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Impact of simulated *in vitro* gastrointestinal digestion on phenolic compounds and the antioxident potential of olive pomace

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Abstract

The study's aim was to investigate the impact of laboratory-imitated digestion, including mouth, gastric, and intestinal phases of olive pomace on the stability, bioaccessibility, and recovery of phenolic compounds as well as antioxidant ability. The total flavonoid content (TFC) and total polyphenol content (TPC) were extracted using water or 50% and 100% methanol, ethanol, and acetone. The digested mixture after each phase of digestion was centrifuged and used to assess recovery, bioaccessibility, and polyphenolic stability. Compared to other solvents, 100% methanol and ethanol extracts showed the highest values of TPC, TFC, half-maximal inhibitory concentration (IC_{50}) of 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) IC_{50}. The recovery rates of TPC, TFC, DPPH IC_{50}, and ABTS IC_{50} decreased in a descending order during the gastrointestinal phases as follows: mouth > stomach > intestines. After gastric (27.20%) and intestinal (26.79%) phases, the TPC bioaccessibility index in olive pomace increased significantly, which was statistically similar to the oral phase (21.20%). For TFC, the bioaccessibility rate did not change significantly after mouth and intestinal phases. There were no significant differences in flavonoids and antioxidant scavenging activities among the three phases of digestion. The pellet fractions had higher phenolic levels and better free radical scavenging activity in all phases of digestion than chyme-soluble fractions. TPC or TFC had a significant and positive relationship with Pearson correlation coefficient (r = 0.891–0.994) with DPPH and ABTS scavenging rates in oral, gastric, and intestinal digestion phases. Overall, our research could pave way for the industrial application of olive pomace waste as a possible food ingredient to generate functional foods with beneficial health effects.

Keywords: antioxidant activity; bioaccessibility; olive pomace; phenolics; stability

Introduction

The Mediterranean region highly values the olive plant for its significant economic, environmental, and social benefits, making it one of the most well-known fruit plants (Nunes *et al.*, 2021). In 2020, the global production of olive oil was 3.2 million metric tons, with the Mediterranean nations producing 90% of the total production (Mili and Bouhaddane, 2021). The olive oil business is fast rising, making it one of the most important agro-food economic sectors in Saudi Arabia. Every year, the region of Al-Jouf in Saudi Arabia produces

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10,000 tons of oil and 15,000 kg of table olives, resulting in thousands of tons of olive pomace (OP), which is a good source of bioactive substances (Alshammari and Shahin, 2022; Skaltsounis *et al.*, 2015). Indeed, olive oil's organoleptic properties, combined with recent developments in health properties, result in an increase in olive oil production and consumption (Banias *et al.*, 2017). Small producers adopt conventional pressing methods, while large-scale factories use two- and three-phase centrifugation techniques to produce olive oil (Qdais and Alshraideh, 2016). Olive pomace is a significant byproduct of olive oil production and is known for its high phenolic content (Malapert *et al.*, 2018). These phenolic compounds have uses in the medicinal, food, and cosmetic industries (Rodrigues *et al.*, 2017). However, about 4 million tonnes of olive pomace are produced for every 5.8 million tons of processed olives, accounting for roughly 65% of the initial weight (Moreno-Maroto *et al.*, 2019). Although olive pomace contains many more polyphenols than oil, many phenolics (98%) stay in byproducts after oil production (Mrabet *et al.*, 2019; Radić *et al.*, 2020). Sugars (polysaccharides) and dietary fibers (10% hemicellulose, 15% cellulose, and 27% lignin), protein, unsaturated fatty acids, minerals, and polyphenols are the main components of olive pomace (Ribeiro *et al.*, 2021). The phenolic components of olive pomace are made up of oleocanthal, oleacein, oleuropein, hydroxytyrosol (about 70% of the total phenolics), and tyrosol (Rubio-Senent *et al.*, 2012). Many fruit byproducts, such as olive pomace, contain substantial quantities of bioactive compounds and nutrients that have yet to be explored completely, but they are mostly treated as underutilized agro-waste by the food industry, primarily used in limited cases for animal feed or discarded, causing environmental pollution (Lai *et al.*, 2017).

Currently, industry and consumers are exploring new sources of natural antioxidants from plants because of their antioxidative, anti-inflammatory, and anticarcinogenic potential for prevention of chronic diseases (Zhang *et al.*, 2015). Dietary fibers aid in the transportation of phenolic substances throughout the gastrointestinal digestive system and protect it from oxidative destruction. Furthermore, they can impede enzyme diffusion and entrap both bound and unbound phenolic compounds, thus limiting their bioaccessibility (Jakobek and Matić, 2019). Ribeiro *et al.* (2021) discovered that fatty acids and dietary fiber could transport phenolic molecules throughout the gastrointestinal tract, making both absorbable and non-absorbable fractions having more antioxidant abilities.

In the *in vitro* gastrointestinal digestive system, the plant matrix undergoes mechanical and biochemical disintegration. This releases bioactive compounds in the gut or upper intestinal followed by their absorption and exerting their biological effects (González-Aguilar *et al.*, 2017). Despite the initial loss of more phenolics during digestion, the stomach releases bound phenolics, allowing for their recovery. Bacterial enzymes in the large intestine, where fermentation of fibrous material takes place, release even unreleased phenolics from food, with significant health benefits and assisting in maintaining a healthy gut (Padayachee *et al.*, 2017). Accordingly, *in vitro* gastrointestinal digestion models have been used in research to figure out how the food matrix and food components affect the bioavailability of bioactive substances from different food sources (González-Aguilar *et al.*, 2017; Jakobek and Matić, 2019). It is important to use solvents and technologies that are safe for the environment, can extract bioactive compounds efficiently in a shorter time, and are inexpensive (Galanakis, 2012). This allows for the acquisition of natural components for the development of new food items while simultaneously boosting the olive oil sector's financial and ecological status and promoting a sustainable agricultural framework.

We conducted this research to assess phenolic compounds and antioxidant ability of olive pomace using different solvents. We also investigated the influence of laboratory gastrointestinal digestion on the recovery, bioaccessibility, and stability as well as antioxidant activities of phenolic compounds. The findings of this study would assist in elucidating the possible health advantages of olive pomace bioactive components.

Materials and Methods

Materials

Olive pomace was obtained from an olive oil factory at Al-Jouf olive fields (National Agricultural Development Company [NADEC], Saudi Arabia). The pomace was dried in shade, milled into powder using a grinder (Gold Mill, GM-203, South Korea), placed in polyethylene bags, and kept at 4°C for further use. All the chemicals used were of the highest standard.

Methods

Olive pomace extract preparation

The extract of olive pomace was prepared following the method adopted byAl-Farsi and Lee (2008). Exactly 3 g of powdered material was mixed with 100 mL (1:50 w/v) of double distilled water or 50% and 100% organic solvents (methanol, ethanol, or acetone) and left to stand overnight at 45°C, with constant stirring. The mixture was then filtered using Whatman filter paper No. 1. The extraction process was repeated twice, and the filtrates were combined. A rotary evaporator

(Heidolph Instruments, Laborota 4003 Control, Schwabach, Germany) was employed to remove solvent from the filtrate (60°C; at reduced pressure), followed by freeze-drying. The dried extract was further used for analyzing total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity.

Determining total phenolic content

The total phenolic content of pomace extracts was determined by following the method referred by Waterhouse (2002) using Folin–Ciocalteu reagent. The results are given as gallic acid equivalents (GAE) mg/gram (gm) of sample.

Determining total flavonoid content

The TFC of pomace extracts from different solvents was analyzed according to the method referred by Kim *et al.* (2003). The results are given as milligram (mg) catechin equivalents (CE)/gm of sample.

Determining antioxidant activity

Determining DPPH radical-scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging rates of various samples were measured as per Chang ShangTzen *et al.* (2001). The DPPH radical scavenging rate was calculated as follows:

$$\text{DPPH scavenging } (\%) = \frac{[A_0 - A_1]}{A_0} \times 100,$$

where A_0 is the absorbance of blank sample and A_1 is the absorbance of sample extract. The level of antioxidants required to drop the initial DPPH rate by 50% is called the half-maximal inhibitory concentration (IC_{50}).

Determining ABTS radical scavenging capability

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical scavenging rate of the extract was determined according to the method described by Thaipong *et al.* (2006). The findings are given as Trolox equivalent (TE) per gram, based on the Trolox calibration curve. The level of antioxidants required to lower the initial ABTS free radical concentration by 50% is known as IC_{50}.

Simulated in vitro *gastrointestinal digestion of olive pomace*

The simulated *in vitro* gastrointestinal digestion method was performed as described by Gong *et al.* (2019), with some modifications. About 5 g of olive pomace was mixed with 3.5 mL of saliva-simulating fluid amylase (20 U/mL), 25 µL $CaCl_2$ (0.3 m/L), and 0.5 mL of water. The mixture was incubated for 30 min in a shaking water bath at 37°C. After incubation, 7.5 mL of simulating gastric fluid, 1.6 mL of pepsin solution (25,000 U/mL), 5 µL of $CaCl_2$ (0.3 mM), and 0.75 mL of water were added to the mouth-digested fluid mixture, and the pH was adjusted to 3 using 1 mL of HCl. The mixture was then incubated for 30 min in a shaking water bath at 37°C. Following this, the gastric chyme was mixed with 11 mL of simulated intestinal fluid, 5 mL of pancreatin (800 U/mL), 2.5 mL of bile salt (160 mM), 40 µl of $CaCl_2$ (0.3 mM), and 1.25 mL of water. After adjusting the pH at 7 (1 mL of NaOH), the mixture was incubated for 2 h at 37°C in a shaking water bath. At the end of the digestion, the mixture was cooled on ice before transferring to a dialysis tube at a 1-kD cutoff. The digested samples were dialyzed against NaCl (10 mM) for 24 h and freeze-dried for further analysis. Following each phase of digestion, triplicate samples were withdrawn. The samples were subjected to centrifugation (8,000 rpm, 12 min, 4°C) to separate the chyme soluble fraction (CSF) from the chyme insoluble fraction (CIF) or pellet. Both fractions were lyophilized, and TPC and antioxidant activity were determined.

Recovery index and bioaccessibility index

To assess the impact of food matrix composition on the simulated gastrointestinal digestion of phenolic compounds, the recovery and bioaccessibility percentages were calculated using the previously described method (Ortega *et al.*, 2011). At each digestion phase, the following formula was used to calculate the amount of phenolic compounds in the whole digested fraction (CSF+CIF) of olive pomace:

$$\text{Recovery index } (\%) = \frac{PC_{DF}}{PC_{TF}} \times 100,$$

where PC_{DF} is the total phenolic amount (mg) in CSF and CIF (CSF+CIF) and PC_{TF} is the total phenolic amount (mg) determined in the food matrix.

The proportion of phenolic molecules solubilizing in CSF after intestinal dialysis is known as bioaccessibility. This index represents the proportion of phenolic compounds that enter the circulatory system. The index is calculated using the following equation:

$$\text{Bioaccessibility index } (\%) = \frac{PC_S}{PC_{DF}} \times 100$$

where PC_S is the total phenolic content in CSF after the dialysis phase (mg), and PC_{DF} is the total phenolic content in the digested sample (CSF+CIF) after duodenal digestion (mg).

GC-MS analysis of olive pomace

The composition of olive pomace 80% methanol extracts from undigested and digested samples was measured using a gas chromatography apparatus linked with a mass spectrometer (GC-MS; Turbomass; Perkin Elmer Inc., Waltham, MA, USA). Helium (0.8 mL/min) served as a

mobile phase and the Innowax FSC column (60 m × 0.25 mm; 0.25-µm film thickness) as a stationary phase for analysis. A 40:1 split ratio was used while injecting the sample volume (0.1 µL). The GC oven was set to 60°C for 10 min, increased to 220°C for 10 min at a rate of 4°C/min, and finally increased to 240°C at a rate of 1°C/min. The temperature of the transfer line was 280°C whereas the injector was set at 250°C. The MS detection range was 35–450 m/z, and the ionization energy was 70 eV. The compounds were detected by comparing the retention time to reference standards and in the Wiley GC/MS Library (McLafferty *et al.*, 1989) and Adams Library (Adams, 2007).

Statistical analysis

The data were statistically analyzed using the SPSS software package 19.0 (SPSS Inc. Chicago, IL, USA). All tests were performed in triplicate, and the results were given as mean±standard deviation. One-way ANOVA was used to determine significant differences between groups of different solvent extracts and digestion phases. Differences between mean values were deemed significant at $p \leq 0.05$, according to Tukey's test. Pearson correlation analysis was applied to find the association between total phenolic and flavonoid contents and the antioxidant activity.

Results

Total phenolic, flavonoid, and antioxidant activity of different olive pomace solvent extracts

Table 1 shows the IC_{50} of DPPH and ABTS as well as TPC and TFC in different olive pomace extracts. The pomace extract's TPC varied significantly ($p < 0.05$) depending on the solvent type: 100% methanol extract had the highest TPC (84.6 mg GAE/100 g), followed by 100% ethanol extract (72.1 mg GAE/100 g). In contrast, the remaining solvent extracts had significantly lower TPC, with the following order: water > 50% aqueous acetone > 50% aqueous methanol > 50% aqueous ethanol > 100% acetone. The 50% aqueous methanol extract contained considerably ($p \leq 0.05$) higher phenolic levels than the aqueous ethanol extract but was lower than the aqueous acetone extract. This could be because water extracts include more non-phenolic components than other extracts, such as carbohydrates and terpenes.

The solvents exhibited a significant ($p \leq 0.05$) variation in TFC content. The 100% methanol and ethanol extracts had significantly higher TFC than other solvent extracts. Except for 100% methanol and ethanol extracts, the following trend showed a decrease in TFC: 100% acetone > 50% aqueous methanol > 50% aqueous ethanol > 50% aqueous acetone. Furthermore, aqueous methanol extracts exhibited statistically similar TFC as aqueous ethanol and acetone; however, the difference was not significant. The effect of solvents on TFC was comparable to that of TPC.

In terms of TFC, 100% ethanol and 100% water extracts followed 100% methanol extract. The extracts' DPPH IC_{50} values were concentration-dependent (Table 2). The DPPH scavenging rate of 100% methanol extract was highest (IC_{50} = 83.6 µg/mL) and that of 100% ethanol extract was second highest. The extract from 50% aqueous acetone had the lowest DPPH IC_{50} (280 µg/mL). The effect of solvent extraction on ABTS IC_{50} led to the results comparable to those of DPPH IC_{50}.

Recovery and bioaccessibility indices

Figure 1 displays the TPC, TFC, DPPH IC_{50}, and ABTS IC_{50} recovery indexes of olive pomace following simulated gastrointestinal digestion (mouth, stomach, and

Table 1. Total phenolic (mg GAE/100 g dry weight [DW]), flavonoid (mg QCE/100 g DW) contents, and DPPH and ABTS IC50 values (µg/mL) of olive pomace extracted with different solvents.

Solvents	TPC	TFC	ABTS	DPPH
Methanol	84.6 ± 2.05^{a}	10.7 ± 0.91^{a}	49.4 ± 4.86^{f}	78.3 ± 2.11^{g}
Ethanol	72.1 ± 2.23^{a,b}	9.4 ± 1.15^{a}	60.9 ± 3.11^{e}	83.6 ± 1.52^{f}
Acetone	28.1 ± 1.4^{c}	5.5 ± 0.51^{b,c}	215 ± 1.55^{d}	147 ± 1.931^{d}
Water	66.1 ± 1.36^{b}	6.2 ± 0.53^{b}	60.4 ± 1.61^{e}	99.4 ± 1.24^{e}
Methanol:water (50:50)	64.8 ± 1.66^{b}	4.17 ± 0.25^{c,d}	250 ± 2.55^{c}	180 ± 1.53^{c}
Ethanol:water (50:50)	57.6 ± 2.04^{b}	4.07 ± 0.32^{d}	239 ± 2.35^{b}	209 ± 1.93^{b}
Acetone:water (50:50)	67.7 ± 1.86^{a,b}	3.7 ± 0.35^{d}	333.2 ± 1.77^{a}	280 ± 2.46^{a}

*Different letters in the same column indicate that the mean difference is significant at $p \leq 0.05$.
ABTS 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid = ; DPPH = 2,2-diphenyl-1-picrylhydrazyl ;TPC = total phenolic contents; TFC = total flavonoid contents

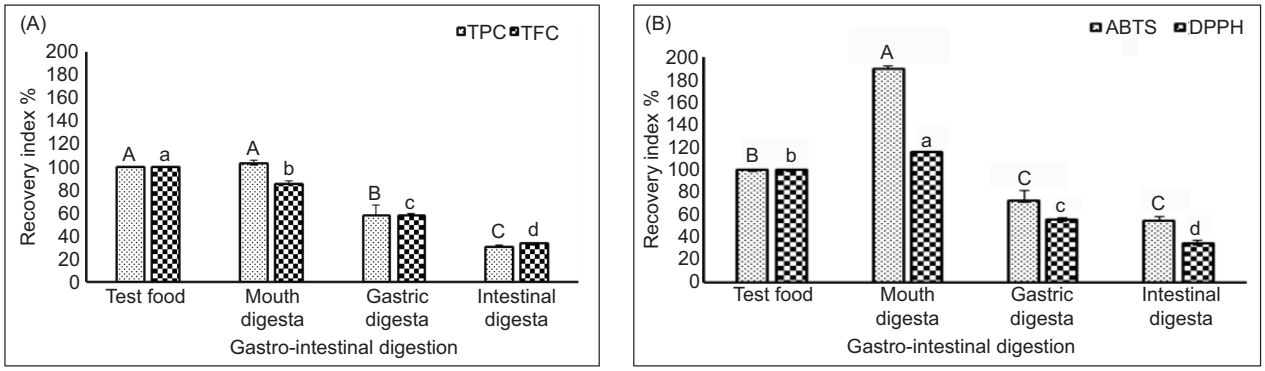

Figure 1. Recovery index (A and B) of TPC, TFC, ABTS, and DPPH after simulated gastro-intestinal digestion (mouth, stomach, and intestinal phases) of olive pomace.

intestines). The values for the test matrix obtained using methanol as an extracting solvent were assumed to represent 100% of TPC or TFC of undigested sample. There was a significant drop in the recovery indexes of bioactive compounds, that is TPC and TFC, as digestion progressed from mouth to the small intestine, the last phase. The percentages of TPC, TFC, DPPH, and ABTS after mouth digestion were 102.6, 85.05, 190.33, and 116.1%, respectively, after gastric digestion lowered to 57.91, 59.94, 80.13, and 55.81%, respectively. At the end of the intestinal phase, there was a significant decrease ($p < 0.05$) in the recovery of TPC, TFC, DPPH, and ABTS to 30.7, 33.92, 55.31, and 55.30%, respectively.

Figure 2 shows that the bioaccessibility index for TPC, TFC, DPPH, and ABTS varied considerably ($p \leq 0.05$). The oral phase lowered the bioaccessibility indexes of TPC, TFC, DPPH IC_{50}, and ABTS IC_{50} ($p \leq 0.05$) to 21.20, 27.50, 21.37, and 22.12%, respectively. After gastric (27.20%) and intestinal (26.79%) phases, the TPC bioaccessibility index in olive pomace increased significantly, which was statistically similar, compared with that of oral phase (21.20%). For TFC, the bioaccessibility rate did not change significantly after mouth and intestinal phases. There were no significant differences in flavonoids and antioxidant scavenging activities among the three phases of digestion.

Phenolic compounds change during simulated *in vitro* gastrointestinal digestion

Figure 3 shows changes in TPC and TFC and antioxidant activity (DPPH IC_{50} and ABTS IC_{50}) in CIF, and CSF during each phase of simulated digestion (mouth, stomach, and intestine). In general, there was a significant

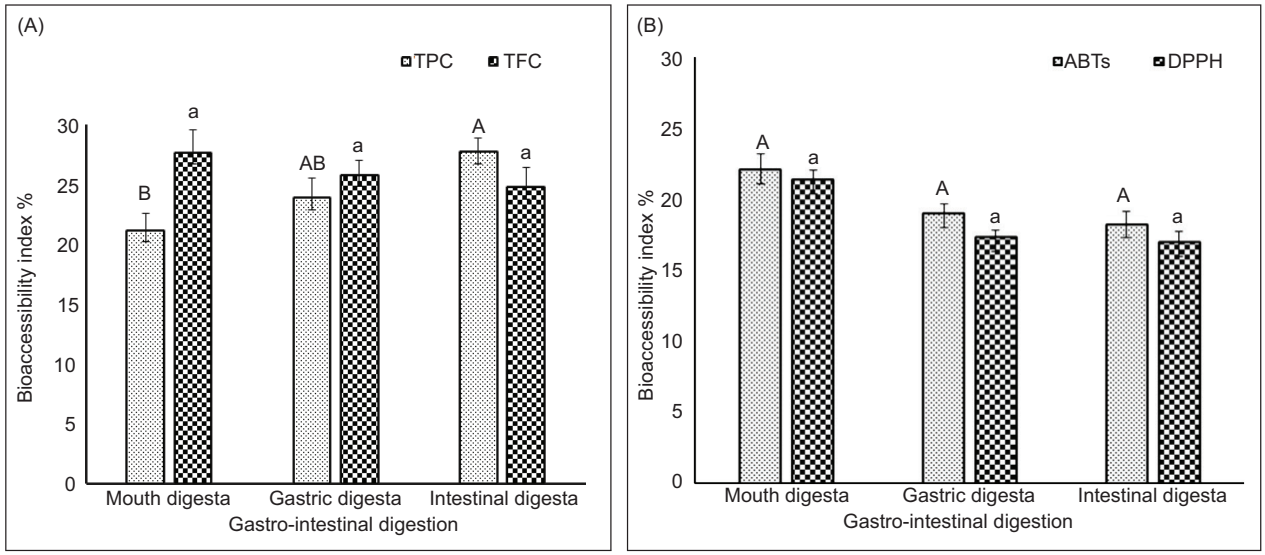

Figure 2. Bioaccessibility index (A and B) of TPC, TFC, ABTS, and DPPH after simulated gastro-intestinal digestion (mouth, gastric, and intestinal phases) of olive pomace.

decrease in bioactive compounds with the progress of digestion from the mouth to the intestinal phase. In the mouth digestion phase, TPC levels rose from 84.0% to 86.83%, augmenting by 2.64%, while TFC decreased from 10.7% to 9.1%, dropping by 9.85%, compared to the undigested (control) sample. Both DPPH and ABTS scavenging rates experienced a spike of 28.90% and 92.97%, respectively, which was comparable to the changes in phenol. This suggests that amylase enzymes release phenolics from olive pomace. TPC and TFC decreased by 42.08% and 27.1.0%, respectively, in the gastrointestinal phase. After intestinal digestion, both DPPH and ABTS decreased by 44.19% and 19.87%, respectively, and TPC and TFC dropped further to 69.30% and 66.18%, respectively. We observed a similar trend with DPPH and ABTS, as they decreased to 65.47% and 44.70%, respectively.

Figure 3 shows the bioactive content of CSF and CIF. In general, the phenolic compounds and antioxidant activity values in CIF were much higher than in CSF. The TPC in CIF after mouth, gastric, and intestinal digestion was 86.43, 37.23, and 18.77 mg GAE/100 g, compared to 18.4, 11.77, and 7.20 mg GAE/1000 g in CSF, respectively. Similarly, after digestion in the mouth, stomach, and intestines, CIF had a higher flavonoid content than CSF, with values of 6.6, 4.6, and 2.6 mg CE/100 g compared to 2.5, 1.6, and 0.9 mg CE/100 g, respectively. After digestion in the mouth, stomach, and intestines, both CIF and CSF exhibited distinct differences in their proportions of DPPH and ABTS scavenging. In oral, gastric, and intestinal digestion phases, CIF had significantly higher ABTS scavenging percentages (73.17, 32.03, and 22.30%, respectively) than the CSF (20.80, 7.53, and 5.0%, respectively). The DPPH scavenging percentages also differed significantly between CIF and CSF at the three digestion phases.

TFC, TPC, and antioxidant activity correlations at different digestion phases

The correlation coefficients between DPPH and ABTS scavenging rates, and TPC and TFC at various phases of gastrointestinal digestion are listed in Table 2. A strong association (r = 0.891–0.994) was discovered between DPPH and ABTS scavenging rates, and TPC and TFC in the oral, stomach, and intestinal phases at either $p \leq 0.05$ or $p \leq 0.01$. Furthermore, all *in vitro* digestion phases demonstrated a robust link between antioxidant activity and TPC.

Identification of olive pomace phenolic compounds at different phases of simulated gastrointestinal digestion

The GC-MS profiles of undigested olive pomace and chyme from oral, gastric, and intestinal digestion phases are shown in Table 3 and Figure S1. Olive pomace had

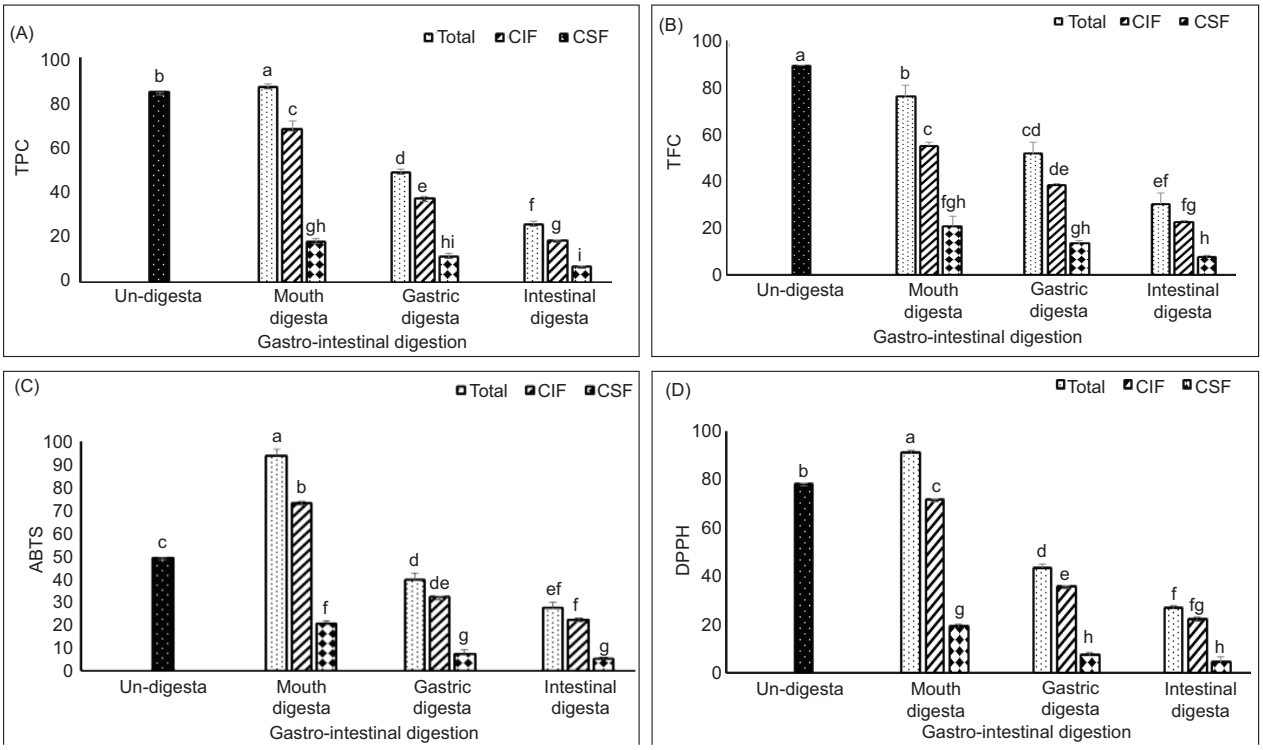

Figure 3. Total phenolic (mg GAE/ 100 g DW), flavonoid (mg QCE /100 g DW) contents, DPPH IC50 (µg/mL) and ABTS IC50 (µg/mL) of olive pomace extracted with different solvents.

Table 2. Correlation coefficients between TPC, TFC, and the antioxidant activity of olive pomace under different phases of gastrointestinal digestion.

	Mouth chyme		Gastric chyme		Intestinal chyme	
	TPC	TFC	TPC	TFC	TPC	TFC
Mouth chyme						
DPPH	0.990**	0.967**	0.994**	0.991**	0.993**	0.991**
ABTS	0.989**	0.964**	0.994**	0.989**	0.993**	0.989**
Gastric chyme						
DPPH	0.981**	0.943**	0.989**	0.982**	0.994**	0.983**
ABTS	0.991**	0.945**	0.990**	0.974**	0.980**	0.970**
Intestinal chyme						
DPPH	0.931**	0.891*	0.949**	0.954**	0.982**	0.967**
ABTS	0.991**	0.959**	0.988**	0.978**	0.977**	0.975**

*Correlation is significant at 0.05 level (2-tailed); **correlation is significant at 0 level.
TPC = total phenolic contents; TFC = total flavonoid contents.

seven distinct peaks with higher peak area percentages. In all, 14, 17, and 10 peaks were discovered in oral, gastric, and intestinal phases.

Discussion

We conducted this study aiming to optimize the extraction of phenolic compounds with high antioxidant capacity using different solvent systems. We also investigated the effect of simulated gastrointestinal digestion of olive pomace on the bioaccessibility and recovery of phenolic compounds and antioxidant potential. According to reports, the harvest season has a significant impact on production and composition of olive pomace, which is frequently transferred and discharged in vast open-air spaces for drying (Valta *et al.*, 2015).

Olive pomace extracts obtained using different solvents showed significant ($p \leq 0.05$) variations in TPC, TFC,

Table 3. The GC-MS composition of (a) undigested olive pomace and pomace chymes from the (b) mouth, (c) gastric, and (d) intestinal phases.

No.	Compound name	Peak area (%)	RT (min)	Bioactivity
(a) Undigested olive pomace				
1.	Oxime-methoxy-phenyl	5.339	6.417	It has anti-inflammatory, antimicrobial, antioxidant, and anticancer activities. Moreover, it is a therapeutic agent (Schepetkin *et al.*, 2021; Surowiak *et al.*, 2020).
2.	2H-Pyran-2-one, 5-ethylidenetetrahydro-4-(2-hydroxyethyl)	18.606	23.603	It has antioxidant properties (Osama *et al.*, 2017).
3.	Hexanedioic acid, bis(2-methylpropyl) ester	23.792	7.686	Not reported
4.	Dibutyl adipate	25.254	0.154	It functions as a plasticizer, skin-conditioning agent, and solvent in cosmetic formulations (Andersen, 2006).
5.	Hexadecanoic acid, methyl ester	27.955	6.564	It has anti-inflammatory, cancer preventive, hepatoprotective, antiarthritic, and anti-coronary properties (Singh *et al.*, 2008).
6.	11-Octadecenoic acid, methyl ester	30.718	7.218	It has antioxidant and antimicrobial properties (Rahman *et al.*, 2014).
7.	Methyl stearate	31.096	9.563	It has a role of a metabolite (Lu *et al.*, 2020).
8.	Octadecanoic acid	31.662	9.356	It has potential antibacterial and antifungal activity (Mahadkar *et al.*, 2013).
(b) Mouth chyme				
1.	Xylitol	18.719	6.51	It prevents demineralization of the teeth and bones, otitis media infections, respiratory tract infections, inflammation, and cancer progression (Ahuja *et al.*, 2020).
2.	Ribitol	20.505	4.60	Not reported.
3.	D-(+)-Talose	21.334	6.25	Not reported.
4.	Meso-erythritol	21.781	7.00	Not reported.
5.	-(2-Bromo-4-methylphenoxy) -N'-([1-(4-nitrophenyl)-2-pyrrolidinyl] methylene) acethydrazide2	29.276	3.19	Not reported.
6.	(Z)-13-docosenamide	34.878	9.59	It has a role of a human metabolite and a plant metabolite (El Mihyaoui *et al.*, 2022).

(*continues*)

Table 3. Continued.

No.	Compound name	Peak area (%)	RT (min)	Bioactivity
(c) Gastric chyme				
1.	Silanol	13.49	1.76	It is used as an intermediate in organosilicon chemistry and silicate mineralogy (Chandrasekhar *et al.*, 2004), and it has an antimicrobial activity (Kim *et al.*, 2006).
2.	D-(-)Fructofuranose	19.875	3.28	It has stabilization properties (Waghmode *et al.*, 2020).
3.	D-(+)-Glucose	21.334	9.55	It plays a role in maternal insulin resistance (resulting in hyperglycemia) and preeclampsia (associated with placental insufficiency and hypoxia) (Rice *et al.*, 2015).
4.	Palmitic acid	23.165	2.62	Is the most common saturated fatty acid and is the precursor to longer fatty acids, is used to produce soaps and cosmetics. It increases low-density lipoprotein (LDL) and total cholesterol (Mensink and Organization, 2016).
5.	Oleic acid	25.197	2.62	It inhibits blood coagulation, improves glucose homeostasis, and attenuates inflammation and oxidative stress (Lopez *et al.*, 2021).
(d) Intestinal chyme				
1.	Palmitic acid	23.211	15.75	It increases low-density lipoprotein (LDL) and total cholesterol (Mensink and Organization, 2016).
2.	9-Octadecenoic acid	25.254	21.87	It has anti-inflammatory, anti-androgenic, dermatitigenic, and hypocholesterolemic effects as well as inhibits 5-alpha reductase activity (Krishnamoorthy and Subramaniam, 2014).
3.	9,12-Octadecadienoic acid (Z, Z)	25.511	2.08	Antimicrobial activity, previously acknowledged for their anti-oxidant activity (Pinto *et al.*, 2017).
4.	Alpha-linolenic acid	25.591	2.47	It is associated with a lower risk of cardiovascular disease and a reduced risk of fatal coronary heart disease (Sala-Vila *et al.*, 2022).
5.	Hexadecanamide	26.942	5.40	It alleviates Staphylococcus aureus-induced mastitis in mice by inhibiting inflammatory responses and restoring blood–milk barrier integrity (Bao *et al.*, 2023).
6.	9-Octadecenamide	28.984	20.25	It has analgesic and anti-inflammatory traits (Hadi *et al.*, 2016).
7.	Deoxycholic acid	30.054	2.24	It plays a role of an immunostimulant of the innate immune system, activating its main actors, the macrophages (Vlček, 1972).
8.	Palmitoleamide	31.239	1.02	It regulates memory processes, decreases body temperature and locomotor activity, stimulates Ca^{2+} release to modulate depressant drug receptors in the CNS (Farrell and Merkler, 2008).
9.	13-Docosenamide	34.93	9.07	It is released by bacteria in response to glucose during growth (Tamilmani *et al.*, 2018).

and antioxidant activity, with 100% methanol being on the top, and ethanol and water being at second and third place, respectively. Based on TPC, TFC, and antioxidant activity data, 100% methanol was the best solvent for extracting olive pomace, followed by 100% ethanol. This was probably due to the existence of antioxidant molecules with varied polarity and characteristics that could be or not be soluble in certain solvents (Ali *et al.*, 2019).

It was discovered that solvent composition and polarity impacted the extraction of phenolics and antioxidant potential (Mohammed *et al.*, 2022). Polar solvents often extract antioxidant molecules more effectively than nonpolar solvents. This was because the antioxidant molecule's polar part could interact with solvent through hydrogen bonding. Sultana *et al.* (2009) reported that methanol was effective in extracting phenolic compounds from some medical plants. Similarly, methanol was found to be superior in extracting polyphenol compounds from pumkin flesh, peel, and seeds as well as *Pistacia atlantica* fruits, compared to ethanol and aqueous extracts (Belyagoubi *et al.*, 2016; Hagos *et al.*, 2023). The extraction efficiency of aqueous solvents was lowest ($p \leq 0.05$), while that of pure organic solvents was highest. Despite the difficulty of obtaining a single solvent capable of extracting all phenolic compounds, almost all phenolics could be extracted from a plant matrix using an organic solvent–water solution (50–70%, v/v). The higher the solvent polarity, the greater the extract's antioxidant rate (Kriaa *et al.*, 2012).

It is important when using flavonoids and phenolic acids and matrices to solubilize them to maximize their

bioactivity (Maduwanthi and Marapana, 2021). During gastrointestinal digestion, numerous alterations in flavonoids and phenolics occur, including structural modifications, changes in solubility, and interaction with other molecules. All these changes influence their bioaccessibility (Ribeiro *et al.*, 2021). Food components and features, as well as physiological circumstances, in the gastrointestinal digestive system affect the bioaccessibility of bioactive molecules (Wojtunik-Kulesza *et al.*, 2020). We assumed that the test matrix values from methanol extraction represented 100% of sample's TPC, TFC, or antioxidant activity. We conducted this study to assess the recovery of olive pomace bioactive compounds and their bioaccessibility within the gastrointestinal digestive system. The results revealed that following the phases of gastrointestinal digestion, the olive pomace's TFC, TPC, DPPH IC_{50}, and ABTS IC_{50} were less bioaccessible. This result agreed with that of Reboredo-Rodríguez *et al.* (2021), who discovered a decrease in the TPC and TFC bioaccessibility indices of olive-related products after intestinal phase.

Similarly, a decrease in TFC and TPC bioaccessibility was revealed after intestinal digestion of selected edible green leaves and tomatoes (Gunathilake *et al.*, 2018; Wang *et al.*, 2022). Cianciosi *et al.* (2022) found that pH value and interaction with nutrients, such as fiber, iron, and proteins, impact the solubility and availability of phenolics. In contrast, Helal and Tagliazucchi (2018) reported high bioaccessibility (79.8%) of total phenolic compounds in stirred cinnamon-fortified yogurt after intestinal digestion. In general, the fact that these polyphenols are very unstable in the moderately alkaline environment of the small intestine might explain why their bioaccessibility decreased in intestinal chyme (Reboredo-Rodríguez *et al.*, 2021).

According to Ma *et al.* (2014), stomach digestion occurs when acids and enzymes hydrolyze proteins, fiber, or sugar residues from the test matrix. Similarly, Takahama and Hirota (2018) reported that the action of digestive enzymes could free flavonoids conjugated to carbohydrates and proteins, thereby increasing their levels. The low bioaccessibility of some phenolic compounds may also be due to degradation before reaching their site of absorption (Zhang and Chang, 2019). At certain pH values, some of the phenolic compounds are structurally unstable, which lead to irreversible alterations in phenolic structures (Velderrain-Rodriguez *et al.*, 2016). Temperature, solvent-to-solid ratio, food matrix composition, bound and free polyphenol ratio, and dietary fiber content influence the release of polyphenols from food matrix after digestion (Dima *et al.*, 2020). Ribeiro *et al.* (2021) discovered that after digestion, certain phenolic compounds could transform into distinct metabolites with unique properties and bioaccessibility, especially in the intestinal phase. These findings support the notion that the gastrointestinal digestive system can function as both phenolic releaser and phenolic-damaging agent. It's possible that foods that are high in insoluble fiber and certain components of the food matrix may lower the bioavailability and antioxidant rates of phenolics after *in vitro* gastrointestinal digestion (Phan *et al.*, 2017).

In this study, olive pomace contained a high amount of insoluble dietary fiber (52.3%) (data not shown), making its phenolic compounds less stable and soluble. The study examined the stability of phenolics and antioxidant activity of olive pomace during gastrointestinal digestion simulations, revealing higher levels of antioxidant activity, TPC, and TFC in the mouth phase and lower levels in the gastric and intestinal phases. Furthermore, the amount of phenolics and the antioxidant ability in pellet fraction was significantly higher than in chyme-soluble fraction.

CSF's phenolics indicate their solubilization, probably because of enzyme activity or agitation that may help breakdown of high-molecular weight compounds into extractable small molecules. The phenolics released during the stomach digestion phase can dissolve in gastric chyme and, when absorbed in the small intestine, may exert antioxidant effects. During the intestinal phase, the levels of TPC, TFC, DPPH, and ABTS decreased significantly. The findings are comparable to those of Reboredo-Rodríguez *et al.* (2021), who revealed a significant decrease in the phenolic levels of olive-related products following intestinal digestion.

Apple varieties and *Quercus ilex* leaves also showed a significant decrease in TPC after intestinal digestion (Bouayed *et al.*, 2011; Sánchez-Gutiérrez *et al.*, 2022). According to Li *et al.* (2022), higher TPC after gastric digestion, compared to intestinal digestion, may be due to changes in pH caused by the acidic medium (gastric digestion), which promotes compound breakdown, as opposed to the alkaline medium presented in the small intestine that promotes the destruction or transformation of phenolics. Andrade *et al.* (2022) suggested that bacteria in the colon could break down high-molecular weight polyphenolics into low-molecular weight phenolics and make metabolites that are more active biologically. Therefore, phenolic compounds that get into blood circulatory system are those that might have bioactivity with positive influences on the organism.

The amount of phenolics and flavonoids influences antioxidant activity. However, because of chemical modifications during gastrointestinal digestion, the antioxidant capacity of these phenolic compounds may vary. For example, modifications to the structure and molecular weights of phenolics during digestion may enhance their antioxidant activity (Ketnawa *et al.*, 2022). The ABTS assay is very sensitive to changes in pH, similar to those

that occur during *in vitro* digestion; this could explain drastic drop after oral and stomach phases (Zhu *et al.*, 2021). Further, the molecular structure of phenolic substances, pH, and how they interact with phenolics, dietary fiber, proteins, and other food components that are released during digestion alter polyphenol solubility and availability as well as antioxidant capacity in the intestine.

We found a strong and favorable link (r = 0.891–0.994) between antioxidant ability (DPPH and ABTS rates), TPC, and TFC in the mouth, stomach, and intestinal chymes. This finding agreed with that of Carbonell-Capella *et al.* (2015). They observed a strong association between total phenolic content and antioxidant capacity of beverages made of oats and exotic fruits after laboratory gastrointestinal digestion. Similarly, Chen *et al.* (2014) also found a link between the TPC and antioxidant capability of fruits before and after *in vitro* digestion. Moreover, Kriaa *et al.* (2012) found that the total amount of antioxidants, phenolics, and flavonoids in three different types of date palms correlated linearly with a very high correlation coefficient. In contrast, Huang *et al.* (2014) demonstrated little association between the antioxidant potential and TPC of germinated soybeans. A strong correlation between TPC and antioxidant ability shows that phenolic molecules play an important role in antioxidant potential (Gullon *et al.*, 2015).

The GC-MS data showed an increase in the number of peaks in mouth, gastric, and intestinal digestion phases of olive pomace, compared to the control. The GC-MS results of gastric chyme revealed more peaks than those of undigested and intestinal digestion samples. This suggested that the stomach digestion phase released phenolic substances from the food matrix despite their relative instability with flavonoids demonstrating greater stability (Mahadkar *et al.*, 2013), while flavonoids demonstrated greater stability (Bouayed *et al.*, 2011). Notably, chyme dissolved phenolic chemicals produced in the stomach, potentially absorbing them and exerting their antioxidant properties in the small intestine (Bouayed *et al.*, 2011). After digestion, certain chemicals that were conjugated to proteins or fibers in the original matrix, and were released by enzymatic digestion and pH shift, manifested in greater quantities after digestion.

In a similar manner, Reboredo-Rodríguez *et al.* (2021) reported that the freedom of conjugated phenolics from the food matrix was responsible for the increment of their peaks after gastric digestion phases. It is worth noting that instead of quantifying the number of prominent peaks at each phase of digestion, it is crucial to employ high-performance *liquid chromatography*–diode array detector (HPLC-DAD) to compare these phenolic compounds with standard compounds. This approach allows for the detection, quantification, and determination of the subsequent fate of these molecules following the digestion phase.

Furthermore, the COVID-19 pandemic hindered the completion of the planned hind gut fermentation process for the undigested portion after intestinal phase. The biological activities of olive pomace collected from various global locations were investigated earlier (Bucciantini *et al.*, 2021; Nunes *et al.*, 2018). According to this research, the phytochemicals identified in olive pomace before and after digestion display antioxidant, antibacterial, anti-inflammatory, and anticancer effects. Researchers have found that phytochemicals offer a wide range of medical benefits, such as protecting against liver damage and cancer, reducing inflammation and blood sugar levels, and raising antioxidant levels (Abdallah *et al.*, 2023; Bucciantini *et al.*, 2021; Rodrigues *et al.*, 2023). We anticipate that olive plants grown in Saudi Arabia would have some biological activities that are somewhat different from those grown in other geographical regions because location and climate significantly impact the content of primary and secondary metabolites in plants.

Conclusions

The study's findings demonstrated that olive pomace is a beneficial source of bioactive molecules such as fibers and phenolic compounds with antioxidant potential. The simulated gastrointestinal effect on olive pomace led to higher amounts of bioactive substances compared to the undigested pomace. Despite their low accessibility in the intestinal phase, these compounds can prevent oxidative stress in the intestine, thereby promoting health benefits. This research suggested that olive pomace could be a viable bioactive source for functional ingredients in food applications, promoting a healthy lifestyle.

Author Contributions

Writing—original draft (Haya F. Alhuthayli); methodology (Magdi A. Osman and Haya F. Alhuthayli); formal analysis (Haya F. Alhuthayli, Magdi A. Osman, and Mohammed A. Mohammed); data curation and investigation (Haya F. Alhuthayli); conceptualization, visualization, and supervision (Magdi A. Osman and Fahad M. Al-Jasass), review, writing, and editing (Magdi A. Osman and Mohammed A. Mohammed)

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Supplementary

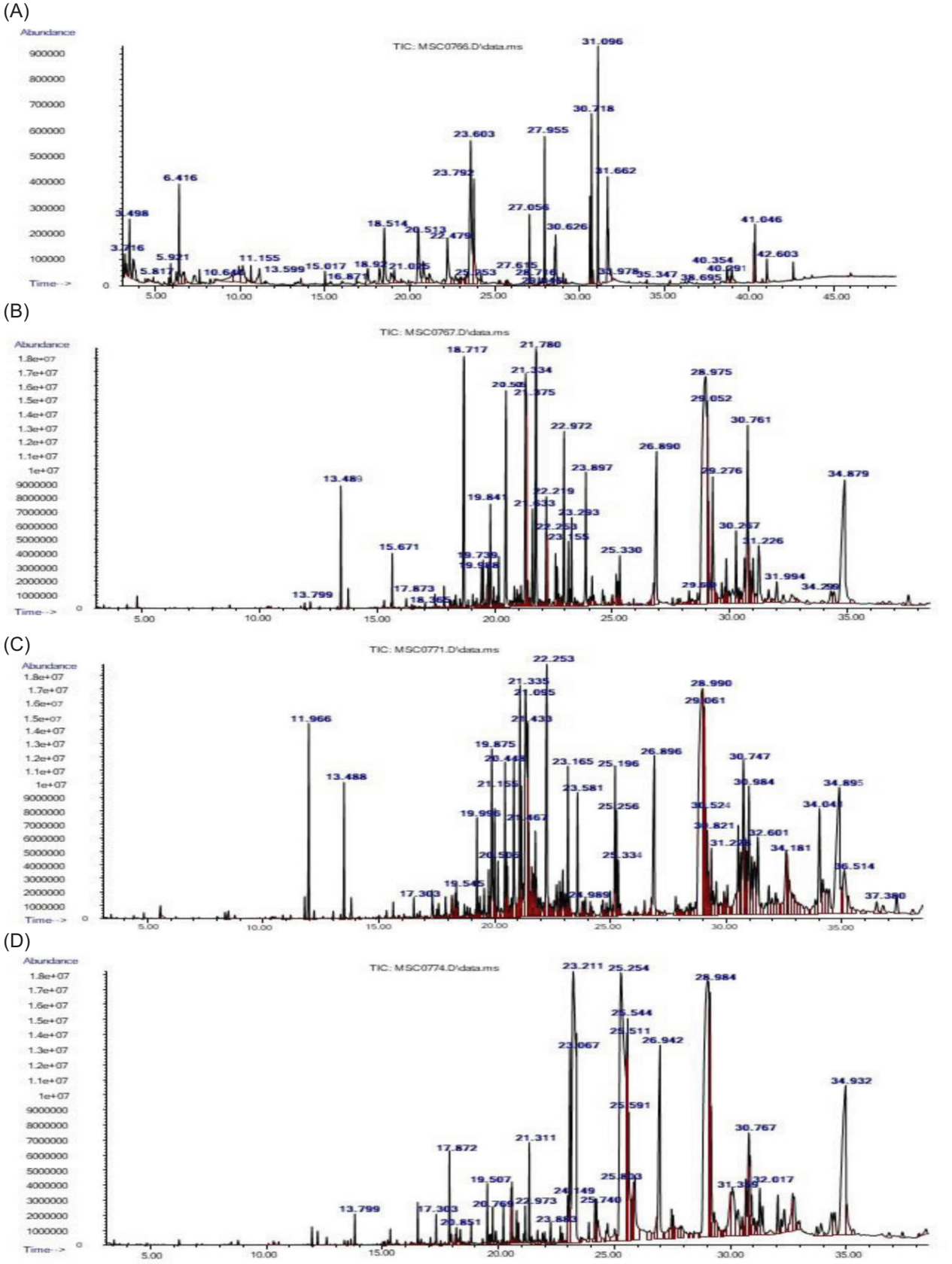

Figure S1. GC-MS chromatograms of (A) undigested olive pomace and pomace chymes from the (B) mouth, (C) gastric, and (D) intestinal phases.