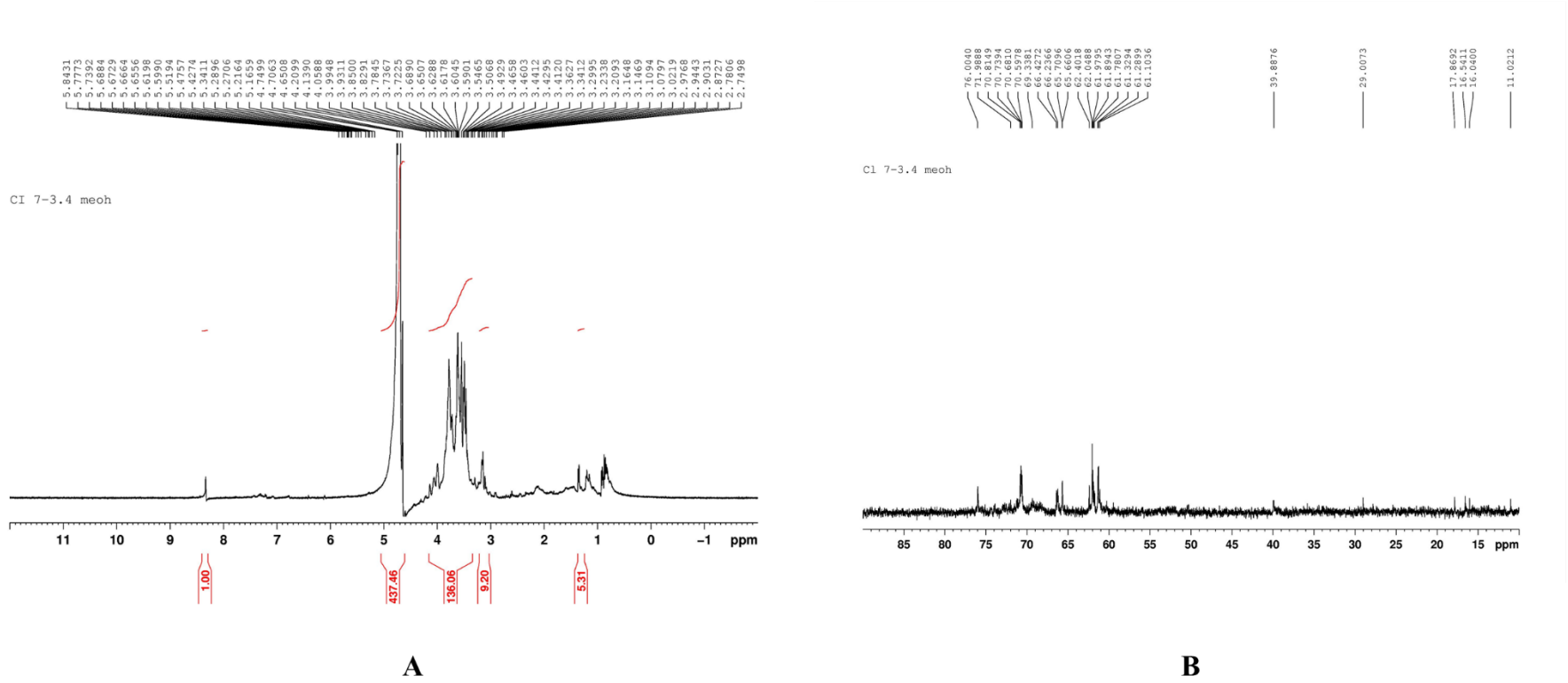
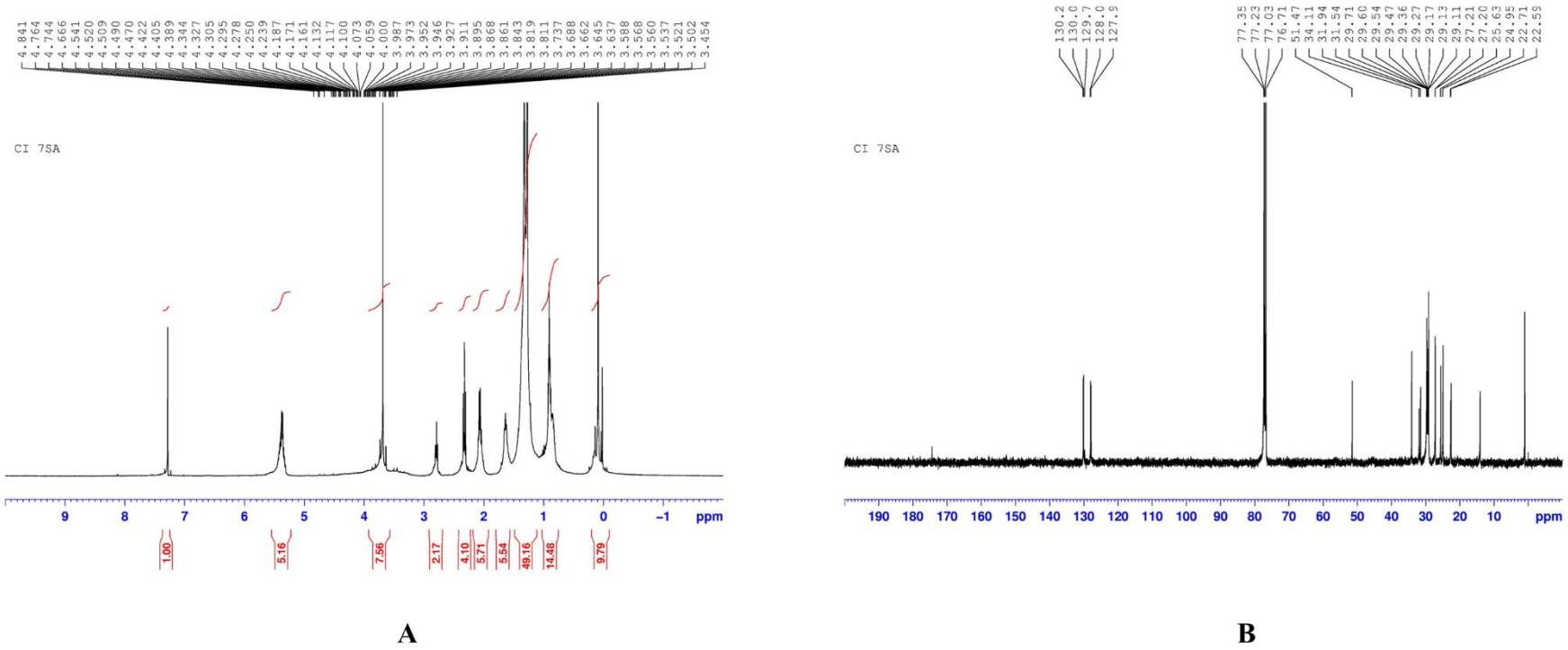


Figure 4.20. Characterization of polar metabolites in chloroform 3&4 fractions by (A) ^{1}H NMR and (B) ^{13}C NMR.

NMR characterization of chloroform 5 fraction

Chloroform 5 fraction was washed with the chloroform-water mixture (Section 3.8.2.2). Subsequently, two subfractions were isolated through this process: the chloroform-soluble subfraction (non-polar) and the water-soluble (polar) subfraction. The non-polar subfraction appeared to be relatively pure in comparison to the polar one. Consequently, it underwent further characterization using NMR analysis.

The **^{1}H NMR Spectrum (400 MHz, $CDCl_3$)** and **^{13}C NMR Spectrum (100 MHz, $CDCl_3$)** spectra (Figure 4.21A-B) obtained for chloroform soluble subfraction of non-polar metabolites were found to be very similar to the spectra obtained for Chloroform 1 fraction (Figure 4.16). Therefore, it was expected that this metabolite may belong to the class of phospholipids. The ^{1}H NMR and ^{13}C NMR spectra of the non-polar subfraction (Chloroform 5) were then compared with the spectra of L-alpha-Phosphatidylcholine from the Biological Magnetic Resonance Databank (Figure 4.22). The matching spectra strongly indicated that the metabolite present in our subfraction was **L-α-Phosphatidylcholine** [187].



A **B**

Figure 4.21. Characterization of non-polar metabolites in chloroform 5 fraction by (A) ^{1}H NMR and (B) ^{13}C NMR.

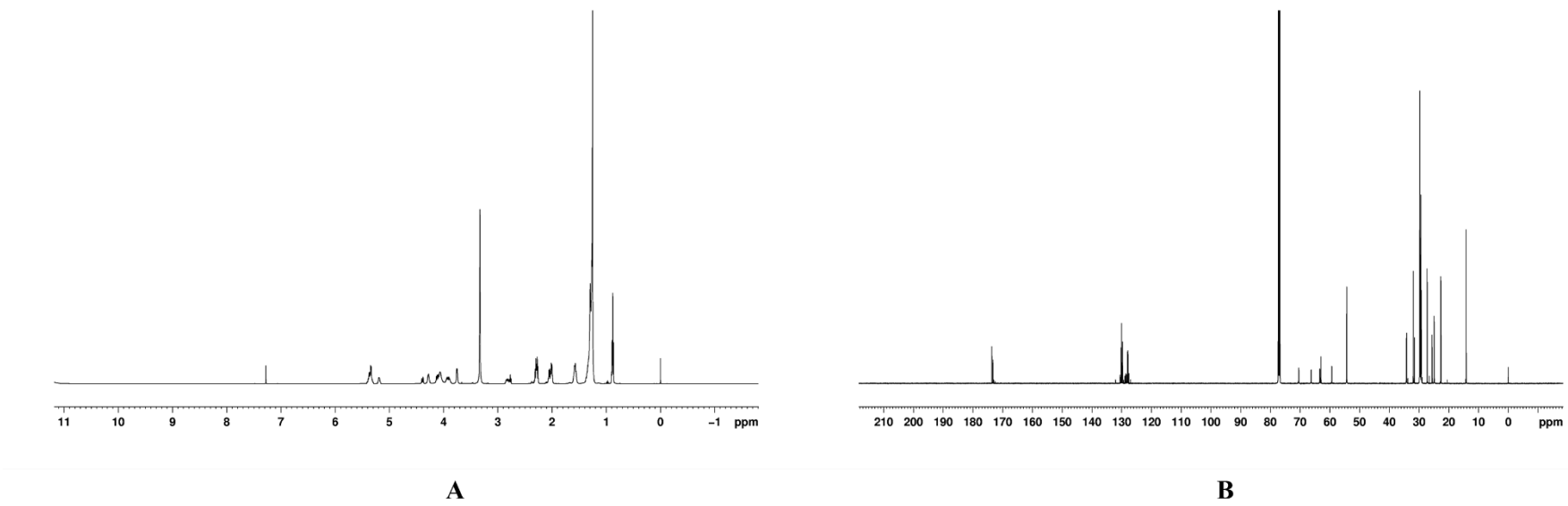


Figure 4.22. (A) ^{1}H NMR and (B) ^{13}C NMR spectra of standard L-α-Phosphatidylcholine [187].

4.4.2.2. Purification and identification of chloroform insoluble extract

Purification of chloroform insoluble extract

Polar metabolites in the chloroform-insoluble extract of the Sünter wheat variety were purified using a combination of MPLC (RP-18), TLC, and PTLC chromatography. To achieve this, the extract was initially applied to an MPLC column containing LiChroprep RP-18. Subsequently, twenty-four fractions were collected. These fractions were applied separately to a TLC plate and examined under UV-366 nm and 254 nm light, but no band appeared. However, some of the bands exhibited fluorescence at UV-366 nm light after acid charring. As a result, the UV-active fractions (UV-366) were combined and collected into seven main fractions (A-G) (Figure 4.23). The B, D and F fractions were chosen for further purification due to the presence of several molecules in the TLC profile.

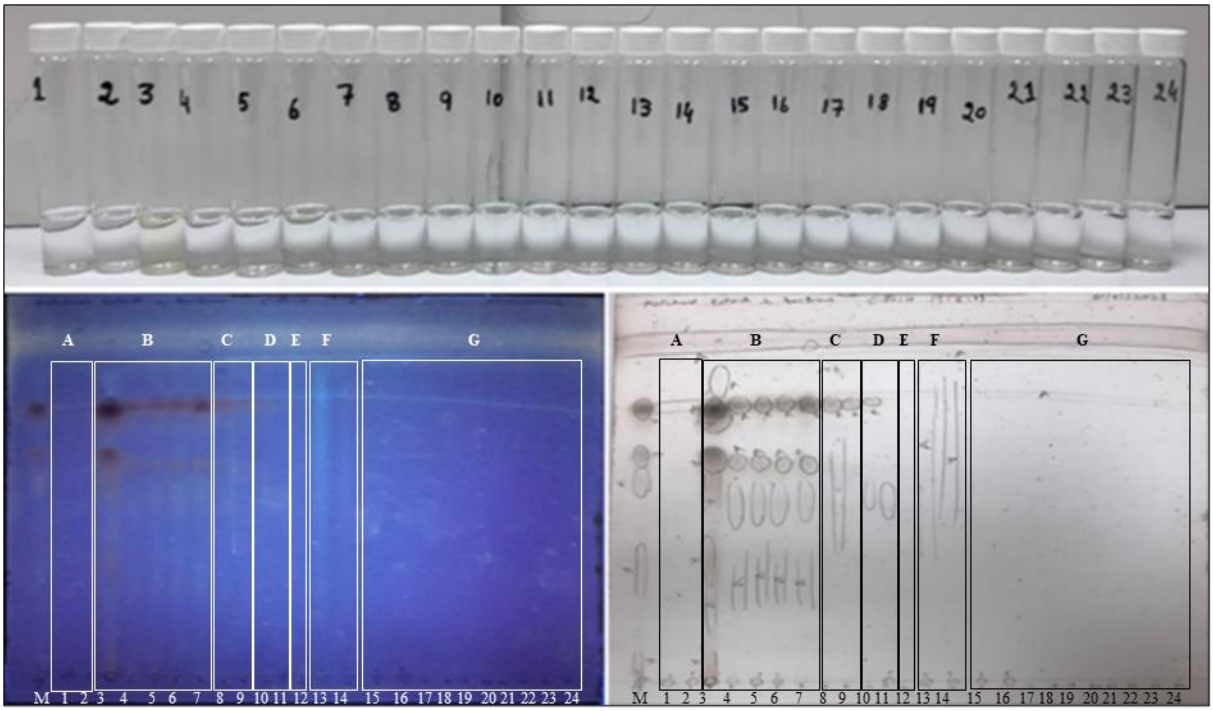


Figure 4.23. TLC of fractions obtained from chloroform-insoluble extract (images captured under UV-366 nm and normal light after acid charring).

The **chloroform-insoluble fraction B** was applied to PTLC plate, and nine distinct bands were observed. Among these bands, the third band displayed a comparatively brighter appearance than the remaining bands (Figure 4.24). Due to high UV-activity of this band, it was scrapped from the plate and characterized by NMR analysis.

Figure 4.24. TLC analysis of chloroform-insoluble B fraction (images captured under UV-366 nm and normal light after acid charring).

NMR characterization of chloroform insoluble B fraction (3rd band)

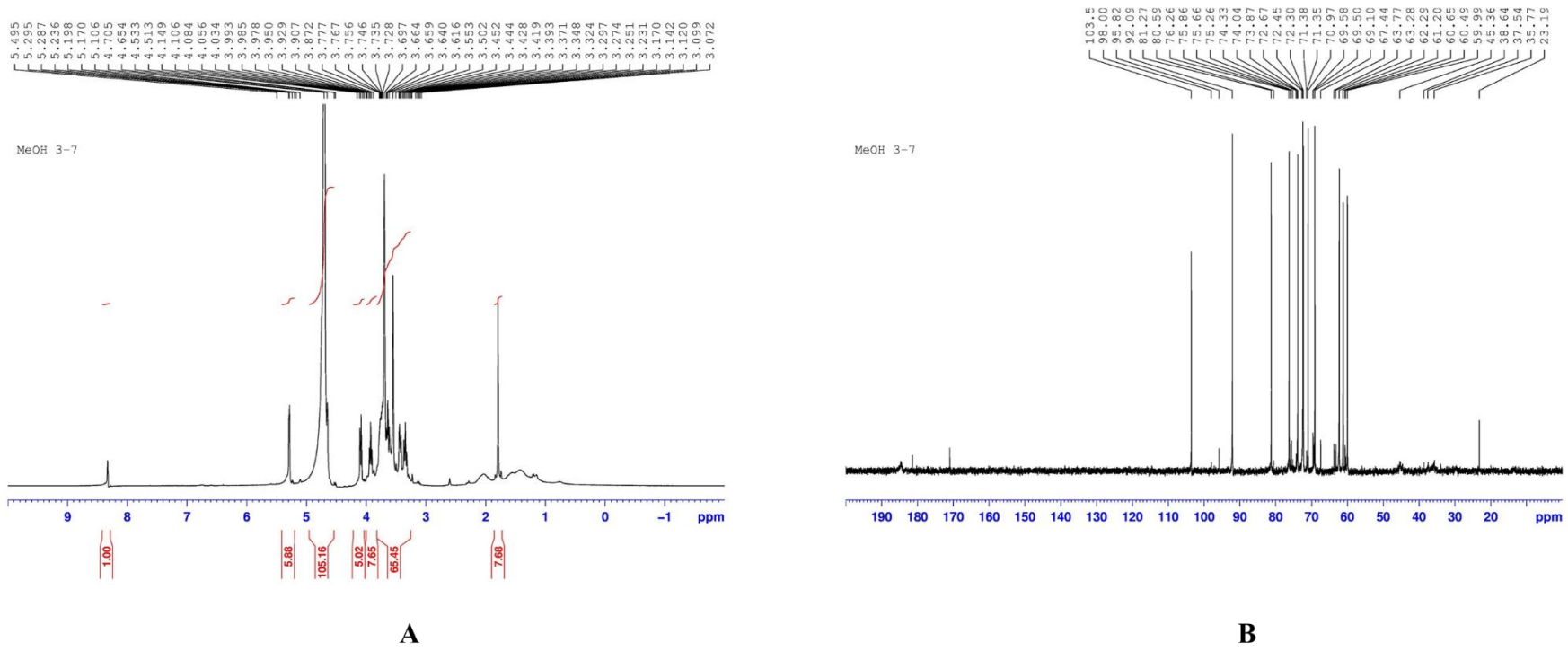
^{1}H NMR Spectrum (400 MHz, D_2O)

In the ^{1}H NMR spectrum (Figure 4.25A), peak signal at 4.7 ppm belongs to D_2O solvent. Other peak signals between 4.09-3.34 ppm correspond to sugars molecules (**sucrose**), which have been confirmed in literature [188].

^{13}C NMR Spectrum (100 MHz, D_2O)

Furthermore, in the ^{13}C NMR spectrum (Figure 4.25B), it is documented in the literature that the signals in the range of 103.6-60.0 ppm belong to the sucrose molecule [189]. This confirms the presence of **sucrose** ($C_{12}H_{22}O_{11}$) in the sample under analysis.

Additionally, the ^{1}H and ^{13}C NMR spectra of this metabolite (Figure 4.26) were found to be quite similar to the spectra of the standard sucrose molecule [187].



A **B**

Figure 4.25. Characterization of the chloroform insoluble B-3 sub-fraction by (A) ^{1}H NMR and (B) ^{13}C NMR spectra.

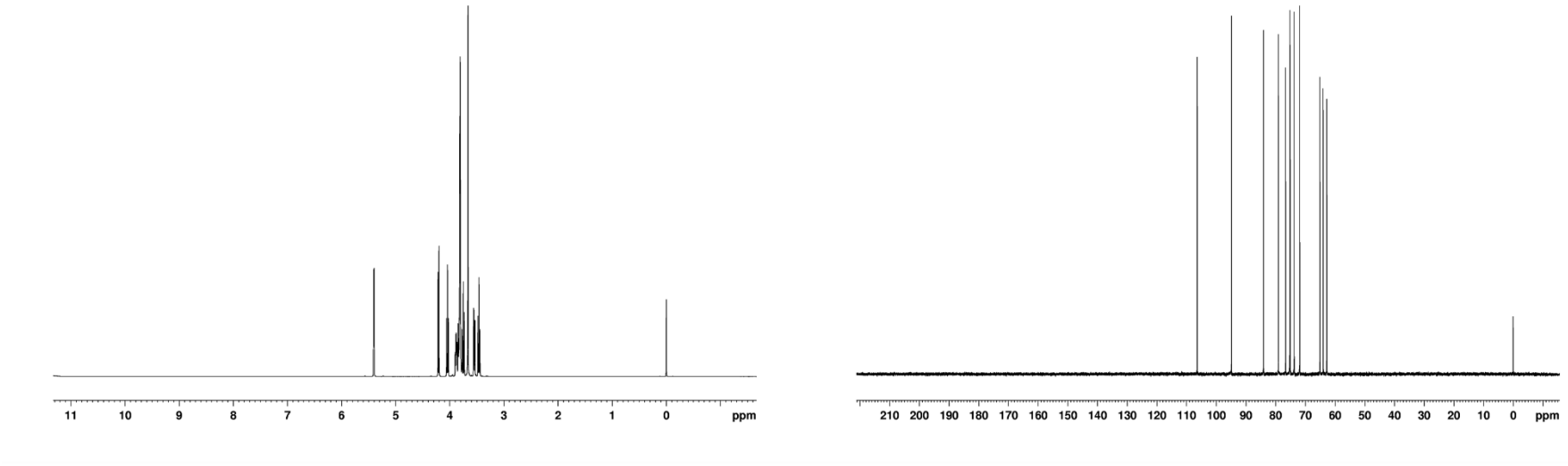


Figure 4.26. (A) ^{1}H NMR and (B) ^{13}C NMR spectra of standard sucrose [187].

The **chloroform insoluble fraction D** was also applied to PTLC plate, and 7 bands were obtained. Subsequently, 4 to 7 bands were scrapped from the plate and (Figure 4.27) and combined before NMR characterization.

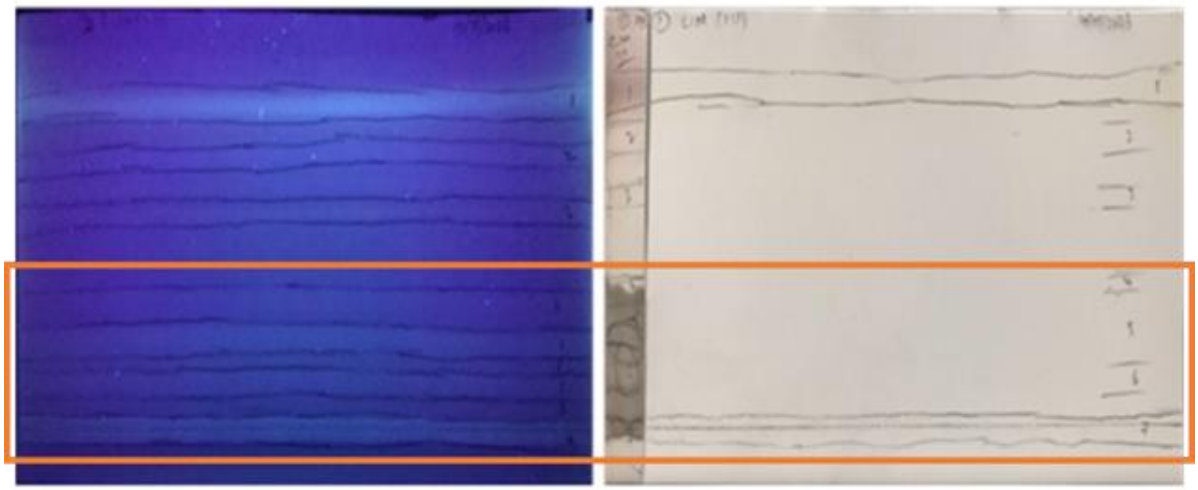


Figure 4.27. TLC analysis of chloroform-insoluble D fraction (images captured under UV-366 nm and normal light after acid charring).

NMR characterization of the 4-7 band of the chloroform insoluble D fraction

^{1}H NMR Spectrum (400 MHz, D_2O)

In the ^{1}H NMR spectra (Figure 4.28A), the obtained profiles have resemblance to those of sugar molecules. It has been confirmed by the literature that the peak signals between 4.09-3.34 ppm correspond to glucose and its derivatives [190].

^{13}C NMR Spectrum (100 MHz, D_2O)

Apart from the signals at 53.3 and 38.6 ppm in the ^{13}C NMR spectrum (Figure 4.28B), the rest of the profile is identical to the carbon NMR of glucose ($C_6H_{12}O_6$) [189].

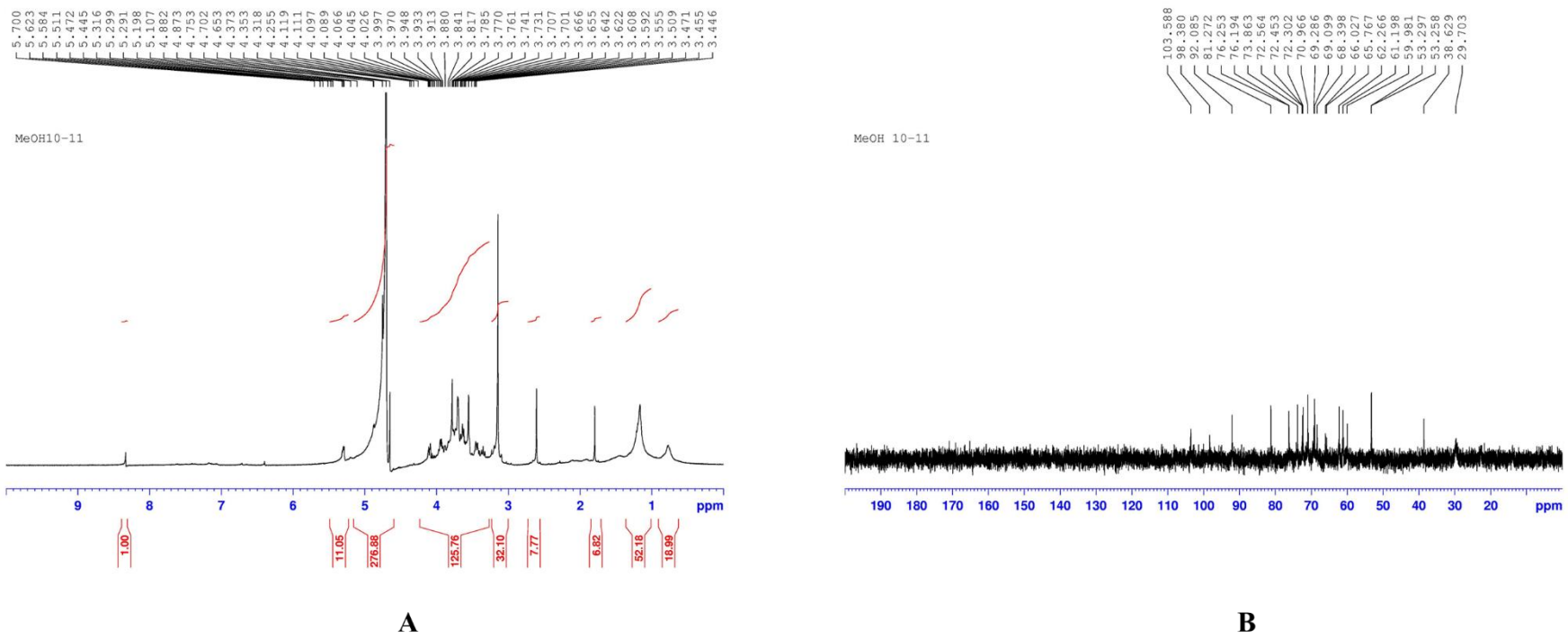


Figure 4.28. Characterization of 4-7 subfraction of the chloroform insoluble D fraction by (A) ^{1}H NMR and (B) ^{13}C NMR spectra.

Proton and carbon NMR characterization of F fraction insoluble in chloroform

A pure molecule was observed in the TLC layer for the chloroform insoluble F fraction, which was combined mix of 13 & 14 MPLC fractions (Figure 4.29). Therefore, this fraction was subjected to NMR without further purification.

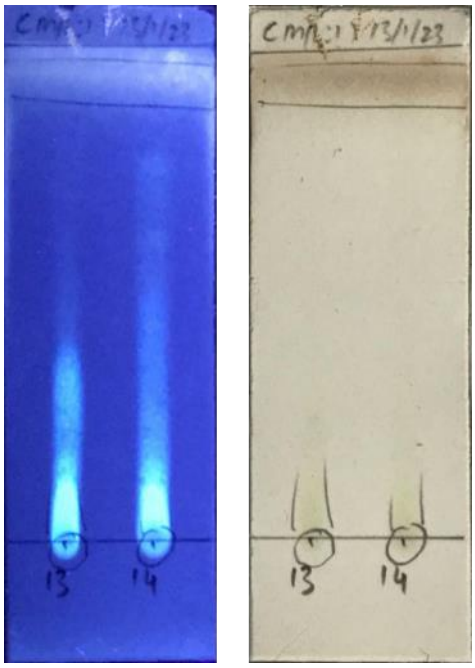


Figure 4.29. TLC analysis of chloroform insoluble F fraction (images were captured under UV-366 nm and normal light after acid charring).

^{1}H NMR Spectrum (400 MHz, D_2O)

In the ^{1}H NMR spectrum (Figure 4.30A), the profile between 4.50-3.20 ppm is indicative of sugars. However, upon comparison with the peaks present in the malic acid spectrum found in the literature, it is assumed that the peaks at 4.35 and 2.6 may correspond to malic acid ($C_4H_6O_5$) [191].

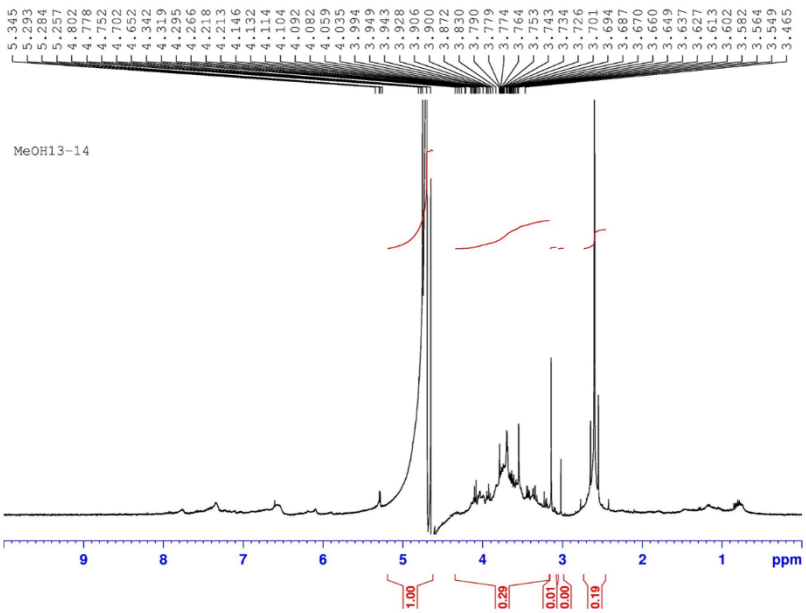


Figure 4.30. Characterization of the chloroform insoluble F fraction by ^{1}H NMR.

4.4.3. Results for ^{1}H and ^{13}C NMR characterization of purified isolates

All purified isolates obtained from hexane, chloroform soluble and chloroform insoluble extracts were characterized by using both ^{1}H and ^{13}C NMR. Here, Table 4.12 and 4.13 represent the distribution as well as differences among individual or group of peaks for ^{1}H and ^{13}C NMR spectra.

Table 4.12. ^{1}H NMR (400 MHz) characterization data (δ) for purified isolates.

No.	Hexane A1	Hexane A4	Chloroform soluble 1	Chloroform soluble 2	Chloroform soluble 3&4	Chloroform soluble 5	Chloroform insoluble B	Chloroform insoluble D	Chloroform insoluble F
1			**9.72** (s, 1H)		**8.35-8.32** (s, 1H)				
2	**7.28** (s, 1H)	**7.26** (s, 1H)	**7.28** (s, 1H)	**7.28** (s, 1H)		**7.28** (s, 1H)			
4	**5.42-5.33** (m, 1H)	**5.54-5.23** (m, 15H)	**5.40-5.17** (m, 8H)	**5.38** (s, 1H)		**5.45-5.30** (m, 5H)	**5.30-5.28** (m, 6H)	**5.30-5.28** (m, 11H)	
6		**4.36-4.27** (d, J=4 Hz, 3H)	**4.30-4.07** (m, 14H)		**4.71** (s, 437H)				**4.50-3.20** (1H, m)
7		**4.20-4.12** (d, J =4 Hz,					**4.09** (d, J=8Hz, 5H)	**4.09** (d, J=8Hz, 10H)	
8							**3.93** (t, J=8Hz, 8H)	**3.93** (t, J=8Hz, 8H)	
9							**3.70** (s, 45H)	**3.70** (s, 45H)	
10							**3.66 -3.58** (m, 5H)	**3.66 -3.58** (m, 5H)	
11				**3.85-3.82** (m, 2H)	**3.95-3.25** (m, 136H)		**3.55** (s, 10H)	**3.55** (s, 10H)	
12			**3.63** (s, 47H)	**3.78-3.64** (m, 5H)		**3.69** (s, 8H)	**3.44** (dd, J=4Hz, 4Hz,	**3.44** (dd, J=4Hz, 4Hz,	
13					**3.22-3.08** (m, 9H)		**3.34** (dt, J=8Hz, 8Hz,	**3.34** (dt, J=8Hz, 8Hz,	**3.17-2.88** (2H, m)
15		**2.90-2.70** (m, 7H)				**2.79** (t, J=8Hz,		**2.60** (s, 8H)	**2.75-2.45** (1H, m)
16	**2.36-2.26** (m, 1H)	**2.40-2.30** (t, J=8Hz, 8H)	**2.30-2.22** (m, 49H)	**2.45-2.18** (m, 3H)		**2.32** (t, J =8Hz,			
17	**2.08-2.00** (m, 1H)	**2.17-2.00** (m, 15H)		**2.11-1.94** (m, 3H)		**2.09-2.04** (q, J=8Hz,			
18		**1.75-1.57** (s, 11H)	**1.58** (s, 220H)	**1.92-1.78** (m, 2H)		**1.66-1.62** (m, 6H)	**1.80** (s, 8H)	**1.80** (s, 8H)	
20	**1.36-1.20** (m, 43H)	**1.50-1.20** (m, 63H)	**1.4-1.09** (m, 276H)	**1.47-1.17** (m, 18H)		**1.41-1.25** (m, 49H)		**1.22-1.17** (m, 27H)	
21	**1.04** (s, 5H)	**1.05-0.80** (m, 13H)		**1.02** (s, 3H)					

Table 4.13. ^{13}C NMR (125 MHz) characterization data (δ) of purified isolates.

No.	Groups	Hexane A1	Hexane A4	Chloroform soluble 1	Chloroform soluble 2	Chloroform soluble 3&4	Chloroform soluble 5	Chloroform insoluble B	Chloroform insoluble D
1	**C=O**	-	173.3-172.9	174.3-172.8	-	-	-	-	-
2	**C=C**	139.7-122.6	130.2-127.8	129.9	121.7	-	130.2-127.9	-	-
3	**-**	-	-	-	-	-	-	103.6-92.1	92.1
4	**RCH_2O-**	77.2-56.0	77.4-62.1	77.4-62.1	71.8-56.8	76.0-61.1	-	81.3-60.0	81.3-60.0
5	**CH_3CO-**	50.0-25.6	34.2-25.6	51.4-27.1	50.1-26.0	-	51.5-25.6	-	53.3-38.6
6	**R_2CH_2**	25.1-18.8	24.9-22.6	24.9-21.9	24.3-18.8	17.9-16.0	25.0-22.6	23.2	-
7	**RCH_3**	14.2-11.9	14.1	14.1-13.9	14.2-11.9	-	14.1	-	-

4.4.4. GC-MS Identification for various extracts of Sünter

GC-MS analysis was conducted to gain further insights into the composition of the whole Sünter flour and its various extractions. All MS chromatograms were presented in Figures 4.31-4.35. All molecules were identified through comparison with the NIST and Wiley MS libraries.

4.4.4.1. Whole wheat extract of Sünter variety

The GC-MS results of the whole Sünter extract (Section 3.9.1) revealed a higher presence of sugars and sugar alcohols. The corresponding GC-MS chromatogram is given in Figure 4.31.

L-Alanine, L-Valine, Glycine, Butanedioic acid, 2-Butenedioic acid, Malic acid, D-(+)-Arabitol, D-(-)-Ribofuranose, Citric acid, D-(-)-Fructose, D-Ribose, D-Glucose, D-Mannitol, Palmitic Acid, Myo-Inositol, Linoleic acid, Oleic acid, D-Glucuronic acid, 1-Monopalmitin, Sucrose, 1-Monolinolein, Maltose, Galactinol were identified in whole Sünter extract.

4.4.4.2. Fraction B of hexane extract

The GC-MS results of fraction B (Section 4.4.1) demonstrated a higher presence of fatty acids and phytosterols. The corresponding GC-MS chromatogram is given in Figure 4.32. The following metabolites were detected in the B fraction: palmitic acid, linoleic acid, oleic acid, and stearic acid as fatty acids; ethylene glycol, 1,3-propanediol, glycerol, 1-monopalmitin, and glycerol monostearate as derivatives of lipids; and campesterol and β-sitosterol as sterols. Most of these compounds were confirmed with GC-FID by using reference standards (Section 4.3.2 and Section 4.3.3).

4.4.4.3. Fraction C of hexane extract

The GC-MS results of fraction C (Section 4.4.1) resulted in a higher presence of fatty acid esters. The corresponding GC-MS chromatogram is given in Figure 4.33. The C fraction contained palmitic acid, linoleic acid, oleic acid, and stearic acid as fatty acids, along with 1-monolinolein and glycerol monostearate as derivatives of lipids.

4.4.4.4. Chloroform soluble extract

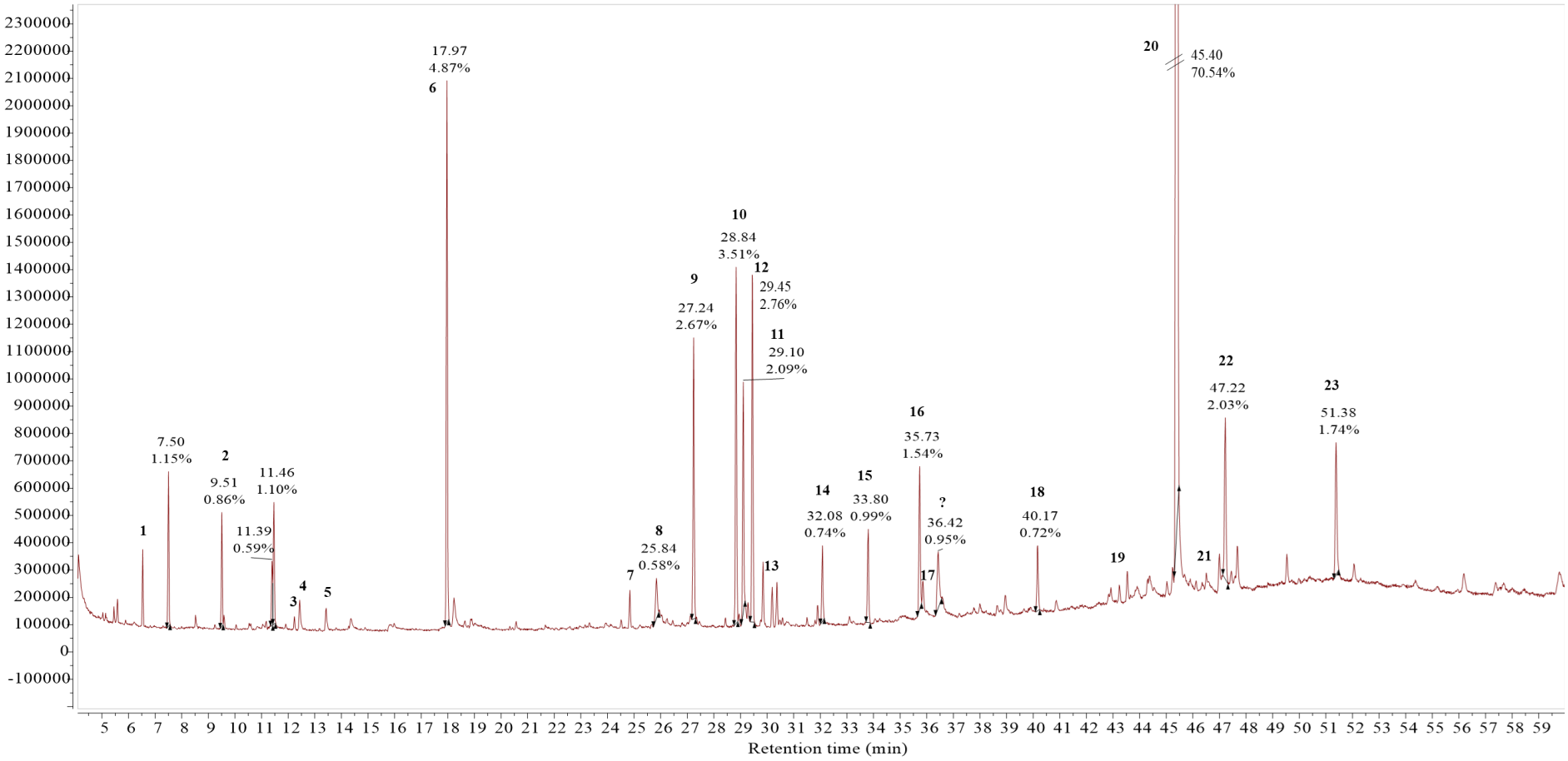
The GC-MS results of the chloroform soluble extract (Section 4.4.2.1) showed a higher presence of phospholipids derivatives, sugars, and sugar alcohols. The corresponding GC-MS chromatogram is given in Figure 4.34. Ethanolamine, Glycerol, D-(+)-Arabitol, Phosphoric acid, 2-trimethylsilyloxy-1-[(trimethylsilyloxy)methyl]ethylbis(trimethylsilyl) ester, Phosphoric acid, bis(trimethylsilyl) 2,3-bis[(trimethylsilyl)oxy]propyl ester, α-D-Glucopyranoside, β-D-Glucopyranoside, Methyl galactoside, Methyl galactoside, D-Glucose,

D-Galactose, Linoleic acid, Myo-Inositol, Glyceryl-glycoside, α-D-galactopyranoside were detected in chloroform soluble extract. Some of the above molecules were confirmed with the help of ^{1}H and ^{13}C NMR analyses.

4.4.4.5. Chloroform insoluble extract

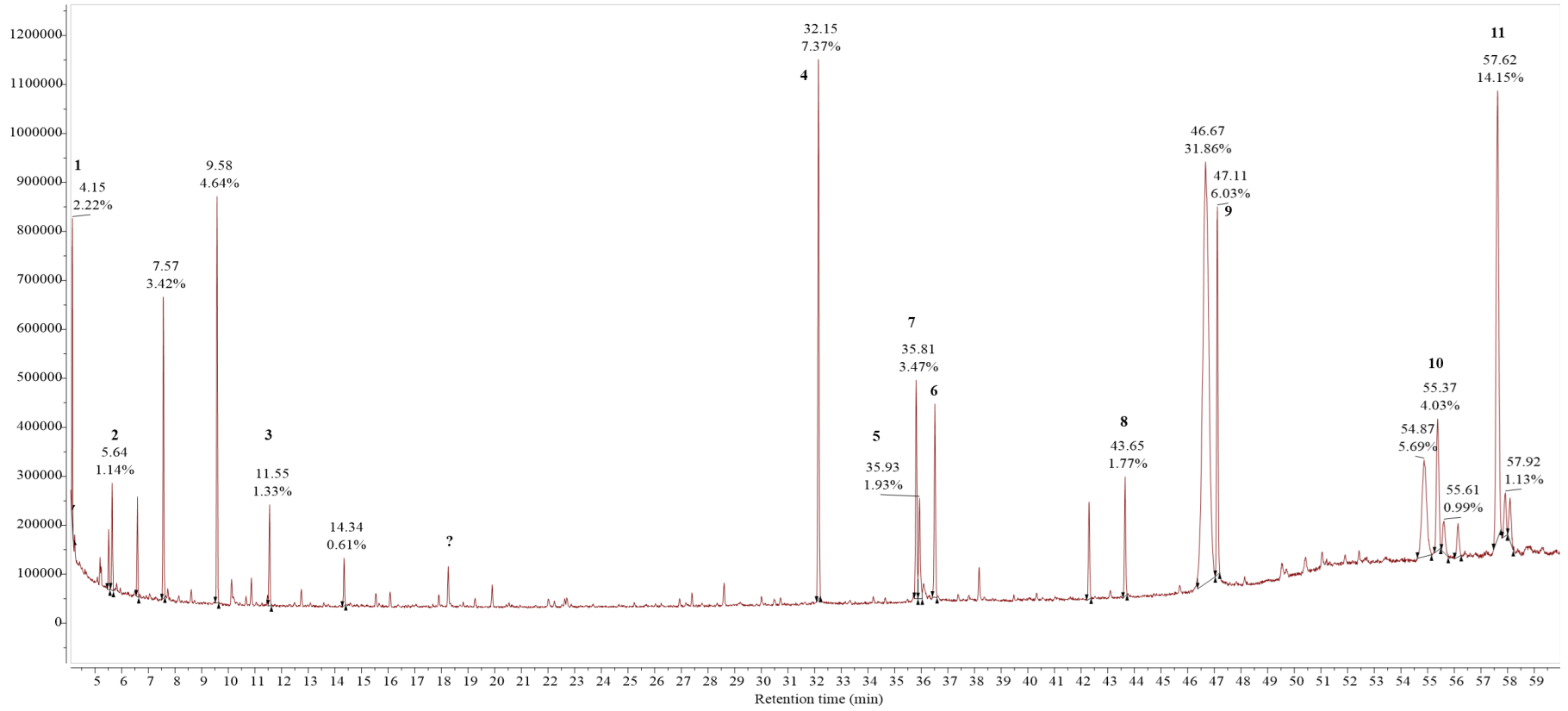
The GC-MS results of the chloroform insoluble extract (Section 4.4.2.2) revealed a higher presence of sugars and sugar alcohols. GC-MS chromatogram is given in Figure 4.35. The Glycerol, Malic acid, D-(−)-Fructose, D-Galactose, β-D-Glucopyranose, D-Mannose, D-Glucose, D-Mannitol, α-D-(+)-Talopyranose, Myo-Inositol, Rafinose, Sucrose, Galactinol were detected in chloroform insoluble extract. corresponding.

Overall, all molecules identified by GC-MS in this study have previously been documented in wheat through numerous publications [47, 162, 192, 193]. This also indicates the significance of local wheat varieties in relation to bioactive compounds and their potential for commercialization, which is comparable to that of global wheat varieties.



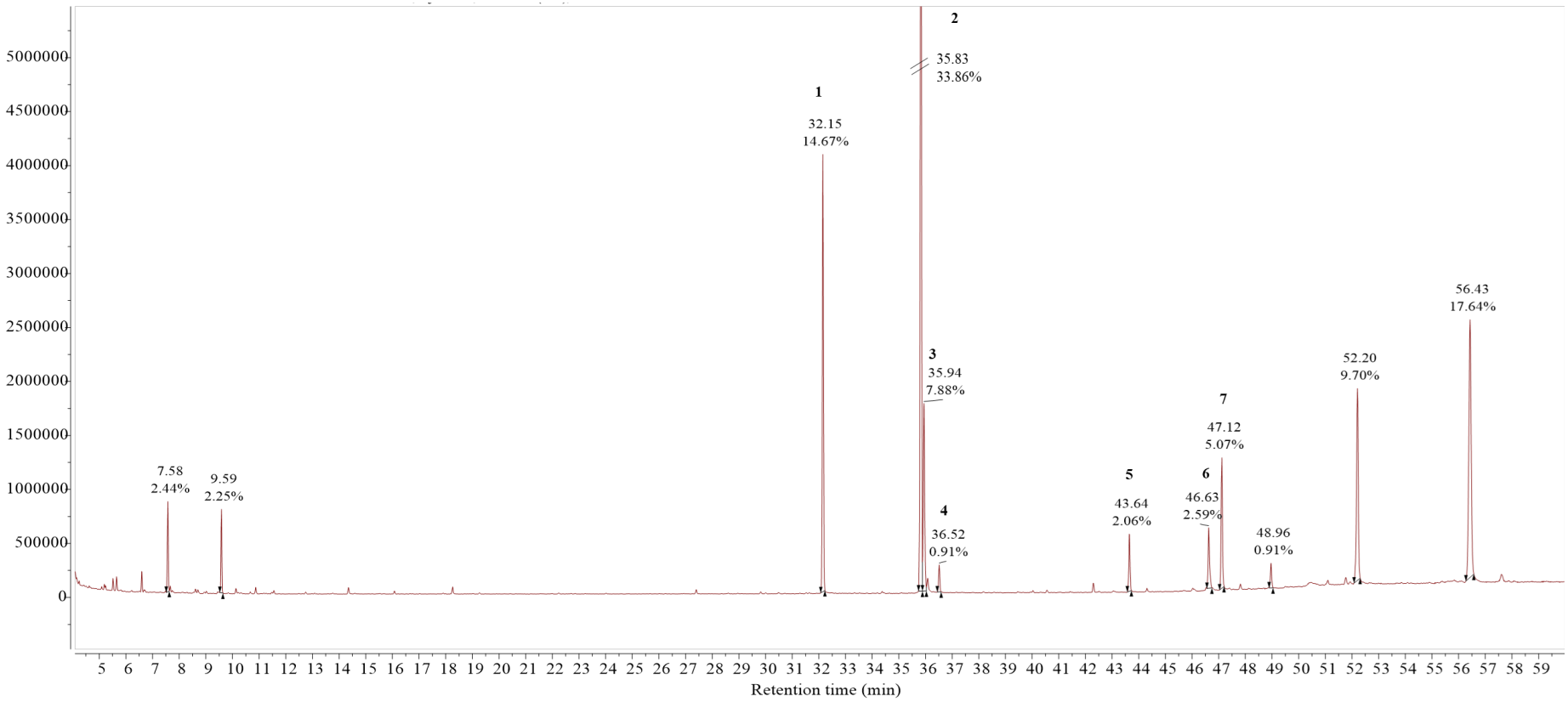
Peak **1**. L-Alanine, **2**. L-Valine, **3**. Glycine, **4**. Butanedioic acid, **5**. 2-Butenedioic acid, **6**. Malic acid, **7**. D-(+)-Arabitol, **8**. D-(-)-Ribofuranose, **9**. Citric acid, **10**. D-(-)-Fructose, **11**. D-Ribose, **12**. D-Glucose, **13**. D-Mannitol, **14**. Palmitic Acid, **15**. Myo-Inositol, **16**. Linoleic acid, **17**. Oleic acid, **18**. D-Glucuronic acid, **19**. 1-Monopalmitin, **20**. Sucrose, **21**. 1-Monolinolein, **22**. Maltose, **23**. Galactinol.

Figure 4.31. GC-MS chromatogram of the whole Sünter extract.



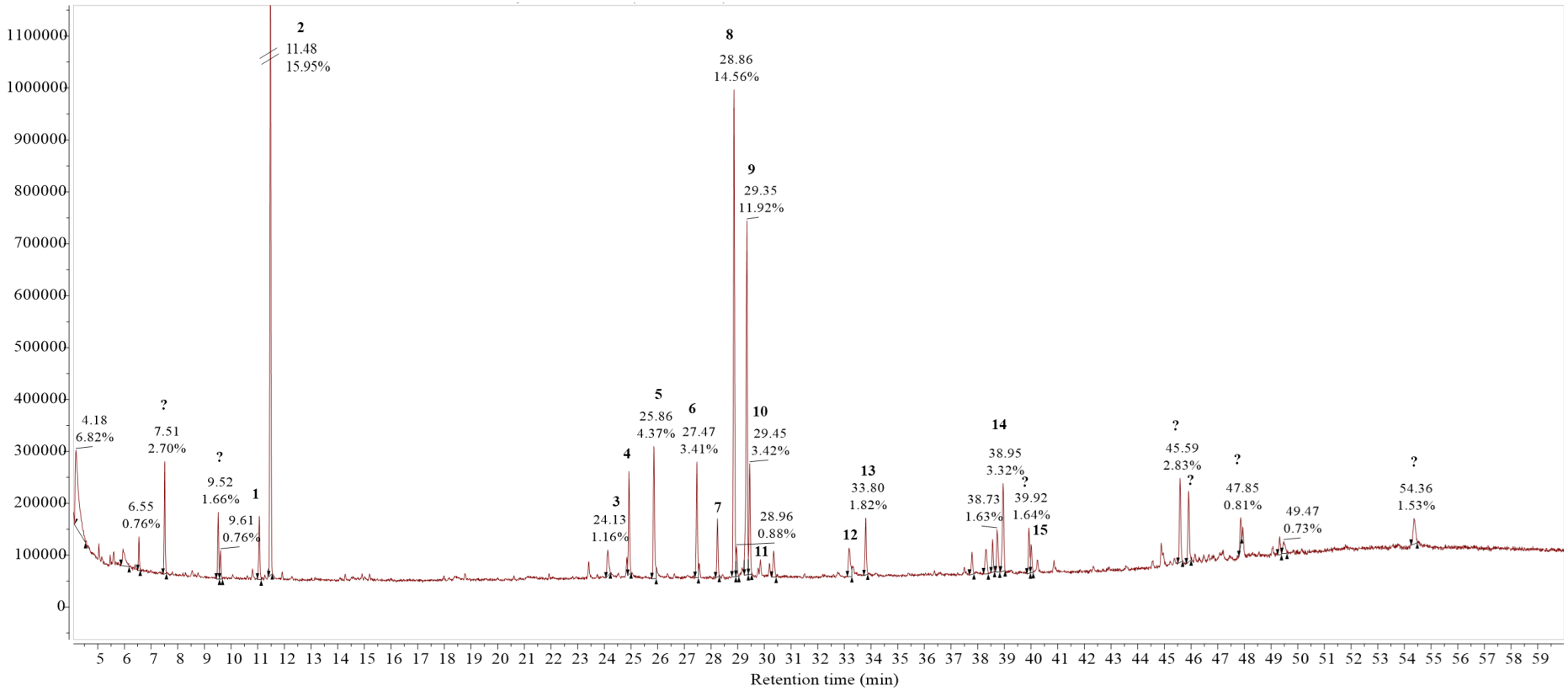
Peak **1**. Ethylene glycol **2**. 1, 3-propanediol, **3**. Glycerol, **4**. Palmitic acid, **5**. Linoleic acid, **6**. Oleic acid, **7**. Stearic acid, **8**. 1-monopalmitin, **9**. Glycerol monostearate, **10**. Campesterol, **11**. β-sitosterol.

Figure 4.32. GC-MS chromatogram of fraction B.



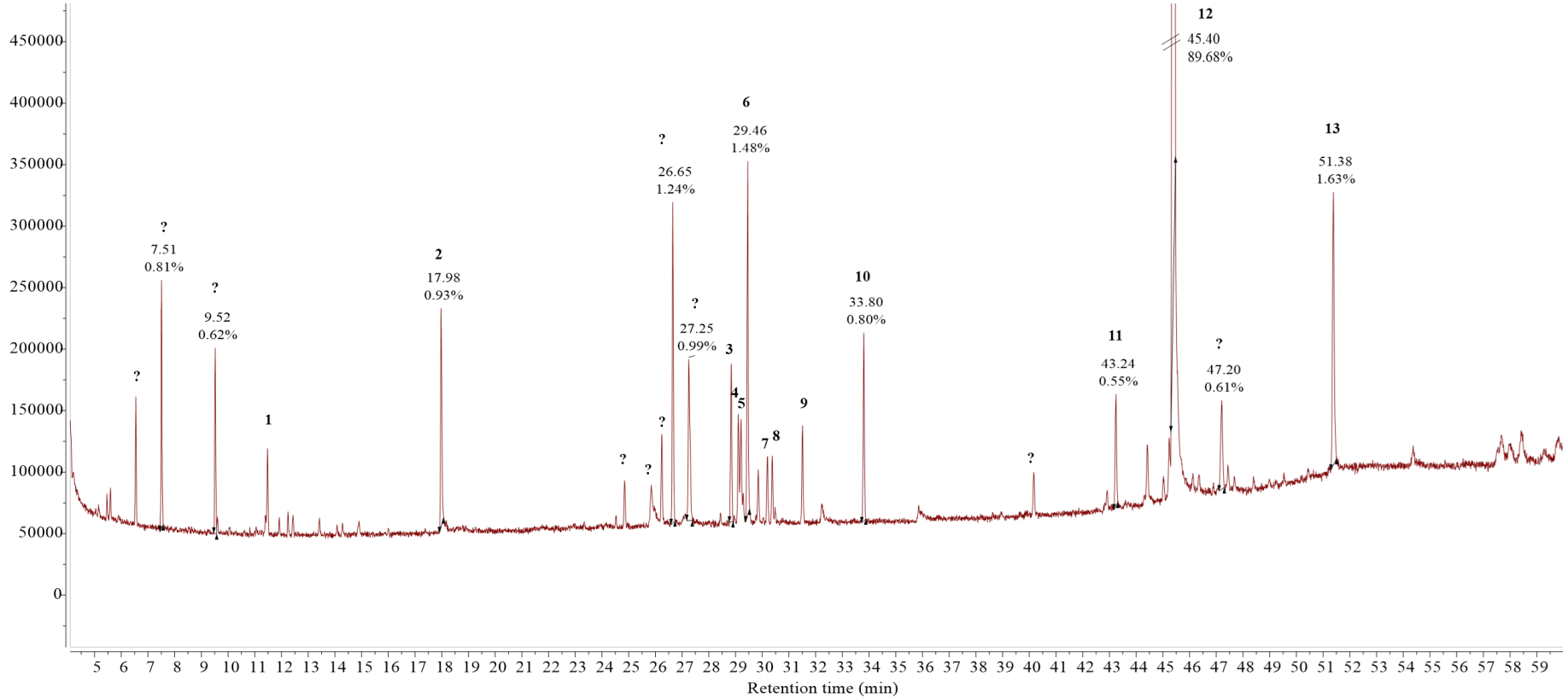
Peak **1**. Palmitic acid **2**. Linoleic acid, **3**. Oleic acid, **4**. Stearic acid, **5**. 1-Monopalmitin, **6**. 1-Monolinolein, **7**. Glycerol monostearate.

Figure 4.33. GC-MS chromatogram of fraction C.



Peak **1**. Ethanolamine. **2**. Glycerol, **3**. D-(+)-Arabitol, **4**. Phosphoric acid 2-trimethylsilyloxy-1-[(trimethylsilyloxy)methyl]ethylbis(trimethylsilyl) ester, **5**. Phosphoric acid, bis(trimethylsilyl) 2,3-bis[(trimethylsilyl)oxy]propy l ester, **6**. α-D-Glucopyranoside, **7**. β-D-Glucopyranoside, **8**. Methyl galactoside, **9**. Methyl galactoside, **10**. d-Glucose, **11**. D-Galactose, **12**. Linoleic acid, **13**. Myo-Inositol, **14**. Glyceryl-glycoside, **15**. α-D-galactopyranoside.

Figure 4.34. GC-MS chromatogram of chloroform soluble extract.



Peak **1**. Glycerol, **2**. Malic acid, **3**. D-(-)-Fructose, **4**. D-Galactose, **5**. β-D-Glucopyranose, **6**. D-Mannose, **7**. D-Glucose, **8**. D-Mannitol, **9**. α-D-(+)-Talopyranose, **10**. Myo-Inositol, **11**. Rafinose, **12**. Sucrose, **13**. Galactinol.

Figure 4.35. GC-MS chromatogram of chloroform insoluble extract.

4.5. Heatmap Analysis

The data was also integrated into the Heatmap to compare wheat varieties both within and among wheat genotypes (Figure 4.36). Firstly, data were normalized between 2 and -2 levels. Then, Heatmaps were obtained from normalized data of all individual metabolites with their TPC, TFC, total phytosterols, total steryl ferulates, ABTS, DPPH, TKW, ash content and extraction yields. As indicated by Figure 4.36 (A and B), all wheat varieties in monococcum and dicoccum genotypes were observed to have higher fatty acid, phytosterols, and steryl ferulate contents. Despite the lowest TKW, the monococcum genotype exhibits a high yield of hexane extract, which is likely attributed to its high bran content. Additionally, the monococcum wheat varieties can be considered favorable for health because of their higher levels of unsaturated fatty acids compared to other wheat genotypes. Mergüze and Siyez-4 wheats have a higher content of ρ-coumaric acid compared to other wheat varieties of monococcum genotype, whereas Atasiyez wheat exhibits significant levels of soluble-free, insoluble-bound, and total phenolic contents.

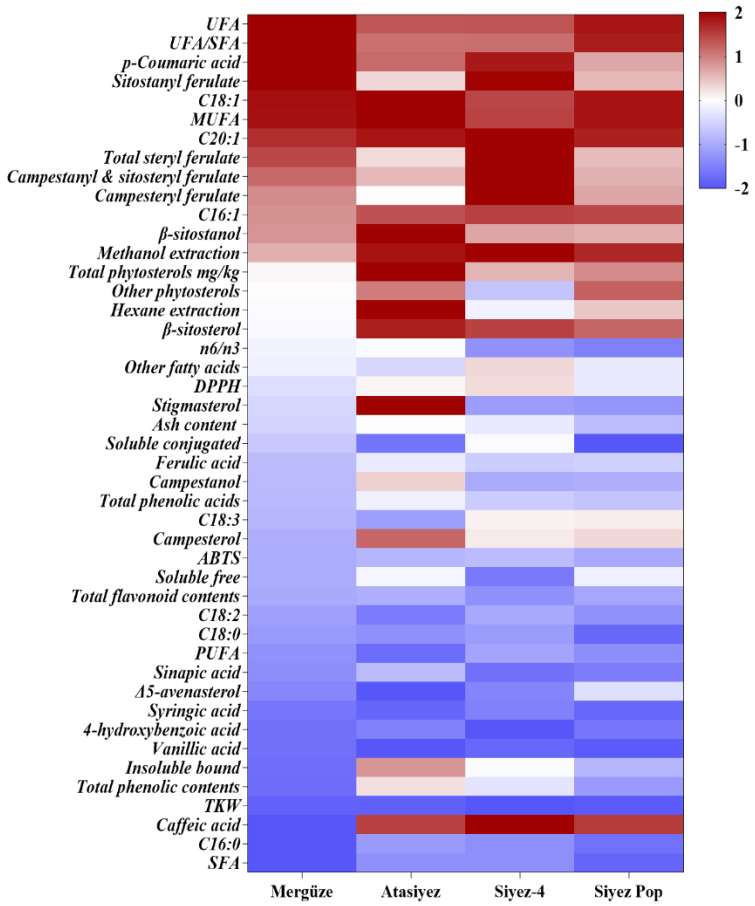
In dicoccum genotype, Kafkas wheat has higher caffeic acid and soluble-free phenolic contents while Kavılca kırmızı and Gacer varieties were found to be rich in terms of soluble-conjugated phenolic compounds. Gacer wheat also exhibits high DPPH based antioxidant capacity as compared to other varieties of dicoccum group. The dicoccum genotype, characterized by a low TKW, exhibits the least hexane extract yield, therefore have low phytosterol and steryl ferulate contents compared to other genotypes. On the other hand, both monococcum and dicoccum genotypes exhibit high concentrations of oleic acid, eicosenoic acid, and MUFAs.

In durum wheat, the Sarı buğday variety has higher ferulic acid and SFA profile that is relatively similar to that of the commercial Eminbey wheat (Figure 4.36C). Both wheat varieties have higher amounts of ferulic acid and total flavonoid contents as compared to Mirzabey 2000 and Karakılçık wheats. On the other hand, PUFAs and linoleic fatty acids were abundant in Karakılçık wheat as compared to other varieties of durum genotypes.

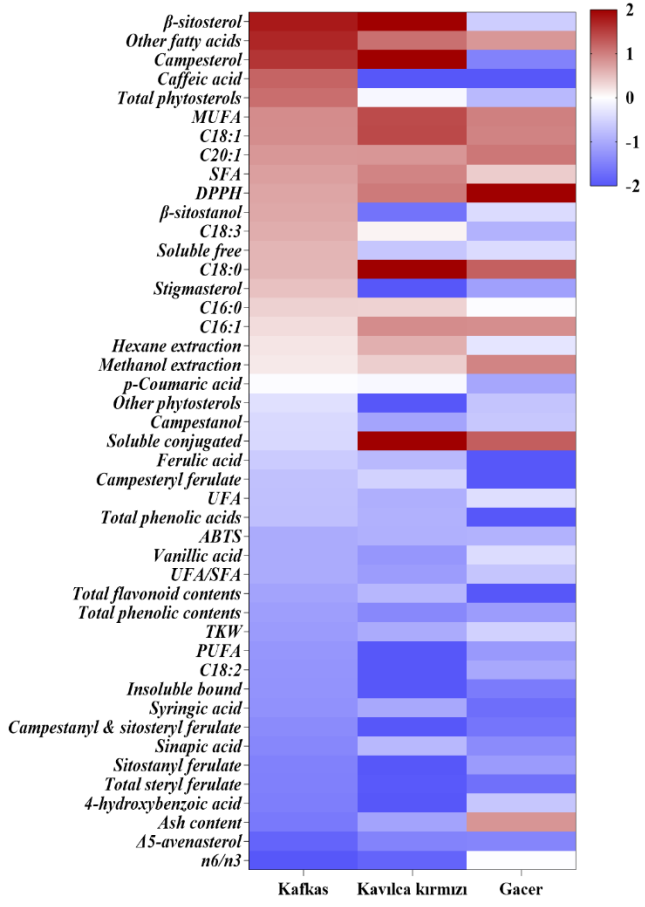
As shown in Figure 4.36D for aestivum wheat varieties, Sünter wheat has higher ferulic acid, sinapic acid, insoluble-bound, total phenolic, and total flavonoid contents compared to all wheat varieties. Zerun also exhibits a similar profile for insoluble and total phenolic contents, with higher quantities of vanillic and 4-hydroxybenzoic acids compared to Sünter wheat. In addition, AK-702 wheat demonstrated a high concentration of Δ5-avenasterol. On the other hand, Spelt S. başak wheat was observed with high total phytosterols and steryl ferulates in

comparison to other wheat varieties in aestivum genotype. In commercial varieties, Bayraktar 2000 exhibited high levels of PUFAs including linoleic acid and linolenic acid, as well as stearic acid, soluble and bound phenolic contents, whereas Demir 2000 wheat was found to be rich in syringic and caffeic acids.

Notwithstanding its high TKW values, the durum genotype exhibits a low hexane extraction yield and amount of steryl ferulates. However, the aestivum genotype has high phytosterol and steryl ferulate contents, regardless of its low yield of hexane extraction. Both durum and aestivum genotypes were also found to be rich sources of palmitic acid, linoleic acid, α-linolenic acid, saturated fatty acids, and PUFAs.


UFA
UFA/SFA
p-Coumaric acid
Sitostanyl ferulate
C18:1
MUFA
C20:1
Total steryl ferulate
Campestanyl & sitosteryl ferulate
Campesteryl ferulate
C16:1
β-sitostanol
Methanol extraction
Total phytosterols mg/kg
Other phytosterols
Hexane extraction
β-sitosterol
n6/n3
Other fatty acids
DPPH
Stigmasterol
Ash content
Soluble conjugated
Ferulic acid
Campestanol
Total phenolic acids
C18:3
Campesterol
ABTS
Soluble free
Total flavonoid contents
C18:2
C18:0
PUFA
Sinapic acid
Δ5-avenasterol
Syringic acid
4-hydroxybenzoic acid
Vanillic acid
Insoluble bound
Total phenolic contents
TKW
Caffeic acid
C16:0
SFA
2
1
0
-1
-2
Mergüze
Atasiyez
Siyez-4
Siyez Pop

A


β-sitosterol
Other fatty acids
Campesterol
Caffeic acid
Total phytosterols
MUFA
C18:1
C20:1
SFA
DPPH
β-sitostanol
C18:3
Soluble free
C18:0
Stigmasterol
C16:0
C16:1
Hexane extraction
Methanol extraction
p-Coumaric acid
Other phytosterols
Campestanol
Soluble conjugated
Ferulic acid
Campesteryl ferulate
UFA
Total phenolic acids
ABTS
Vanillic acid
UFA/SFA
Total flavonoid contents
Total phenolic contents
TKW
PUFA
C18:2
Insoluble bound
Syringic acid
Campestanyl & sitosteryl ferulate
Sinapic acid
Sitostanyl ferulate
Total steryl ferulate
4-hydroxybenzoic acid
Ash content
Δ5-avenasterol
n6/n3
2
1
0
-1
-2
Kafkas
Kavılca kırmızı
Gacer

B

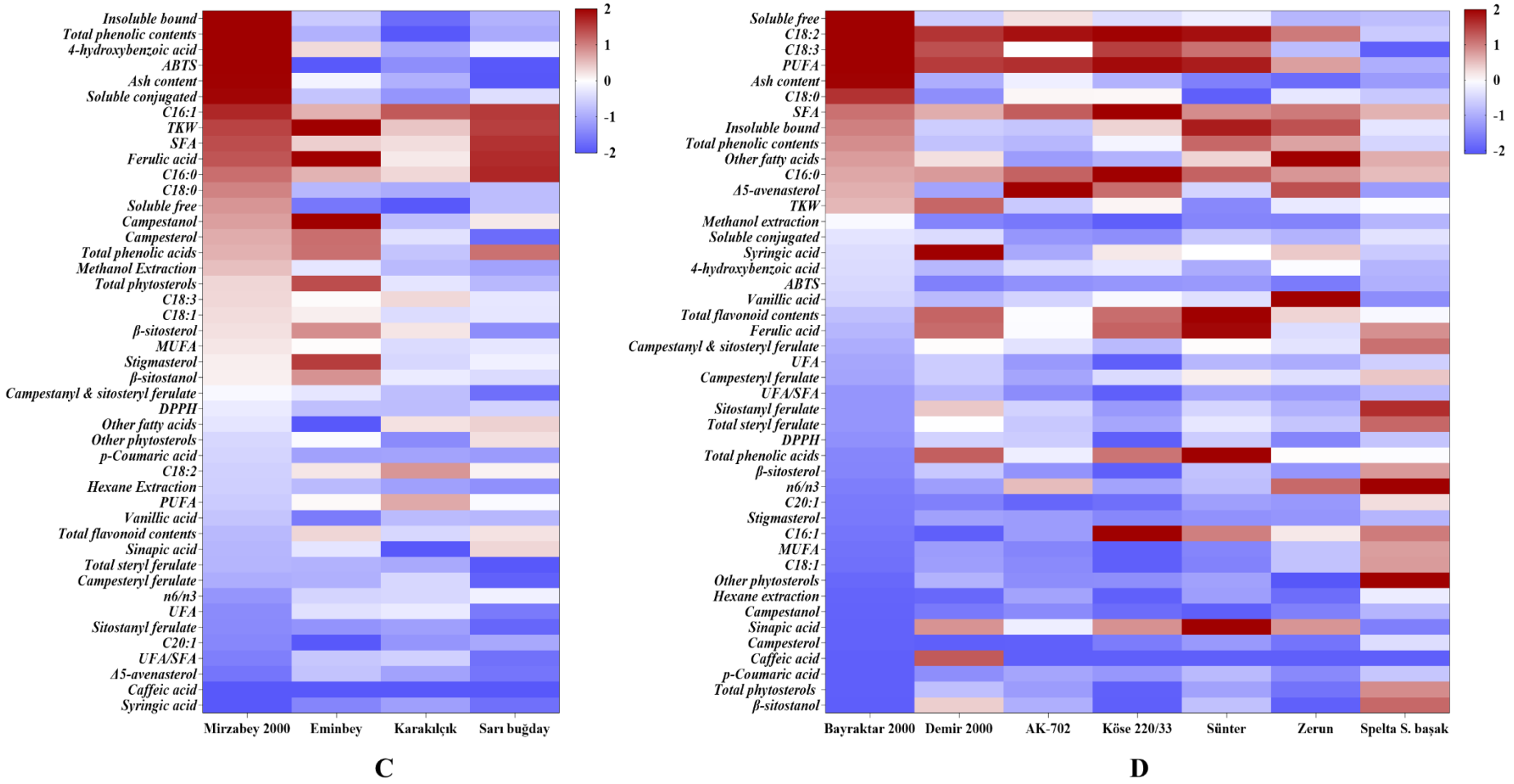


Figure 4.36. Heatmap analysis of wheat genotypes, (A) monococcum, (B) dicoccum, (C) durum, and (D) aestivum.

5. CONCLUSIONS

Wheat is a significant source of carbohydrate, protein, starch, fat, and dietary fiber, which play a vital role in human nutrition. Additionally, it contains numerous bioactive phytochemicals with antioxidant properties. These phytochemicals include phenolic compounds such as phenolic acids, flavonoids, as well as essential fatty acids, phytosterols, and steryl ferulates. The local Turkish wheat varieties have recently garnered attention from both researchers and farmers due to their health-promoting benefits and the limited literature available for these varieties. For this, a special emphasis was given to the identification and characterization of bioactive compounds in local wheat varieties.

In this study, it was observed that local wheat genotypes exhibited significantly high levels of phenolic acids, flavonoid content, and antioxidant capacity, comparable to those found in commercial wheat varieties. Upon evaluating the phenolic compounds and antioxidant capacity, it was noted that dicoccum genotype had the highest levels of soluble-free and soluble-conjugated phenolic contents. Among the wheat varieties, AK-702 and Sünter wheat have been found with the highest levels of soluble-free phenolic substances. The soluble-free phenolic compounds are present in small quantities in wheat, but they hold great importance for the human body due to their high digestibility and ability to cross intestinal barriers [194]. On the other hand, the aestivum genotype showed the highest levels of insoluble-bound phenolic, total phenolic, and total flavonoid contents. The bound-phenolic compounds are effective against colon and prostate cancer due to their ability to survive the adverse acidic conditions of human stomach [16]. The seven different phenolic acids were identified in all the wheat varieties studied, with ferulic acid being the most abundant. The antioxidant capacity of monococcum and dicoccum wheat was found to be higher than that of the durum and aestivum genotypes. Moreover, some local wheat varieties, such as Sünter and Sarı buğday, have been observed to exhibit phenolic compounds and antioxidant potentials comparable to those found in commercial wheat varieties.

Fatty acids, with linoleic acid being the highest, as well as sterols/stanols, and steryl ferulates, were analyzed using GC-MS, GC-FID, and HPLC-DAD in hexane extracts of all wheat genotypes. Notably, Siyez Pop (monococcum), Gacer (dicoccum), Sarı buğday (durum), and Spelt S. başak (aestivum) varieties were found to be rich in polyunsaturated fatty acids, similar to commercial varieties (Mirzabey 2000 and Demir 2000). Generally, monococcum genotype was observed to have a low content of saturated fatty acids and a high content of unsaturated

fatty acids. According to the HEI-2015 (Healthy Eating Index-2015), a healthy nutritional index, the ratio of unsaturated fatty acids to saturated fatty acids should be above 2.5 for a food to be considered healthy [175]. In the case of wheat samples, this ratio far exceeded the required threshold, classifying these wheat varieties as valuable products in terms of unsaturated fatty acids.

This study revealed that Siyez 4, Karakılçık, Spelt S. başak, Köse 220/33, and Zerun wheat varieties have comparable levels of phytosterols and stanols, with β-sitosterol being particularly abundant, to those of commercial wheat varieties worldwide. The phytosterol contents in these samples were either higher or comparable to those reported in the literature for commercial wheat varieties. The high phytosterol content found in these local wheat varieties highlights their significant potential for commercialization. Phytosterols are bioactive compounds known for their various beneficial effects on human health, including antioxidant, antifungal, and anti-inflammatory properties. Additionally, they play a crucial role in reducing blood pressure and helping to prevent certain types of cancer [84]. In addition, local wheat varieties such as Siyez-4, Karakılçık, Spelt S. başak, and Köse 220/33 displayed ferulate levels comparable to those found in commercial samples. This suggests that the bioactivity of these varieties may be superior to others, considering their steryl ferulate content.

Metabolites such as organic acids, fatty acids, sterols/stanols, steryl ferulates, α-tocopherol, phospholipids, sugars, sugar alcohols, phenolic acids, etc., were analyzed in fractions (hexane, chloroform-soluble, and insoluble) collected through TLC and MPLC separations in the local Sünter variety, which was chosen to represent all wheat samples. The identification of these compounds was carried out using ^{1}H NMR, ^{13}C NMR, GC-MS, GC-FID, and HPLC-DAD techniques. All identified and/or confirmed metabolites were compared with the existing literature on global wheat varieties and summarized in Table 5.1.

Table 5.1. Metabolites identified in Sünter wheat variety.

Group	Component	GC-MS	GC-FID	HPLC-DAD	NMR (^{1}H & ^{13}C)	Literature comparison
Amino acids	Alanine	+				[192, 193]
	Valine	+				[192, 193]
	Glycine	+				[192, 193]
	Proline	+				[192, 193]
	Aspartic acid	+				[193, 195]
Organic acids	Propanoic acid	+				[193, 196]
	2-hydroxybutyric acid	+				[193]
	Malic acid	+			+	[192]
	Citric acid	+				[192]
	Quininic acid	+				[192, 193]
	Gluconic acid	+				[192]
Sugars	Fructose	+				[192, 193]
	Galactose	+				[196]
	Ribose	+				[196]
	Mannose	+				[197]
	Glucose	+			+	[192]
	Glucopyranose	+				[193]
	Maltose	+				[192, 193]
	Sucrose	+			+	[192]
	Manobiosis	+				[197]
	Melibiosis	+				[198]
Sugar alcoholics	Mannitol	+				[192, 193]
	Myo-inositol	+				[192, 196]
Saturated fatty acids	Palmitic acid	+	+			[10, 192]
	Stearic acid	+	+			[10, 192]
	Behenic acid		+			[192, 199]
Unsaturated fatty acids	Palmitioleic acid		+			[192]

	Oleic acid	+	+			[192, 193]
	Elaidic acid		+			[192]
	Linoleic acid	+			+	[192, 193]
	Linolenic acid		+			[192]
	Eicosenoic acid		+			[199]
Fatty acid esters	1-monopalmitin	+				[200]
	1-monolinolein	+				[201]
	Phosphatidylethanolamine diacylglycerols				+	[202]
	Fatty acid sugar ester				+	[186]
Vitamins	α-tocopherol		+			[21]
Sterols/stanols	Campesterol	+	+			[47, 48, 56]
	Stigmasterol	+	+			[47, 48, 56]
	β-sitosterol	+	+			[47, 48, 56]
	Stigmastanol	+	+			[47, 48, 56]
	β-sitostanol	+	+			[47, 48, 56]
	Δ5-avenasterol	+	+			[47, 48, 56]
	α1-sitosterol	+	+			[47, 48, 56]
Steryl ferulates	Campesteryl ferulate			+		[21]
	β-sitosteryl ferulate			+		[21]
	Sitostanyl ferulate			+		[21]
Phenolic acids	4-hydroxybenzoic acid			+		[162]
	Vanillic acid			+		[193]
	Caffeic acid			+		[193]
	Syringic acid			+		[162]
	ρ-coumaric acid			+		[162, 193]
	Ferulic acid			+		[162, 193]
	Sinapic acid			+		[162]
Others	Hexadecane	+				[198]
	Uridine	+				[193]

Overall, the findings indicate that these specific local wheat varieties possess advantageous characteristics in terms of phenolic compounds, phytosterols and steryl ferulate contents, enhancing their potential for offering better bioactivity and health benefits compared to commercial ones. As a result, selected local wheat varieties, including Sünter, Köse 220/33, AK-702, Spelta S. başak, Siyez-4, and Karakılçık, have been identified as having a high potential for bioactive components.

The findings indicated that the local wheat varieties hold promising prospects for becoming commercial varieties, mainly due to their high levels of bioactive compounds. It also highlights the significance of local wheat genotypes in terms of their bioactive properties and provides valuable insight for the selection of such seeds in future breeding programs. Moreover, these findings can be used to increase awareness among local farmers and encourage them to promote the cultivation of local wheat varieties. Likewise, their presence is important to deliver new opportunities for the commercial production of Turkish local wheat varieties and their utilization in functional foods. As consumer health awareness continues to rise, there is a potential for the consumption of these local wheat varieties to increase due to their improved nutrient profiles. Their valuable health properties make them promising candidates for further utilization and promotion in the market.

Additionally, the approach methodology applied in this study provides a useful investigating procedure to study other local and international landraces for exploring bioactive compounds and facilitating breeding programs. Therefore, by adopting similar methodologies in future research, researchers can efficiently assess the bioactive potential of various wheat varieties, aiding in the selection of improved and nutritionally beneficial wheat cultivars for future agricultural and commercial purposes.

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7. ANNEXES

ANNEX 1 – Images of Wheat Genotypes (both with and without husk)

T. monococcum genotypes

Mergüze

With husk **Without husk**

Atasiyez

With husk **Without husk**

Siyez 4

With husk **Without husk**

Siyez Pop

With husk **Without husk**

T. dicoccum genotypes

Kafkas

With husk **Without husk**

Kavılca kırmızı

With husk **Without husk**

Gacer

With husk **Without husk**

T. durum genotypes

Mirzabey 2000

Eminbey

Karakılçık

Sarı buğday

T. aestivum genotypes

Bayraktar 2000

Demir 2000

AK-702

Köse 220.33

Sünter

Zerun

Spelta S. başak

With husk

Without husk

ANNEX 2 – Preliminary Trial and Method Optimization

The preliminary investigation for the research project titled "Identification and Characterization of Bioactive Components in Local Wheat Varieties (*Triticum* Spp.)" has been established through collaboration among the Department of Food Engineering at Hacettepe University, TAGEM, and Department of Horticulture, Michigan State University, USA, which was facilitated by H.Ü. BAP (Project No: FUK-2019-17752) and Erasmus+ World International Credit Mobility Program (K107) (February - June 2019). The aim of this collaboration was to optimize the research methodology and the findings collected from the initial investigations are presented below.

1. Materials and Methods

1.1. Selected Wheat Samples for Method Optimization

Nine different wheat varieties were studied including four local wheat varieties (*Triticum dicoccum* 1178, 1180, 1181, and 1191) obtained from the Turkish Seed Gene Bank (TSGB, Ankara), and five local wheat varieties supplied by the farmers of Bolu, Kayseri, Kastamonu, Canakkale, and Sivas cities. After the removal of the husk, advanced milling processing were applied to whole wheat grains to obtain different fractions (coarse bran, fine bran and flour) in Quality and Technology Department, Ministry of Agriculture and Forestry, Field Crops Research Institute (Ankara) (Table A1).

Table A1. Fractions of Gernik, Siyez and Bread wheat varieties.

Name	**Type**	**Husk (g)**	**Coarse Bran (g)**	**Fine Bran (g)**	**Flour (g)**
Triticum dicoccum 1178	Gernik / TSGB	84.19	111.3	57.02	180.14
Triticum dicoccum 1180	Gernik / TSGB	93.48	152.53	66.86	191.57
Triticum dicoccum 1181	Gernik / TSGB	91.94	163.74	45.87	274.57
Triticum dicoccum 1191	Gernik / TSGB	88.94	160.47	60.60	197.88
Triciticum dicoccum 373/38	Siyez / Kayseri	417.72	508.68	173.57	736
Triciticum monococcum 373IZA	Siyez / Bolu	456.02	492.31	79.60	779.74
Triciticum monococcum 373/37	Siyez / Kastamonu	439.46	447.63	47.13	710.48
Triciticum astivum 373/17	Siyez / Canakkale	*	627.21	101.69	1212.28
Triciticum astivum 118/33	Siyez / Sivas	*	757.99	56.93	1231.9

* Bread wheat varieties have no husk fraction.

1.2. Chemicals

The isolation and characterization of bioactive compounds in local wheat varieties were performed using ACS reagent-grade solvents (Sigma-Aldrich, USA). TLC and PTLC plates (20×20 cm size and thicknesses of 250 and 500 µm, respectively) were obtained (Analtech, Inc. Newark, DE, USA) and the Spectroline CX-20 UV fluorescence analysis chamber (Spectroline Corporation Westbury, NY, USA) was employed for their examination using UV light at wavelengths of 366 and 254 nm. Subsequently, a solution of 10% sulfuric acid in methanol was sprayed onto the plates for their inspection in visible light. Silica gel 60 (35-70 µm particle size) as MPLC filling material was purchased from Merck, USA. For positive control, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), t-butyl hydroquinone (TBHQ), and vitamin C, were purchased from Sigma-Aldrich, USA. All samples and chemical reagents were preserved in the Bioactive Natural Products laboratory at Michigan State University, East Lansing, MI, USA.

1.3. Hexane-Methanol Sequential Extraction

All fractions of local wheat varieties were extracted sequentially with hexane and methanol. The samples were mixed with hexane (1:20 w/v) and shaken for 16 h. Then, the supernatant and the residue were separated from each other by a 15 min centrifugation at 10000 rpm and 4 °C. The obtained supernatant was evaporated using a rotary evaporator at 35 °C. Following hexane extraction, similar extraction was performed with the help of methanol solvent. Both hexane and methanol extracts were stored at −20 °C after calculating their mass balance.

1.4. TLC, MPLC and PTLC

TLC has been used to separate various metabolites from the obtained extracts based on their polarity. In the TLC profiling, generally two types of solvent systems were used to separate the polar and non-poplar components in the extracts: chloroform-methanol (15:1, v/v), hexane-acetone (4:1, v/v), respectively. Similarly, MPLC was performed on the silica column with silica gel 60 (particle size 35-70 µm) in order to separate the non-polar extracts of whole wheat samples. Following the MPLC, selected fractions of extracts were purified into different sub-fractions by PTLC.

First, a fraction of hexane extract was dissolved in a mixture of hexane:acetone (10:1, v/v) (40 mg/1 ml) and the resulting supernatant was applied evenly to two PTLC plates.

A hexane:acetone (6:1, v/v) solvent system was used as mobile phase to separate the individual components and the separated bands were scratched from silica plate and taken into a sintered-crucible. The scratched silica samples were then eluted with the help of approximately 30 ml of chloroform:methanol (4:1, v/v) solvent mixture. The filtrates were individually evaporated (rotary evaporator) and their mass balance was calculated.

1.5. NMR

Two different NMR (Varian Unity ±500, VRX instruments) spectra were recorded; ^{1}H NMR at 500 MHz and ^{13}C NMR at 125 MHz. The chemical shift values of ^{1}H and ^{13}C atoms for $CDCl_3$ were detected at 7.24 and 77.2 ppm, respectively.

1.6. GC-MS

GC-MS was employed to determine the fatty acid composition of triglycerides in hexane extract. For this, the triglyceride mixture was hydrolyzed with 3M KOH in methanol, and then the fatty acid methyl esters were prepared with the help of diazomethane (CH_2N_2) according to the procedure [150]. These methyl esters were analyzed in a GC capillary column (Agilent J&W VF-5ms GC column, 30m × 0.25 mm, 0.25 µm film thickness) attached to a 10 m EZ-guard column. Analysis conditions; sample volume (1 µL), split ratio of 10:1, carrier gas as helium, flow rate of 1.5 ml/min and oven temperature was arranged from 240 °C, 40 °C/min increase rate to 320 °C for 15 min.
The GC-MS chromatogram was recorded on the Thermo DSQ-II GC/single quadrupole mass spectrometer (Thermo Electron Corporation, Austin, TX USA). Fatty acid methyl ester peaks were identified by comparing the retention time of FAME-37 standard mix (Supelco-37 FAME Mix, 47885-U, Bellefonte, PA, USA).

1.7. MTT Antioxidant Analysis

Advanced redox-based antioxidant analysis was performed to determine the proton scavenging ability of hexane and methanol extracts of wheat samples (Table 1.1). MTT analysis [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] is a colorimetric analysis applied to measure the activity of mitochondrial enzymes found in healthy cells by monitoring the absorption of purple formazan (570 nm) formed as the enzymatic reduction product of MTT (410 nm). DMSO was used to prepare stock solutions of positive controls (1 mg/ml Vitamin C and TBHQ) and test extracts (10 mg/ml). Whereas ultrapure water (Milli-Q, 18.2 MΩ cm) was used for the preparation of MTT stock solution (1 mg/ml). A small amount test samples (10 µL) were mixed with

190 µl of MTT stock solution and vortexed for 1 min. The resulting mixture was incubated overnight at 37 °C. Then 200 µL DMSO was added to the mixture again and vortexed for 1 minute. About 200 µL of the final mixture was transferred to the plate (96 well cell culture plate). The absorbance of each sample was measure in two parallel at 570 nm in the Bio-Tek Elx800 universal microplate reader [128].

1.8. Lipid Peroxidase (LPO) Inhibitor Analysis

Lipid peroxidation inhibitor analysis for antioxidant activities of hexane and methanol extracts (Table 1.1) was also performed using Large Unilamellar Vesicles (LUVs) [153]. Briefly, 1 mg of SLPC (phospholipid) in $CHCl_3$ and 1 mg of the DPH-PA fluorescence probe in 1 ml DMF were transferred to the test tube and the solvent was removed under vacuum. Next, the suspension was formed by adding the MOPS buffer to the obtained residue. After the freeze-thawing process, the suspension was passed through the extruder to obtain the vesicles of the LUVs. Finally, the buffer mixture of HEPES (100 µl), 1M NaCl (200 µl), N_2 sparged water (1.64 ml), DMSO (20 µl) and liposome suspension (20 µl) was used to perform LPO analysis. Here, 0.5 mM $FeCl_2$ (20 µl) solution was used to initiate peroxidation reaction and changes in fluorescence were observed at zero, first and third minutes, followed by fluorescence changes up to 21 minutes with 3-min intervals. The peroxidation rate is indicated by the overtime decrease in the relative fluorescence intensity. All test compounds at 100 µg/mL were tested in two parallels in a liposome-test mixture. Liposome-DMSO solvent control was used as blank. Various commercial antioxidants such as tert-butyl hydroquinone (TBHQ), butylated hydroxytoluene (BHT) and butylated hydroxy-anisole (BHA) were used as positive controls.

2. RESULTS

Sequential extraction method (hexane and methanol extracts), TLC, MPLC and PTLC conditions were optimized for the isolation and purification of bioactive compounds in wheat varieties. The results of optimized conditions were given in Trials 1, 2 and 3.

2.1. Trial 1:

In the first trial, all 34 samples (including husk, bran, fine bran and flour) belonging to 9 wheat species were extracted with methanol (Fig. A1). After weighing approximately 10 g of each sample and shaking with 200 ml of methanol (×1) for 16 h. All supernatants and dry extracts were obtained after centrifugation at 10000 rpm (4 °C) for 15 min, and the mass balance was determined (Table A2).

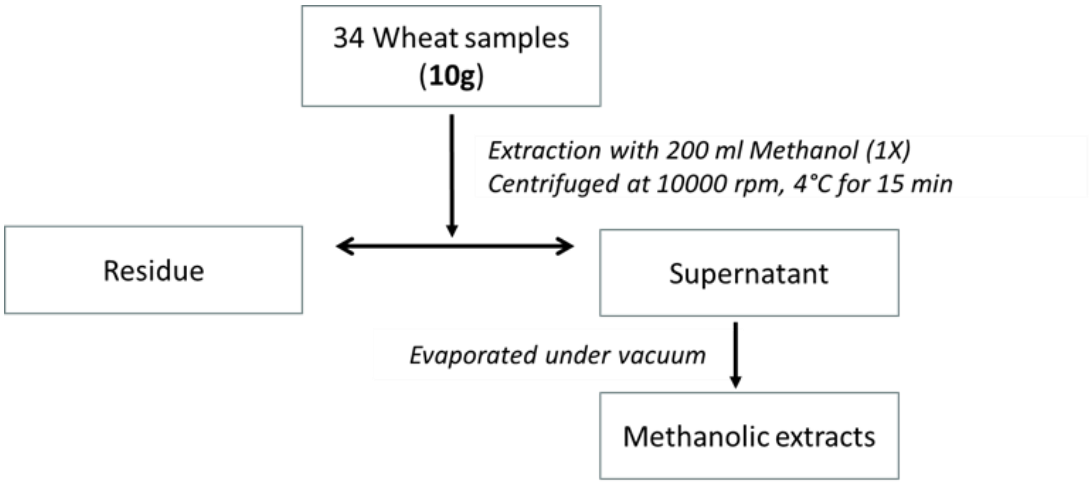


Fig. A1. Methanol extraction flow chart for all wheat fractions.

Table A2. Mass balance of methanol extracts.

Examples	Husk (g)	Extract 1 (mg)	Coarse bran (g)	Extract 2 (mg)	Fine bran (g)	Extract 3 (mg)	Flour (g)	Extract 4 (mg)
Triticum dicoccum 1178	10	272.6	10	589.7	10	613.3	10	239.6
Triticum dicoccum 1180	10.1	383.3	10.1	534.2	9.9	609.8	10	248.2
Triticum dicoccum 1181	10	313	10.3	550.9	9.9	652.6	10	282.9
Triticum dicoccum 1191	9.8	263.8	9.9	565.3	10.2	478.9	10.3	248.2
Triticum monococcum 373IZA	10.3	147	10.2	706.9	10	695.3	10.2	234.1
Triticum dicoccum 373/38	10.1	471.5	10.3	629.8	9.9	569.3	10	243.3
Triticum monococcum 373/37	9.9	83.9	10.1	611.1	10	766.1	9.9	207.7
Triticum aestivum 373/17	-	-	10.1	566.4	10	609.6	10	174.7
Triticum aestivum 111/33	-	-	10	559.1	10	778.2	10	145.4

TLC

Two different TLC solvent systems: chloroform-methanol (15:1), and hexane-acetone (4:1) were used to separate polar and non-polar components in methanol extraction, respectively (Fig. A2).

Based on TLC images, it has been interpreted that all wheat fractions may contain similar compounds in different proportions. The bands that moved quickly with the chloroform solvent have covered very short distances in the acetone solution. This suggests that these components are considered non-polar (such as triglycerides, etc.). It has been understood that the components from both TLC plates preferred organic solvents (chloroform,

hexane), therefore an additional extraction procedure with hexane may increase the yield of bioactive compounds.

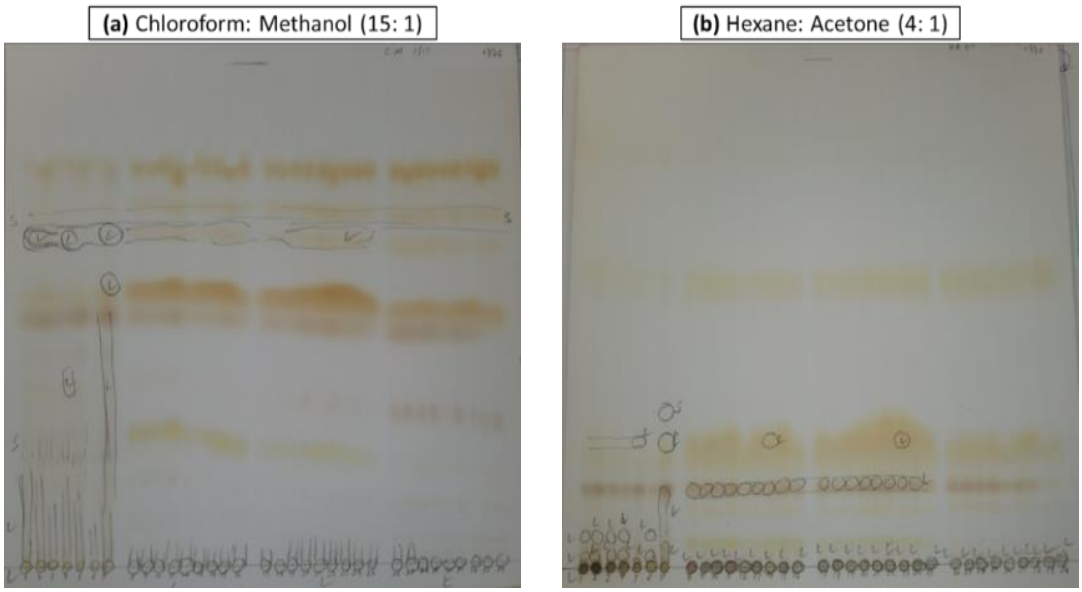


Fig. A2. TLC profiles; (a) chloroform:methanol (15:1) (b) hexane:acetone (4:1) solvents.

MTT Antioxidant Analysis

The results of MTT analysis of methanol extracts from 34 samples were presented in Fig. A3. Significant MTT based antioxidant activity was not observed even by using high concentrations of samples (250 µg/mL). This suggests that potential high-antioxidant activity components were not properly extracted with methanol alone. Therefore, additional initial extraction was employed with hexane in future experiments.

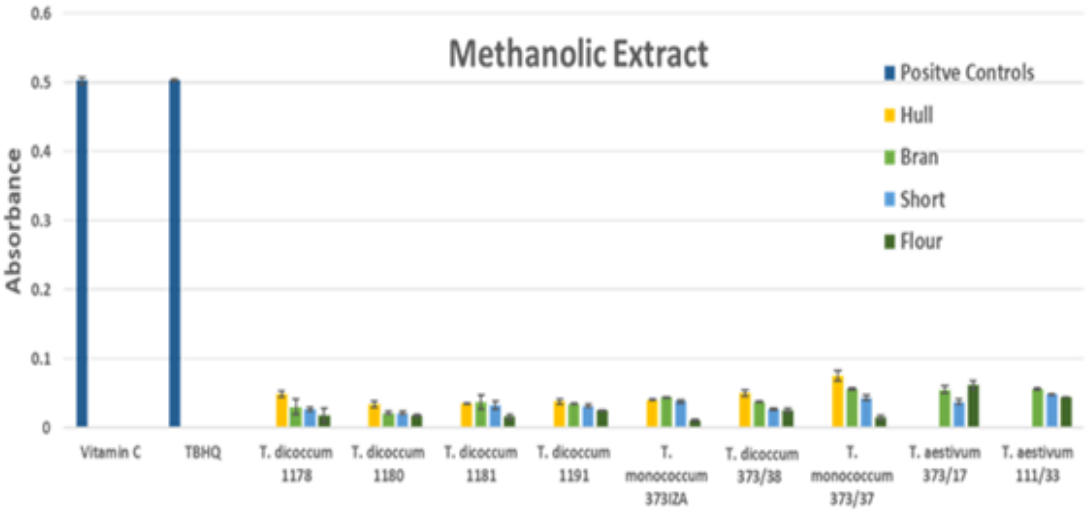


Fig. A3. MTT analysis in methanol extracts.

2.2. Trial 2:

In the second trial, the sample of *T. dicoccum* 1191 which showed the comparatively higher MTT based antioxidant activity, was selected for the purification and identification of bioactive substances. All four fractions of the *Triticum dicoccum* 1191 wheat were sequentially extracted first with hexane (1.4 L, 3×) and then with methanol (1.4 L, 3×) (Fig. A4). After vacuum removal of hexane solvent, 1.33 g of bran, 886 mg of fine bran and 615 mg of flour hexane extract were obtained (Table A3). Similarly, 2.37 g bran, 1.38 g fine bran and 1.06 g flour methanol extracts were collected after removal of methanol solvent. Due to the similar TLC profile, hexane and methanol extracts were also combined to obtain a yield of is 2.83 g /130 g and 4.81g/130 g, respectively.

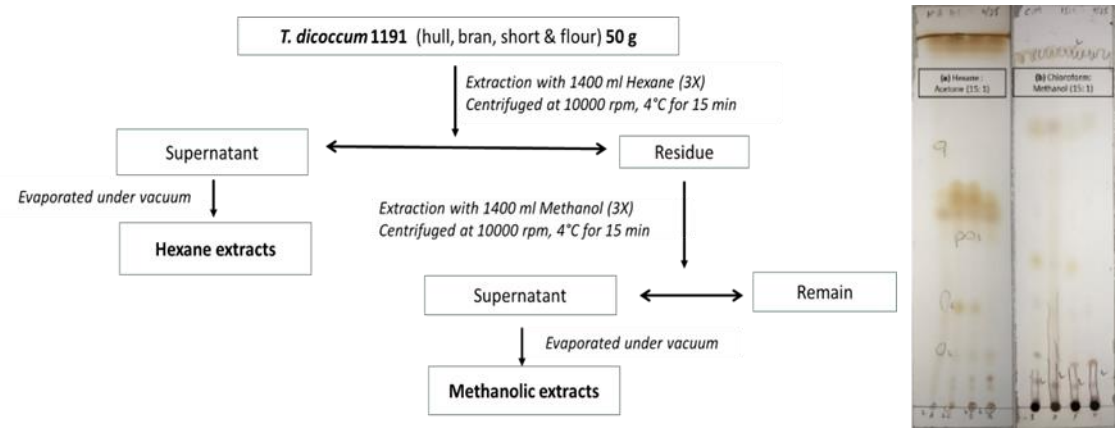


Fig. A4. Sequential extraction and TLC profile of *Triticum dicoccum* 1191.

Table A3. Mass equivalence of hexane and methanol extracts.

***Triticum dicoccum* 1191**	**Sample (g)**	**Hexane Extract (g)**	**Methanol Extract (g)**
Husk	50.8	0.4263	1.6855
Coarse bran	50.4	1.3258	2.3659
Fine bran	30.1	0.8862	1.3799
Flour	50.3	0.615	1.0651

Subsequently, the resulting hexane extracts were purified through MPLC and PTLC, and some fractions of hexane extracts were characterized by using NMR and GC-MS methods. Briefly, a portion (0.93 g) of the hexane extract (*T. dicoccum* 1191) was isolated by using a silica gel MPLC column (ACE, 51×450 mm) with elution of hexane-acetone gradient (10:1, 4:1, and 2:1 v/v) and 12 fractions were collected at a rate of 3 mL/min.

The collected fractions were applied to TLC and the fractions with a similar profile were combined into 3 main groups, A (91 mg), B (415 mg) and C (338 mg) (Fig. A5).

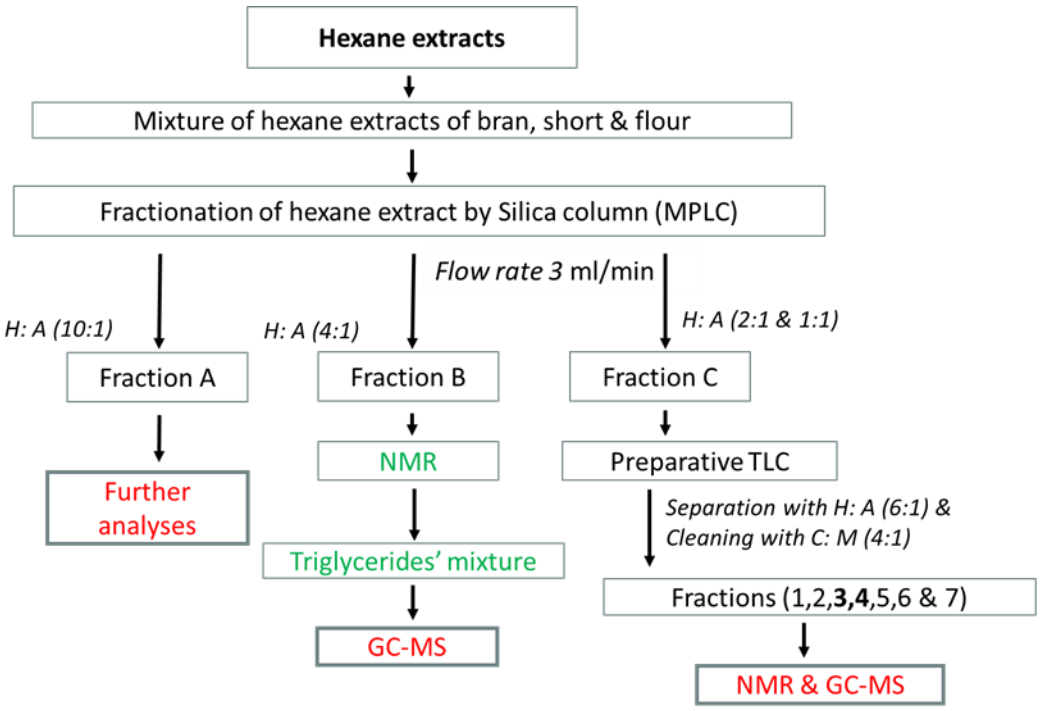


Fig. A5. Workflow-flow chart for the purification and identification of hexane extract.

- Fraction A is stored for further analysis.
- NMR and GC-MS analyses were performed on fraction B. NMR peaks showed a significant proportion of triglycerides and fatty acids. ^{1}H and ^{13}C NMR spectra of fraction B were presented in Figure A6.

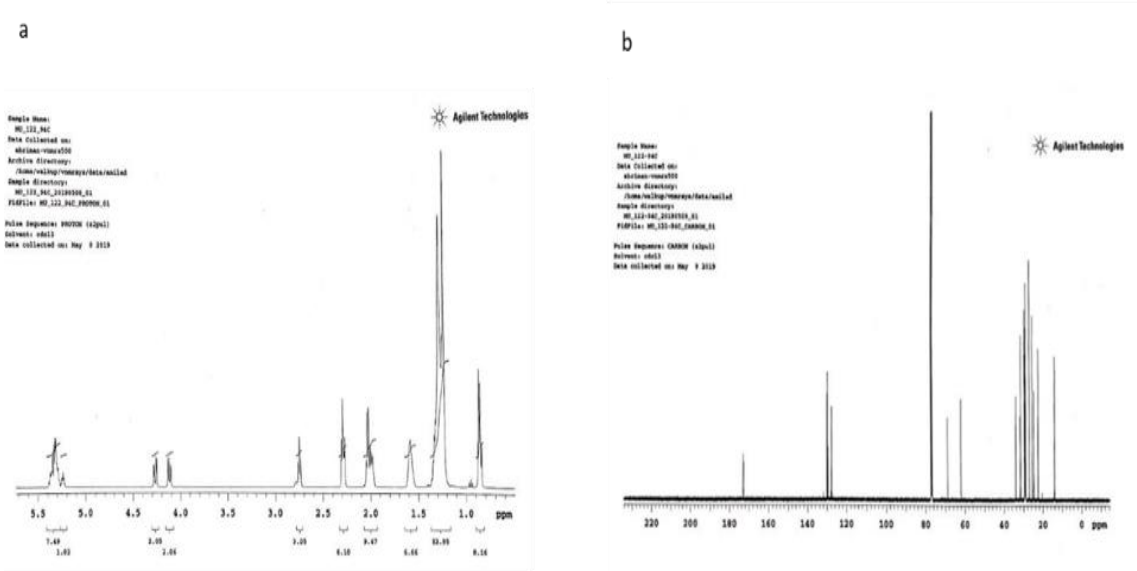


Fig. A6. (a) ^{1}H NMR and (b) ^{13}C NMR of Fraction B.

- The C fraction was separated by PTLC and then the 3rd and 4th sub-fractions were analyzed with GC-MS.

The fraction C (40 mg) was applied twice on the silica plates (250 µm) using a hexane-acetone (6:1, v/v) solvent system. As a result, 7 distinct bands were obtained, which were scraped off and eluted with the help of chloroform-methanol (4:1) solution. After solvent removal, subfractions of C-1 (1.8 mg), C-2 (2.2 mg), C-3 (16.2 mg), C-4 (8.3 mg), C-5 (7.2 mg), C-6 (3.5 mg), and C-7 (2.6 mg) were obtained.

Finally, B fraction and the C-3 sub-fraction were further identified with the help of GC-MS. Fig. A7 shows the chromatogram of fraction B. In this fraction, the presence of palmitic, linoleic and oleic acids was observed as a result of comparison with the FAME-37 standard. The GC-MS chromatogram of the C-3 subfraction is given in Fig. A8, and palmitic, linolenic, linoleic, oleic and stearic acids were identified in the GC chromatogram.

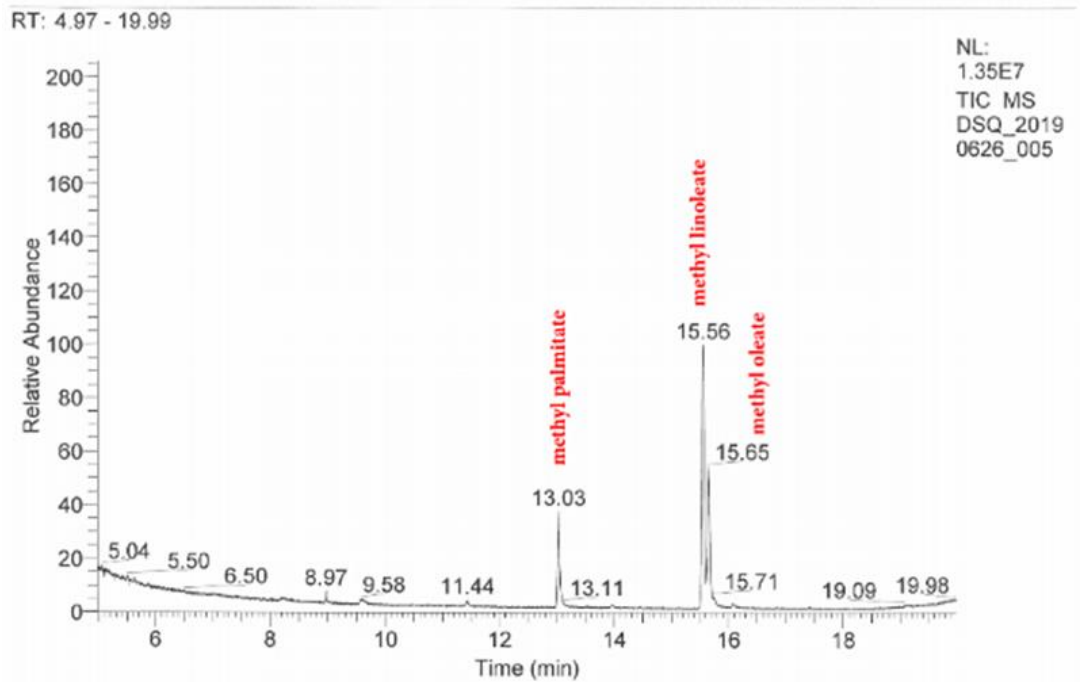


Fig. A7. GC-MS profile of fraction B.

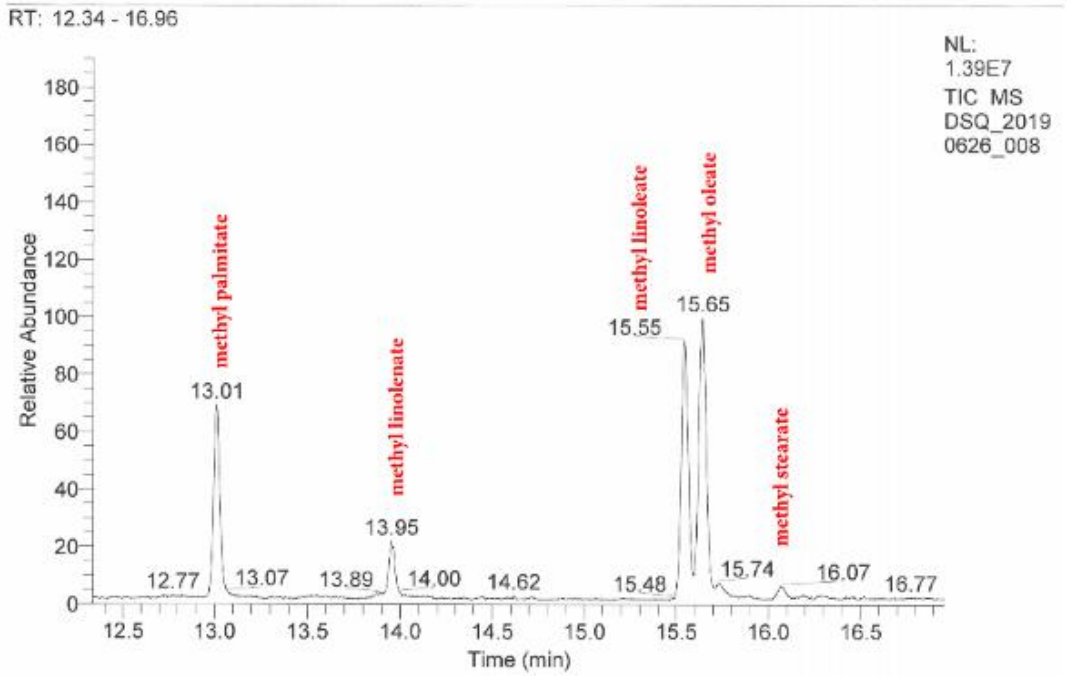


Fig. A8. GC-MS profile of the C-3 subfraction.

2.3. Trial 3:

The third trial involved the hexane-methanol extraction and TLC analysis of whole wheat samples reconstituted by mixing coarse bran, fine bran, and flour fractions of individual wheat varieties. In addition, the antioxidant activity of whole hexane and methanol extracts was determined by *in vitro* antioxidant analyses such as MTT and LPO.

All whole wheat samples were lyophilized and extracted with hexane and methanol (Fig. A9). Briefly, about 10 g of ground sample was mixed with 200 ml of hexane (×1) and extracted for about 16 hours. The mixture was then centrifuged at 10000 rpm (4 °C) for 15 min. The obtained supernatant was evaporated using a rotary evaporator at 35 °C. The resulting residue was then extracted with methanol (×1). Mass balances were calculated for hexane and methanol extracts of all samples (Table A4).

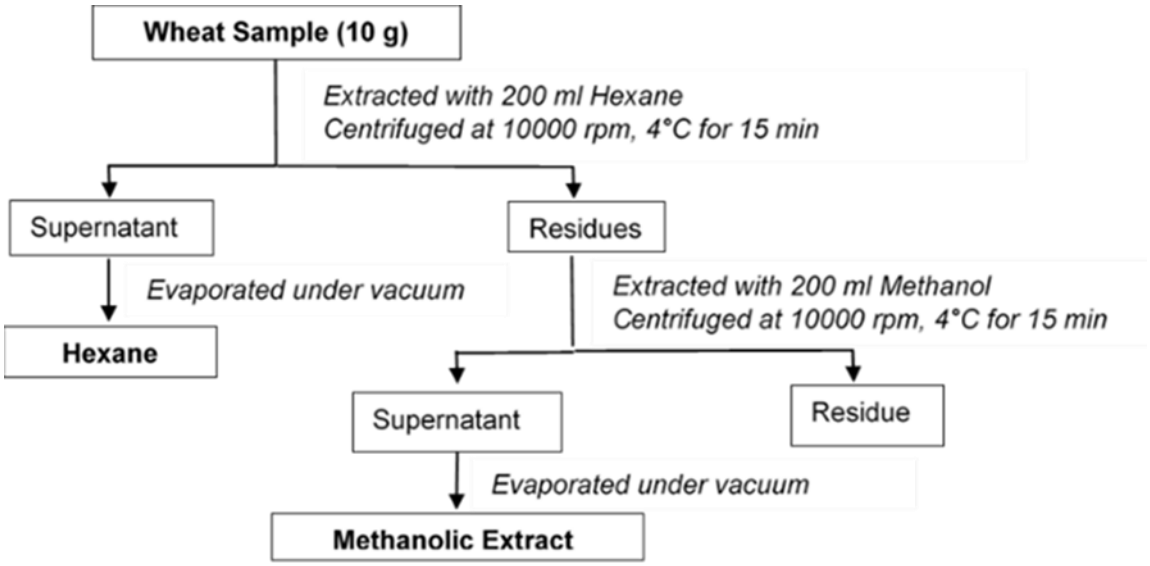


Fig. A9. Flow chart of hexane-methanol sequential extraction of whole wheat samples.

Table A4. Mass equivalence of hexane and methanol extracts.

Whole wheat samples	**Lyophilized sample (g)**	**Hexane extract (mg)**	**Methanol extract (mg)**
Triticum dicoccum 1178	9.2	144.7	202.4
Triticum dicoccum 1180	8.6	111.3	217.2
Triticum dicoccum 1181	9.5	150.9	241.2
Triticum dicoccum 1191	7.8	121.1	171.1
Triticum monococcum 373IZA	7.7	100.7	210.8
Triticum dicoccum 373/38	9.2	145.2	243.4
Triticum monococcum 373/37	7.7	128.4	180
Triticum aestivum 373/17	8	118	175.6
Triticum aestivum 111/33	8.4	98.9	175.8

TLC

TLC analysis of hexane and methanol extracts of whole wheat extracts were performed in both polar (chloroform-methanol, 15:1) and non-polar (hexane-acetone, 4:1) solvent systems (Fig. A10). All extracts exhibited the presence of both non-polar (fatty acids, triglycerides, etc.) and polar (sugars, alcohols, etc.) compounds.

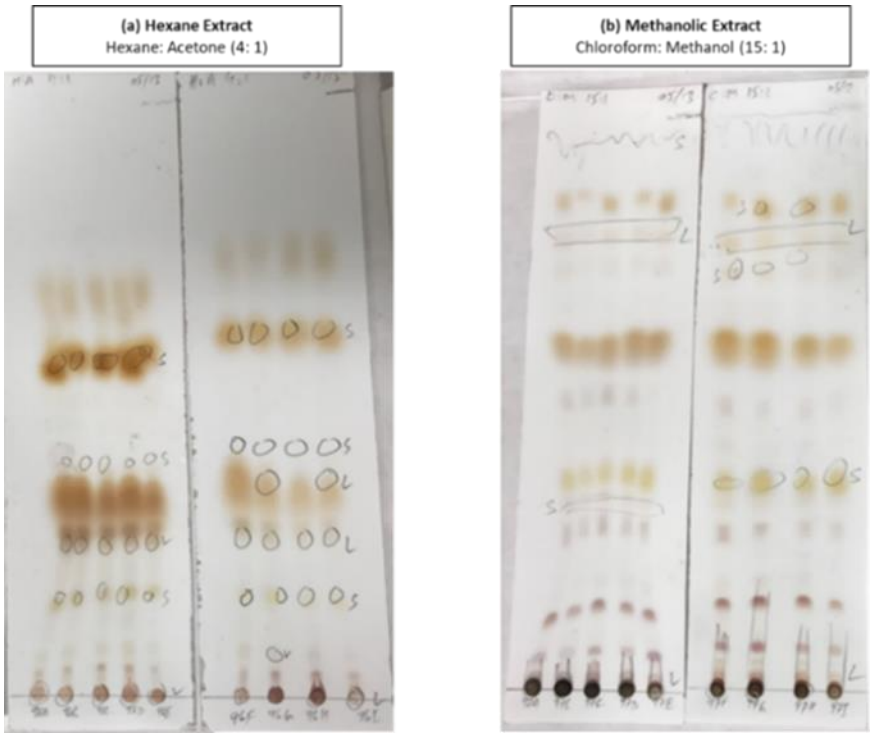


Fig. A10. TLC profiles of hexane and methanol extracts of whole wheat samples.

MTT Antioxidant Analysis

The results of MTT antioxidant analysis of hexane and methanol extracts are given in Fig. A11. Antioxidant activity of methanol extracts was found to be very low as compared to that of hexane extracts with an equal sample concentration of 100 µg/mL.

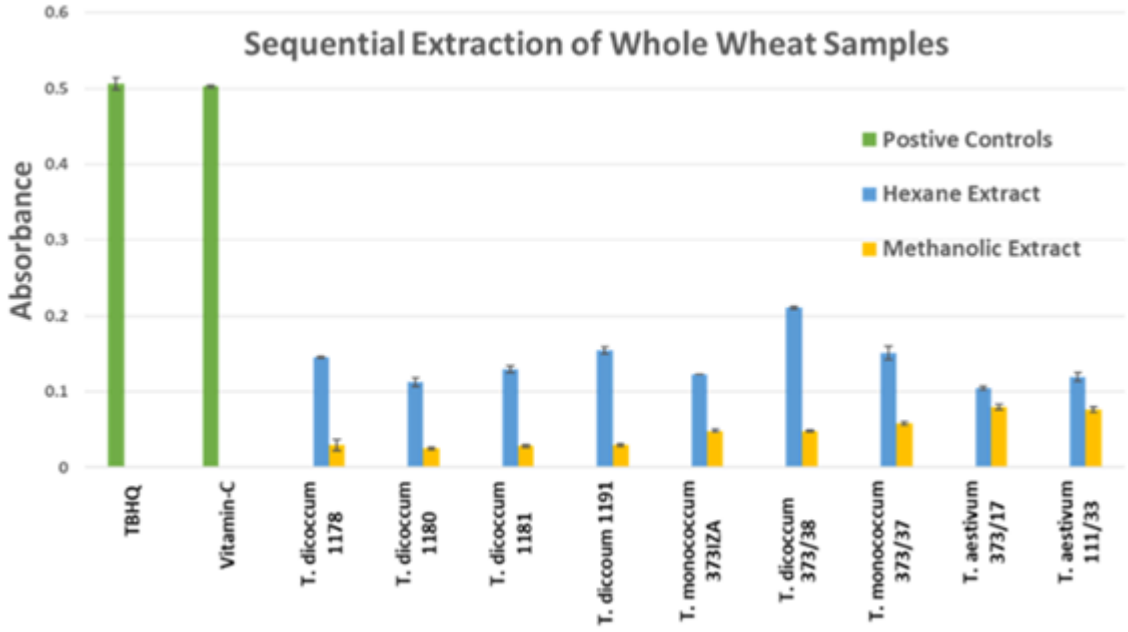


Fig. A11. MTT antioxidant analysis of whole wheat samples (SD±2).

Lipid Peroxidation Inhibitor Analysis (LPO)

In a liposomal model system, the results of comparing the lipid peroxidation inhibitory activities of hexane and methanol extracts with commercial antioxidant controls are presented in Fig. A12. Lipid peroxidation was initiated by Fe (II) and the rate of peroxidation was monitored as a function of time by the decrease in fluorescence intensity.

The hexane and methanol extracts were used at 100 ppm concentrations, whereas commercial antioxidants; BHA, BHT, and TBHQ were used at 1.66 ppm, 2.2 ppm, and 1.8 ppm concentrations, respectively. Similar to MTT results, higher antioxidant activities were observed in hexane extracts of wheat varieties excluding bread wheat samples (*T. aestivum* 373/17 and *T. aestivum* 111/33), where both hexane and methanol extracts have comparatively similar activities.

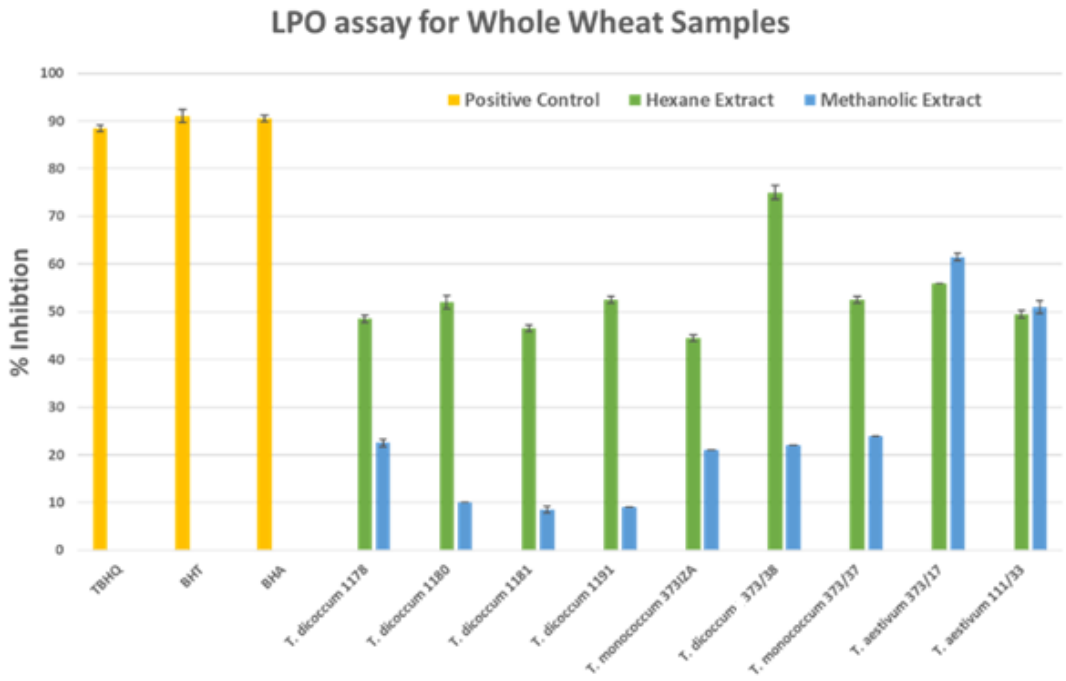
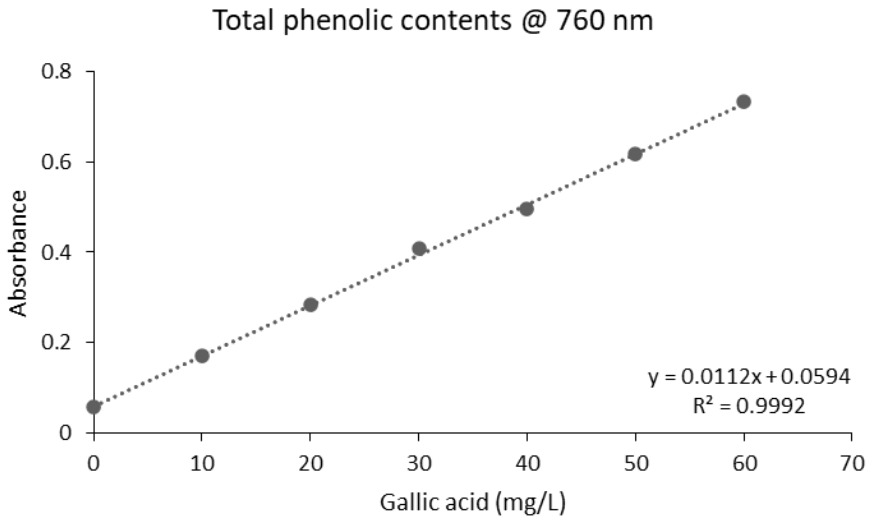


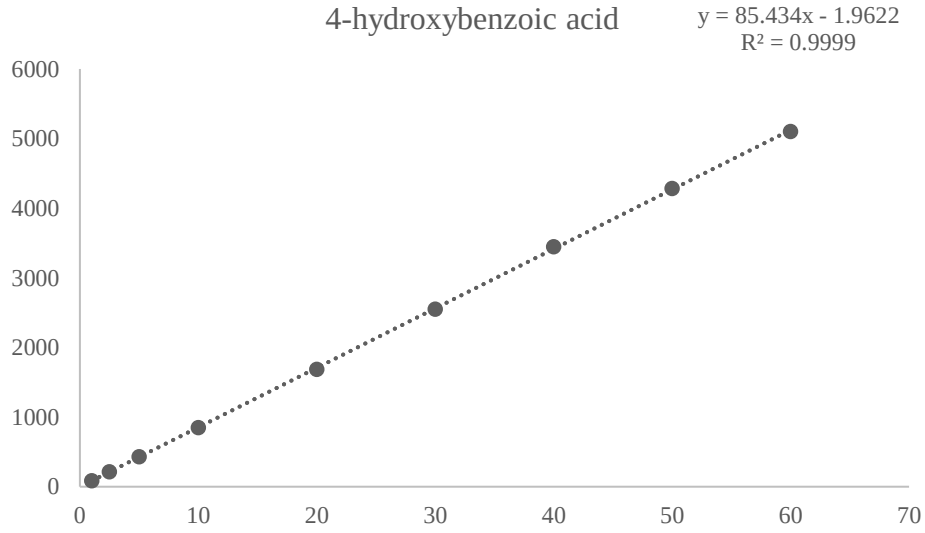
Fig. A12. Results of LPO inhibition analysis of hexane and methanol extracts (SD±2).

ANNEX 3 – Calibration Curves

2.1. Galic acid calibration



2.2. Phenolic acid calibration



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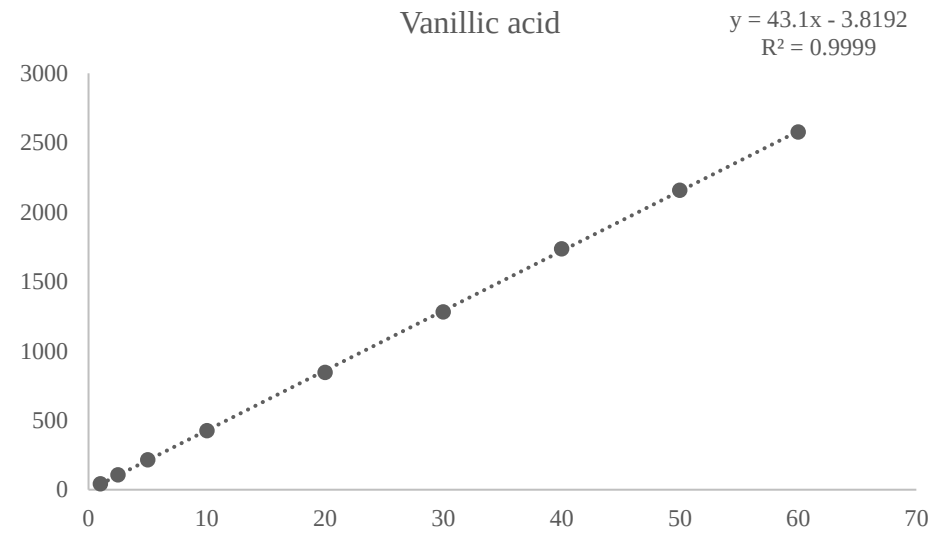
Vanillic acid
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1500
1000
500
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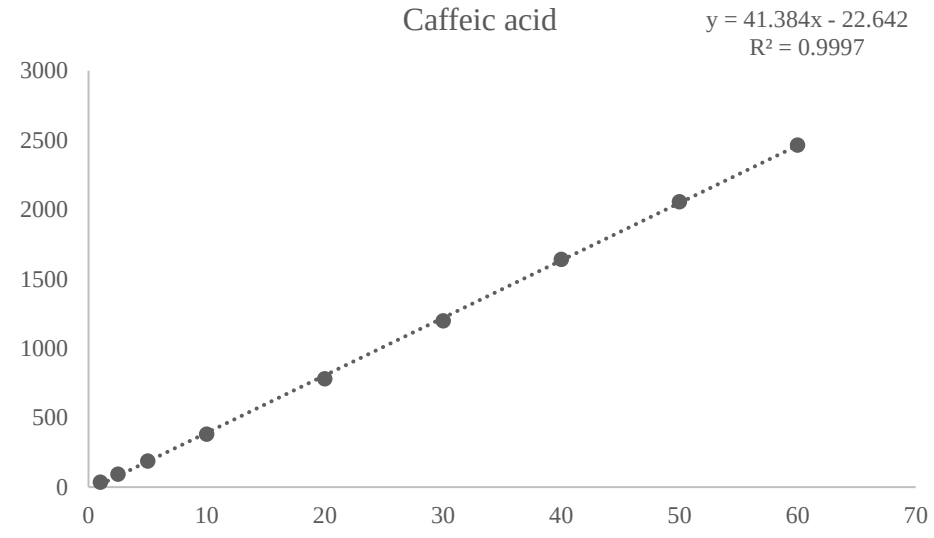
0
10
20
30
40
50
60
70


Caffeic acid
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1000
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0
0
10
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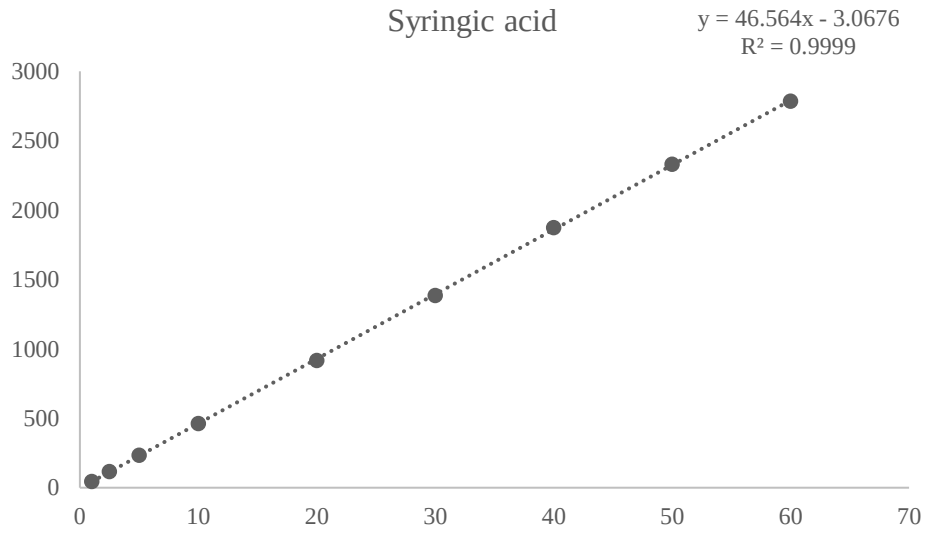
40
50
60
70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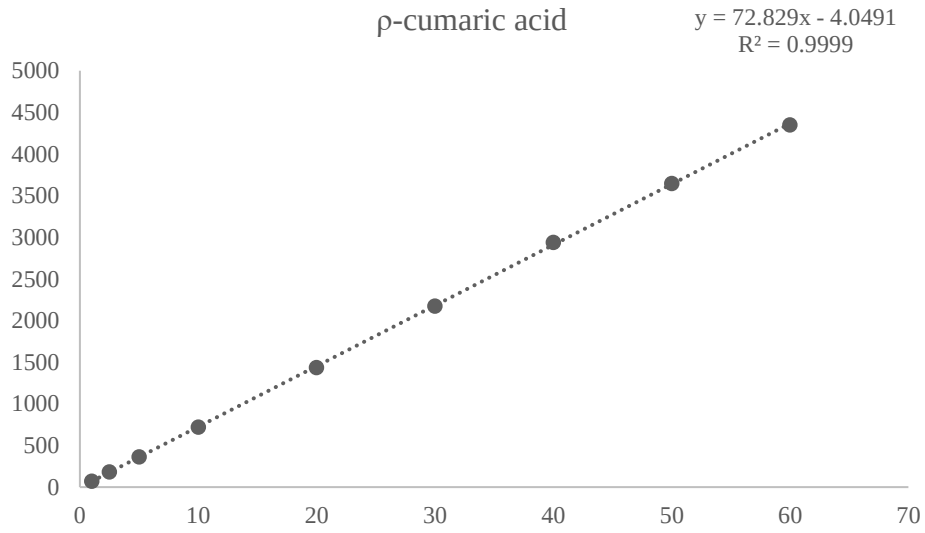
60
70


ρ-cumaric acid
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R² = 0.9999
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30
40
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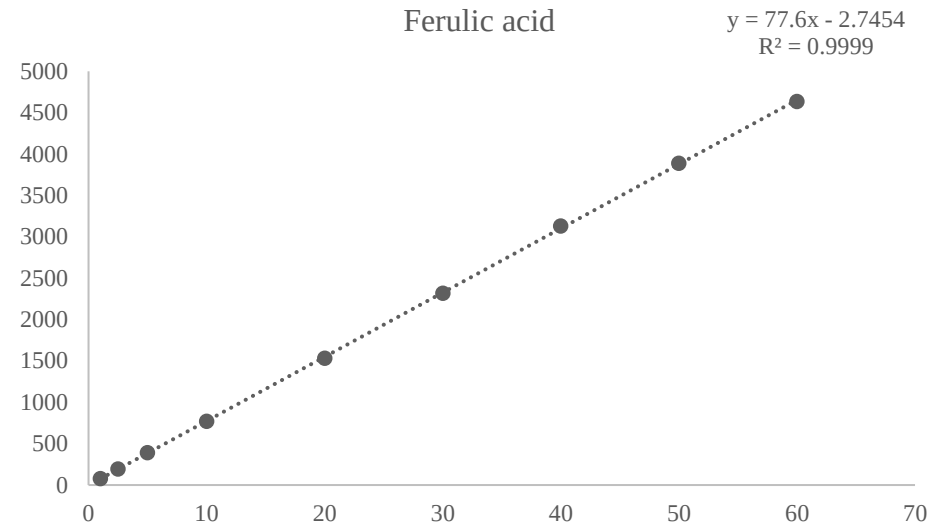
60
70


Ferulic acid
y = 77.6x - 2.7454
R² = 0.9999
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3000
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2000
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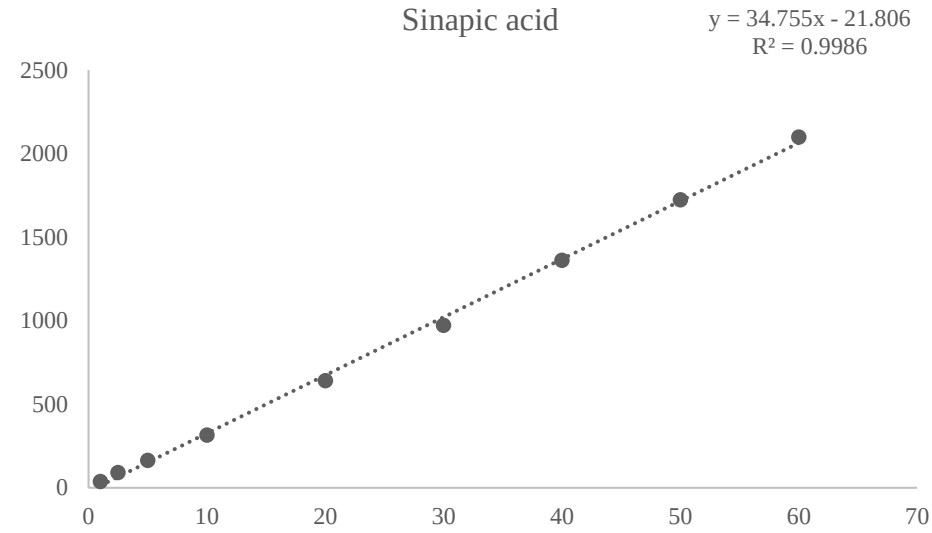
10
20
30
40
50
60
70


Sinapic acid
y = 34.755x - 21.806
R² = 0.9986
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500
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0
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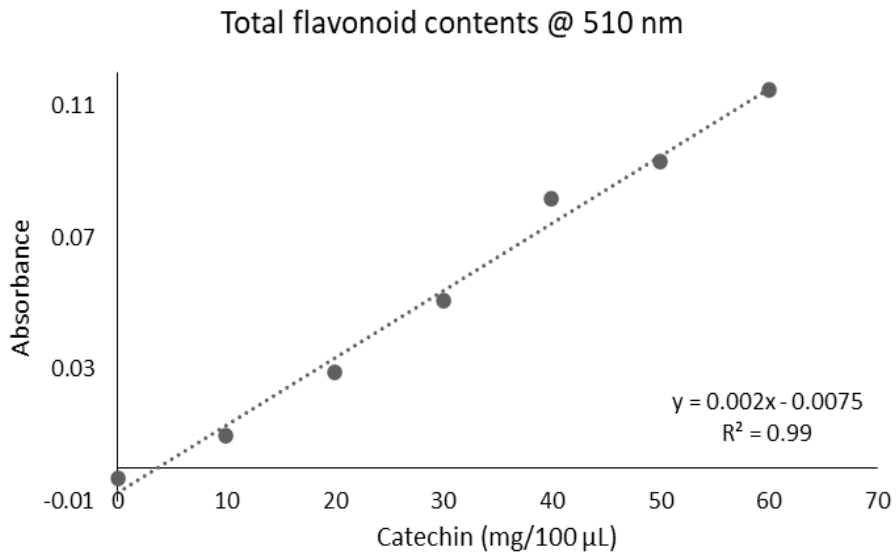
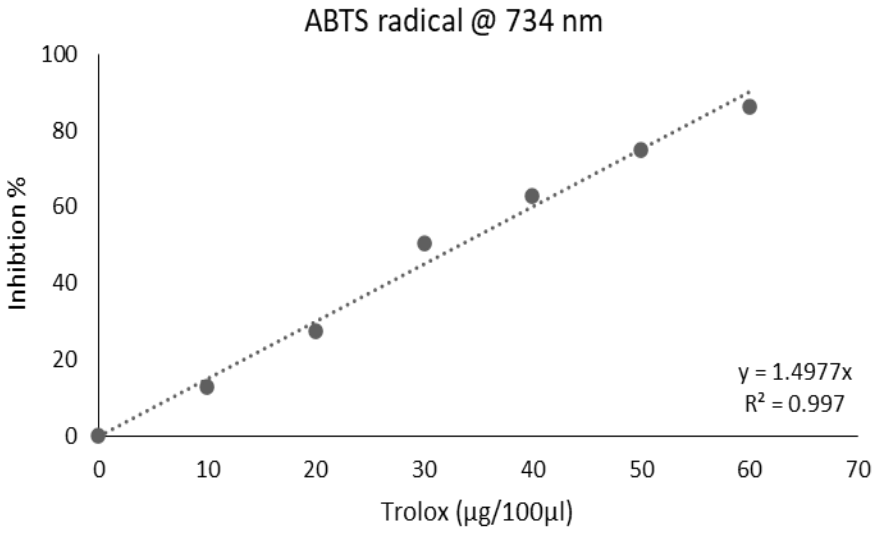
20
30
40
50
60
70

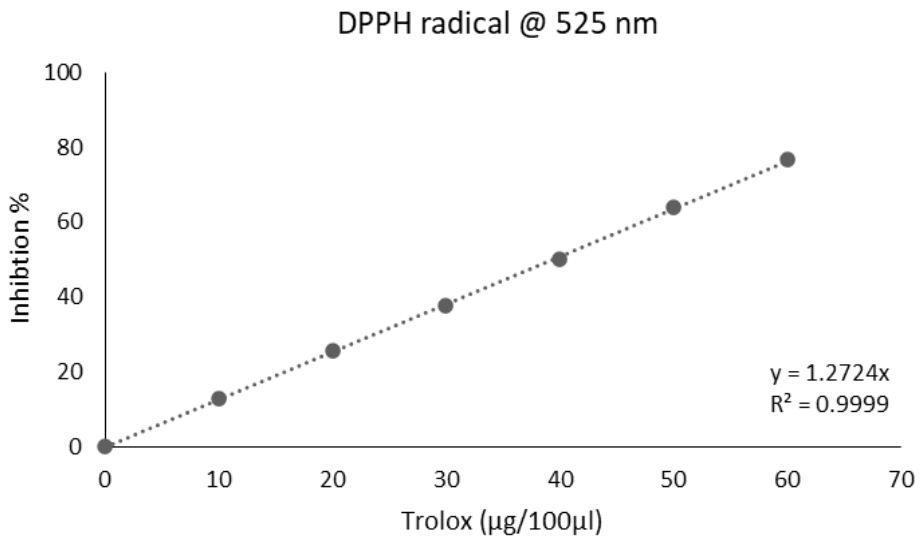
2.3. Catechin calibration



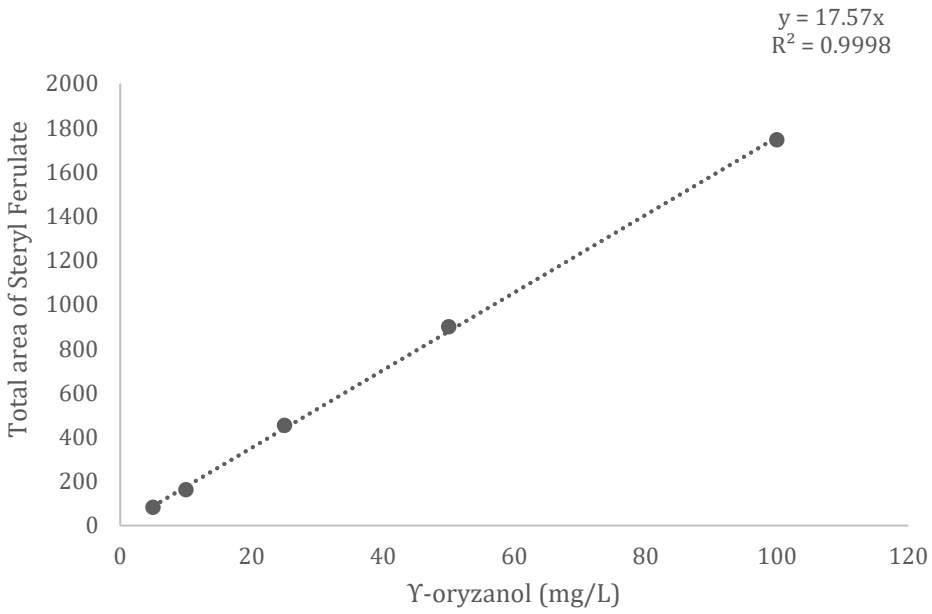
2.4. Trolox calibration for ABTS



2.5. Trolox calibration for DPPH



2.6. γ-oryzanol calibration



ANNEX 4 - Publications Derived from Thesis

Akram, M.U., Yılmaz, R., Salantur, A. and Uygun, U. (2023), Bioactive compounds in lipids of selected wheat genotypes. Int J Food Sci Technol, 58: 6049-6058. https://doi.org/10.1111/ijfs.16713

ANNEX 5 - Poster Derived from the Thesis

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