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Effect of Aqueous Enzymatic Processes on Sunflower Oil Quality

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Abstract The use of enzymes in aqueous vegetable oilseed extraction for simultaneous recovery of high quality oil and protein is gaining recognition. In the present work, five enzyme preparations [Protex 7L by Genencor (Rochester, NY USA), Alcalase 2.4L, and Viscozyme L by Novozymes (Bagsvaerd, Denmark), Natuzyme by Bioproton Pty Ltd (Australia) and Kemzyme by Kemin Europa N·V. (Belgium)] were studied to evaluate their effects on the extraction of oil and protein from sunflower seeds. Preliminary experiments were conducted for the selection of enzymes, optimum enzyme concentration, incubation time and pH. Maximum oil yield (87.25% of the total oil in the seed) was obtained with Viscozyme L, whereas, Protex 7L offered the highest level of protein in the aqueous phase. The comparison of the quality attributes of enzyme-assisted aqueous extracted (EAAE) oil with those of solvent-extracted and control (oils extracted without enzyme treatment) oils revealed no significant ($P > 0.05$) variations for iodine value, density, refractive index, unsaponifiable matter, and fatty acid composition among the extraction methods. The control and EAAE oils also exhibited a better oxidation state. The tocopherol concentration for the oils, produced with the enzymes, was noted to be quite improved relative to the control and solvent-extracted oils. A higher antioxidant activity in terms of total phenolic contents, 2,2′-diphenyl-1-picrylhydrazyl scavenging capacity and inhibition of linoleic acid peroxidation was also observed for the EAAE oils as against control and the solvent-extracted oils.

Keywords Sunflower oil · Enzyme-assisted aqueous extraction · Physico-chemical properties · FAs · Tocopherols · Oxidative stability · Comparison

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Introduction

Sunflower (*Helianthus annuus*) is one of the most widely cultivated and important oilseed crops in the world [1]. Because of its wide adaptability, sunflower is mainly grown in Mediterranean regions such as Southern Europe [2] and western states of the USA [3]. Its common uses include food, medicine, and dyes but the sunflower seed is often pounded into flour and used in cakes, mush, and bread [4]. Sunflower seeds contain a high amount of oil (40%–50%) which is an important source of polyunsaturated fatty acid (linoleic acid) of potential health benefits [5].

The use of hexane, generally employed for oilseed extraction is being questioned because of its toxicity and flammability [6]. Involvement of high temperatures during hexane-extraction may result undesirable effects on the quality of extracted oil due to oxidative deterioration of polyunsaturated fatty acids and development of rancid and off flavours [7]. For this reason, there is considerable interest in seeking suitable alternatives to hexane for oilseed extraction. Furthermore, the crude vegetable oils are refined by different processing steps (degumming, neutralization and bleaching). During these steps, oils are exposed to high temperatures and metallic catalysts [8] leading to losses of valuable components [9]. These steps

also prompt the need of suitable alternative to avoid such losses in oil quality.

Aqueous oil extraction is becoming an important alternative to hexane oil extraction. With this process we can eliminate not only the use of hexane but some refining steps can also be omitted. In this context, another recent development involves the use of enzymes to assist oil extraction from seeds. The challenge of producing high quality oil can be met through the innovative enzymatic extraction process. The enzymatic pretreatment of seeds, prior to oil extraction, helps in degrading seed cell wall components, thus facilitating oil release from the seeds. Some enzyme preparations, having cellulases, hemicellulases, pectinases and proteases activities, are quite effective in different vegetable oil extraction processes [10–14].

An enzyme-assisted aqueous extraction (EAAE) process for sunflower seeds may prove to be an environmently friendly alternative to solvent extraction and may allow the recovery of high-quality protein for human consumption. Very few references are available on the applications of aqueous enzymatic process for simultaneous extraction of oil and protein from sunflower seeds [15, 16]. The present study deals with the evaluation and quantification of the effects of aqueous enzyme applications on the quality of oil during sunflower seed extraction. We studied and compared the physico-chemical properties, fatty acid composition and tocopherol profiles of the enzyme-extracted oil with those of control and solvent-extracted oils.

Materials and Methods

Sunflower (*Helianthus annuus* L.) seeds were acquired from the Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan. All reagents (analytical and HPLC) used were from Merck (Darmstadt, Germany) or Sigma-Aldrich (Buchs, Switzerland). Protex 7L (protease) was provided by Genencor (Rochester, NY USA), Alcalase 2.4L (protease), and Viscozyme L (Multi-enzyme complex containing a wide range of carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase) by Novozymes Bagsvaerd (Denmark), whereas, Natuzyme (mainly cellulase, xylanase, phytase, alpha-amylase, pectinase activities) by Bioproton (Pty Ltd, Australia) and Kemzyme (mainly alpha-amylase, beta-glucanase, cellulase-complex, hemicellulase-complex, protease and xylanase activities) by Kemin Europa N·V., Belgium.

Solvent Extraction

After removal of impurities, the dehulled sunflower seeds were crushed using a coffee grinder. The material that passed through 80-mesh sieve was used for extraction purposes. The ground seed material (25 g) was fed to a Soxhlet extractor fitted with a 0.5-L round-bottom flask and a condenser. The extraction was carried out for 6 h with 0.3 L of *n*-hexane on a water bath. After extraction, hexane was distilled off under vacuum using a rotary evaporator (Eyela, N–N Series, Rikakikai Co. Ltd. Tokyo, Japan) at 45 °C and the oil obtained was stored under refrigeration (4 °C), until used for further analyses.

Aqueous Enzyme-Assisted Extraction

Ground seeds were mixed with distilled water at a ratio of 1:6 w/v [17]. The mixture was boiled for 5 min and allowed to cool down to room temperature. The pH was then adjusted to the optimal level for each enzyme with 0.5 N NaOH and 0.5 N HCl. Then, an optimised amount (% by seed wt) of each of the five enzyme preparations (Protex 7L, Alcalase 2.4L, Viscozyme L, Kemzyme and Natuzyme) was added and the mixture was incubated at 45 °C for 2 h with constant shaking at 120 rpm. The mixture was centrifuged (7000 rpm, 30 °C) for 15 min (Sigma, 3 K 30, Osterode am Harz, Germany) resulting in an oil, creamy and aqueous phase [17]. Using a micro-pipette, the top, oil-rich phase was first withdrawn, followed by the creamy and aqueous phase, leaving the meal at the bottom. The wet meal was mixed, dried overnight in a vacuum oven (VOC-300 SD; EYELA, Tokyo, Japan) at 85–90 °C and ground. The control samples were treated identically, except for the enzyme addition.

Analysis for Protein

The meals, aqueous and creamy phases obtained after oil extraction by solvent and aqueous processes were separately analysed for protein. The protein content (N × 6.25) was determined by the Kjeldahl method according to the AOAC method 954.01[18]. The protein contents of the aqueous and creamy fractions were added together and referred to as protein ACP (aqueous and creamy phase).

Analysis of Extracted Oils

Physical and Chemical Parameters

Iodine value, density, unsaponifiable matter, peroxide and saponification values of the control, solvent and EAAE oils were determined by AOCS standard methods [19]. The colour and refractive index of the oils were determined by a Lovibond tintometer (Tintometer Ltd., Salisbury, Wiltshire, United Kingdom) using a 1-inch cell and Refractometer (RX-7000α, Atago co., Ltd. Japan) respectively. Specific extinctions at 232 and 270 nm were

determined using a spectrophotometer (U-2001, Hitachi Instruments, Inc., Tokyo, Japan). Samples of oil were diluted with iso-octane, and the absorptions at 232 and 270 nm were recorded. Specific extinctions as $\varepsilon^{1\%}{}_{1\ \mathrm{cm}}(\lambda)$ were calculated following the standard IUPAC [20] method.

Fatty Acid (FA) Composition

Fatty acid methyl esters (FAMEs) were prepared according to the IUPAC [20] method 2.301 and were analysed on a Shimadzu (Tokyo, Japan) gas chromatograph, model 17-A, fitted with a methyl-lignocerate-coated (film thickness 0.20 μm) SP-2330 polar capillary column (30 m × 0.32 mm; Supelco Inc., Supelco Park Bellefonte, PA), and a flame ionization detector (FID). Oxygen-free nitrogen was used as a carrier gas at a flow rate of 3.0 mL min^{-1}. Other conditions employed were as follows: initial oven temperature, 180 °C; ramp rate, 5 °C min^{-1}; final temperature, 220 °C; injector temperature, 230 °C; detector temperature, 250 °C; and temperature hold, 2 min before and 10 min after the run. A sample volume of 1.5 μL was injected using split mode (split ratio 1:75). FAMEs were identified by comparing their relative and absolute retention times to those of authentic standards (Sigma-Aldrich Chemical Co. (St. Louis, MO). The quantification was based on an internal standard method using Chromatography Station for Windows (CSW32) data handling software (Data APEX Ltd., Pague 5, The Czech Republic). The FA composition was reported as relative percentage of the total peak area.

Tocopherol Content

Tocopherols (α, γ, and δ) were analysed using an HPLC following the CPFA (Current Protocols in Food Analytical Chemistry) [21] methods. 0.1 g oil sample and 0.05 g ascorbic acid were weighed accurately into a 16 × 125-mm test tube. Five milliliters of 90.2% ethanol and 0.5 mL of 80% aqueous KOH solution were added to the test tube and vortexed for 30 s. The test tube was flushed with nitrogen, capped and incubated in a water bath (70 °C) for 30 min with periodical vortexing. The tubes were placed in an ice bath for 5 min then 3 mL deionised water and 5 mL *n*-hexane were added and vortexed for 30 s followed by centrifugation for 10 min at 1000*g* (room temperature). The upper hexane layer was transferred to another test tube. The aqueous layer and the residue were re-extracted by repeating the same procedure. The upper hexane layers from both the extractions were combined and evaporated to dryness under nitrogen streaming. One milliliter of mobile phase was added to the tube and vortexed 30 s to re-dissolve the extract and then transferred to an HPLC sample vial. A 20-μL sample was injected into a Supelcosil LC-Si column (250 × 4.6 mm, Supelco Inc., Supelco Park, Bellefonte, USA). A mobile phase of ethyl acetate/acetic acid/hexane (1:1:198, v/v/v) was used at the rate of 1.5 mL min^{-1}. The detector monitored UV absorbance at 295 nm. Tocopherols were identified by comparing their retention times with those of pure standards of α-, γ-, and δ- tocopherols, and were quantified on the basis of peak area of the unknowns with those of pure standards (Sigma-Aldrich Chemical Co.). Quantification was based on an external standard method. A D-2500 Hitachi Instruments, Inc., Tokyo, Japan Chromato-integrator model with a built-in computer program for data handling was used for quantification.

Antioxidant Activity

Extraction of Antioxidant Constituents

The antioxidant components from the tested oil were extracted with 80:20 MeOH:H_2O v/v [22]. Briefly, 1 gm of oil was weighed into a test tube and then 3 mL of solvent was added. The test tube was vortexed and then centrifuged at 6000 rpm for 5 min and the supernatant was collected. The same procedure was repeated two more times and the three extracted phases were combined and the final volume was brought to 10 mL with the extraction solvent. The resulting antioxidant solution was then kept in the dark under N_2 until further analysis.

Determination of Total Phenolics (TP)

The amount of TP was calculated using the Folin–Ciocalteu reagent as described by Anwar et al. [23]. 0.5 mL of extract solution (0.05 g/5 mL) was mixed with 0.5 mL of Folin–Ciocalteu reagent and 7.5 mL deionised water. The mixture was kept at room temperature for 10 min and then 1.5 mL of 20% sodium carbonate (w/v) was added. The mixture was heated in a water bath at 40 °C for 20 min and then cooled in an ice-bath; finally absorbance was taken at 755 nm. The amount of TP was calculated using a calibration curve for gallic acid (10–130 ppm). The results were expressed as Gallic acid equivalents (GAE) mg/100 g of oil.

2,2′-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

The antioxidant activity of the extracted oils extracts was assessed by measuring the scavenging abilities to 2,2′-diphenyl-1-picrylhydrazyl stable radicals. The DPPH assay was performed as described by Bozin et al. [24]. The samples (0.5–15.5 μg mL^{-1}) were mixed with 1 mL of 90 μM DPPH solution and filled up with 95% MeOH, to a final volume of 4 mL. The absorbance of the

resulting solutions and the blank were recorded after 1 h at room temperature. Butylated hydroxytoluene (BHT) was used as a positive control. For each sample, three replicates were recorded. The disappearance of DPPH was read spectrophotometrically at 515 nm. Inhibition of free radical by DPPH in percent (%) was calculated in the following way:

$$I(\%) = 100 \times \left(A_{blank} - A_{sample}/A_{blank}\right)$$

where A_{blank} is the absorbance of the control reaction mixture excluding the test compounds, and A_{sample} is the absorbance of the test compounds. IC_{50} values, which represented the concentration of oil extracts that caused 50% neutralization of DPPH radicals, were calculated from the plot of inhibition percentage against concentration.

Percent Inhibition of Linoleic Acid Oxidation

The antioxidant activity of the extracted oils extracts was also determined using inhibition of linoleic acid oxidation, following the method described by Singh and Marimuthu [25] with slight modification. The test samples (50 μL) were dissolved in 1 mL of ethanol, mixed with linoleic acid (2.5%, v/v), 99.5% ethanol (4 mL) and 4 mL of 0.05 M sodium phosphate buffer (pH 7). The solution was incubated at 40 °C for 175 h. The extent of oxidation was measured by the peroxide value using the colorimetric method described by Yen et al. [26]. To 0.2 mL sample solution, 10 mL of ethanol (75%), 0.2 mL of an aqueous solution of ammonium thiocyanate (30%) and 0.2 mL of ferrous chloride solution (20 mM in 3.5% HCl) were added sequentially. After 3 min of stirring, the absorbance was measured at 500 nm, using a spectrophotometer. A control was performed with linoleic acid without oil extracts. Butylated hydroxytoluene (BHT) was used as a positive control. Inhibition of linoleic acid oxidation expressed as a percentage was calculated as follows:

% inhibition of linoleic acid oxidation = 100 − [(Abs. increase of sample at 175 h/Abs. increase of control at 175 h) × 100]

Statistical Analysis

All the experiments were conducted in triplicate and statistical analysis of the data were performed by analysis of variance (ANOVA) using the statistical software STATISTICA 5.5 (Stat Soft Inc, Tulsa, Oklahoma, USA). A probability value at $P < 0.05$ was considered statistically significant. Data are presented as mean values ± standard deviation calculated from triplicate determination.

Results and Discussion

Oil and Protein Contents

The amount of oil recovered under aqueous extraction conditions was higher when enzymes were included; 26.6 to 39.7% vs 18.3% without enzymes. However, the oil yield in enzyme assisted extraction method was significantly ($P < 0.05$) lower than the solvent extraction method (45.5%) (Table 1). The highest oil yield (39.7%) was obtained from Viscozyme L treated seed samples, whereas, the lowest oil content (20.63%) was observed with Alcalase 2.4L. The higher oil contents determined in the aqueous enzyme-assisted extraction process relative to the control can be explained by the better solubilisation and hydrolysis of proteins, which possibly causes a breakdown in the protein network characteristic of the cotyledon cells, and in the protein (oleosin) based membranes that surround the lipid bodies, thereby liberating the oil [27–29].

Analysis of the sunflower oilseed residue obtained by the aqueous extraction process with and without enzymes revealed significantly ($P < 0.05$) lower protein content (13.5%–16.1%) of the residue compared to meal left after solvent extraction (18.7%). The protein content for enzyme-extracted seeds being lower than those solvent-extracted might be due to extraction of protein in aqueous phase in the former case. Of the enzyme-treated seeds, the Protex 7L extracted seed offered the highest protein contents (4.3%) in the ACP, revealing high efficacy of Protex 7L for protein extraction. This is in agreement with the

Table 1 Oil and protein extracted from sunflower seeds

Parameter (%)	Solvent-extracted	Enzymes-assisted					Control
		Protex 7L	Kemzyme	Alcalase 2.4L	Viscozyme L	Natuzyme	
Oil extracted	45.5 ± 0.7^{a}	28.3 ± 0.4 ^{cd}	32.2 ± 0.3^{c}	26.6 ± 0.3^{d}	39.7 ± 0.4^{ab}	35.5 ± 0.6^{b}	18.3 ± 0.3^{e}
Protein (meal)	18.7 ± 0.2^{a}	13.5 ± 0.2^{e}	15.2 ± 0.2^{c}	15.0 ± 0.3^{d}	14.6 ± 0.2^{d}	15.1 ± 0.3 ^{cd}	16.1 ± 0.3^{b}
Protein (ACP)[f]	–	4.3 ± 0.3^{a}	2.4 ± 0.5^{c}	3.1 ± 0.2^{b}	3.7 ± 0.4^{ab}	2.7 ± 0.5^{bc}	1.9 ± 0.4^{d}

Values are means ± SD, calculated as percentage on dry seed weight basis for three sunflower seed samples analysed individually in triplicate
Mean values in the same row followed by the same superscript letters are not significantly different ($P > 0.05$)

[f] Aqueous and creamy phase

findings of Nobrega de Moura et al. [30] who reported an improvement in protein extractability from the soybeans using Protex 7L during EAAE.

Physico-Chemical Properties of Extracted Oils

The physical and chemical parameters of the extracted oils are given in Table 2. A significantly ($P < 0.05$) lower content of free fatty acid (0.64%–0.69% as oleic acid) was observed in the EAAE oils as against solvent extracted oil (0.94) which might be due to accelerated temperature treatment during the solvent extraction. No significant ($P > 0.05$) variations were observed for iodine value, density, refractive index, and unsaponifiable matter of the oils extracted by different means. The colour (1.5–1.7 red units + 15–17 yellow units) of the EAAE sunflower seed oils was comparable with the control (1.5 red units + 15 yellow units) but significantly ($P < 0.05$) lower in red and yellow units than solvent extracted oil (1.9 red units + 19 yellow units). These results were found to be in agreement with the findings of Hanmoungjai et al. [31] and Abdulkarim et al. [32]. Higher colour values in solvent extracted oils may be attributed to the better solubility of pigments in the solvent. The intensity of the colour of vegetable oils mainly depends on the presence of colouring pigments such as chlorophyll, which must be removed during the degumming, refining, and bleaching steps of oil processing. The vegetable oils with a minimum colour index are considered to be more suitable for edible and industrial purposes [33].

The oxidative stability parameters of the extracted oils are presented in Table 3. The specific extinctions at 232 and 270 nm, which revealed the oxidative deterioration and purity of the oils [34] of the EAAE oils, ranging from 3.10 to 3.18 and 0.64 to 0.72, respectively were found to be comparable with that of control (3.13 and 0.70, respectively) but significantly ($P < 0.05$) lower than the solvent-extracted oil (3.28 and 0.82, respectively). The peroxide value of the EAAE sunflower oil (1.25 to 1.37 mequiv/kg) was found to be comparable with control (1.29 mequiv/kg), however, it was significantly ($P < 0.05$) lower than the solvent-extracted oil (1.78 mequiv/kg). The high

Table 2 Physicochemical properties of sunflower seed oils

Parameter	Solvent-extracted	Enzymes-assisted					Control
		Protex 7L	Kemzyme	Alcalase 2.4L	Viscozyme L	Natuzyme	
Refractive index (40 °C)	1.47 ± 0.02^{a}	1.47 ± 0.03^{b}	1.47 ± 0.01^{b}	1.47 ± 0.02^{b}	1.47 ± 0.01^{b}	1.47 ± 0.03^{b}	1.47 ± 0.02^{b}
Density (20 °C g mL^{-1})	0.92 ± 0.03^{a}	0.92 ± 0.02^{a}	0.92 ± 0.02^{a}	0.92 ± 0.03^{a}	0.92 ± 0.02^{a}	0.92 ± 0.04^{a}	0.92 ± 0.03^{a}
Saponification value (mg KOH/g of oil)	190 ± 3^{a}	187 ± 4^{ab}	186 ± 2^{b}	187 ± 5^{ab}	185 ± 4^{b}	187 ± 6^{ab}	187 ± 3^{ab}
Free fatty acids (% oleic acid)	0.94 ± 0.08^{a}	0.69 ± 0.02^{b}	0.65 ± 0.03^{c}	0.66 ± 0.04^{bc}	0.64 ± 0.02^{c}	0.67 ± 0.03^{bc}	0.68 ± 0.04^{b}
Iodine value (g of I/100 g of oil)	127 ± 2^{a}	122 ± 4^{a}	121 ± 3^{b}	124 ± 2^{ab}	121 ± 5^{a}	123 ± 3^{ab}	120 ± 4^{a}
Unsaponifiable matter (% w/w)	0.51 ± 0.02^{b}	0.45 ± 0.03^{b}	0.47 ± 0.02^{ab}	0.47 ± 0.04^{a}	0.45 ± 0.03^{ab}	0.43 ± 0.02^{ab}	0.54 ± 0.04^{ab}
Colour (1-in. cell)							
Red units	$1.9\ r \pm 0.04^{a}$	$1.7\ r \pm 0.03^{ab}$	$1.5\ r \pm 0.02^{b}$	$1.6\ r \pm 0.03^{b}$	$1.6\ r \pm 0.02^{b}$	$1.7\ r \pm 0.02^{ab}$	$1.5\ r \pm 0.02^{b}$
Yellow units	$19\ y \pm 0.5^{a}$	$17\ y \pm 0.4^{ab}$	$15\ y \pm 0.2^{b}$	$16\ y \pm 0.4^{b}$	$16\ y \pm 0.3^{b}$	$17\ y \pm 0.2^{ab}$	$15\ y \pm 0.3^{b}$

Values are means ± SD for three sunflower seed oils analysed individually in triplicate

Mean values in the same row followed by the same superscript letters are not significantly different ($P > 0.05$)

Table 3 Determination of the oxidative state of sunflower seed oils

Parameter	Solvent-extracted	Enzymes-assisted					Control
		Protex 7L	Kemzyme	Alcalase 2.4L	Viscozyme L	Natuzyme	
Conjugated diene $\varepsilon^{1\%}{}_{1\ cm}$ (λ232)	3.28 ± 0.09^{a}	3.14 ± 0.11^{bc}	3.15 ± 0.08^{bc}	3.18 ± 0.13^{b}	3.10 ± 0.12^{c}	3.11 ± 0.10^{c}	3.23 ± 0.14^{ab}
Conjugated triene $\varepsilon^{1\%}{}_{1\ cm}$ (λ270)	0.82 ± 0.04^{a}	0.68 ± 0.03^{bc}	0.62 ± 0.02^{c}	0.72 ± 0.03^{b}	0.69 ± 0.01^{b}	0.64 ± 0.04^{bc}	0.72 ± 0.02^{b}
Peroxide value (mequiv/kg)	1.78 ± 0.06^{a}	1.31 ± 0.13^{c}	1.33 ± 0.08^{bc}	1.25 ± 0.10^{d}	1.37 ± 0.13^{b}	1.32 ± 0.14^{c}	1.36 ± 0.13^{b}
p-anisidine	1.93 ± 0.05^{a}	1.74 ± 0.13^{bc}	1.75 ± 0.06^{bc}	1.76 ± 0.11^{bc}	1.71 ± 0.14^{c}	1.78 ± 0.13^{b}	1.79 ± 0.07^{b}
Induction period (h)	1.82 ± 0.12^{b}	1.94 ± 0.09^{a}	1.93 ± 0.13^{a}	1.95 ± 0.15^{a}	1.93 ± 0.12^{a}	1.96 ± 0.14^{a}	1.84 ± 0.06^{b}

Values are means ± SD for three sunflower seed oils analysed individually in triplicate

Mean values in the same row followed by the same superscript letters are not significantly different ($P > 0.05$)

Table 4 FA composition (grams per 100 g of fatty acids) of sunflower seed oils

FA	Solvent-extracted	Enzymes-assisted					Control
		Protex 7L	Kemzyme	Alcalase 2.4L	Viscozyme L	Natuzyme	
$C_{16:0}$	6.89 ± 0.13^{d}	7.29 ± 0.11^{ab}	7.22 ± 0.06^{b}	7.16 ± 0.09^{bc}	7.11 ± 0.16^{c}	7.35 ± 0.14^{a}	7.24 ± 0.18^{b}
$C_{16:1}$	0.22 ± 0.06^{a}	0.15 ± 0.08^{b}	0.11 ± 0.02^{c}	0.13 ± 0.04^{c}	0.16 ± 0.05^{b}	0.21 ± 0.03^{a}	0.19 ± 0.06^{ab}
$C_{18:0}$	4.31 ± 0.07^{d}	4.76 ± 0.07^{ab}	4.53 ± 0.11^{bc}	4.41 ± 0.07^{c}	4.79 ± 0.12^{a}	4.84 ± 0.08^{a}	4.63 ± 0.11^{b}
$C_{18:1}$	28.32 ± 0.43^{a}	27.39 ± 0.41^{b}	28.12 ± 0.52^{a}	27.64 ± 0.45^{b}	28.14 ± 0.42^{a}	27.76 ± 0.69^{b}	27.58 ± 0.53^{b}
$C_{18:2}$	59.44 ± 1.19^{a}	58.18 ± 1.45^{b}	58.31 ± 1.26^{b}	59.45 ± 0.89^{a}	59.21 ± 1.38^{a}	58.53 ± 1.54^{ab}	58.22 ± 0.58^{b}
$C_{18:3}$	0.13 ± 0.05^{d}	0.18 ± 0.07^{ab}	0.22 ± 0.09^{a}	0.15 ± 0.06^{c}	0.24 ± 0.04^{a}	0.21 ± 0.05^{ab}	0.15 ± 0.07^{c}
$C_{20:0}$	0.21 ± 0.07 ^{cd}	0.24 ± 0.05^{c}	0.28 ± 0.03^{b}	0.19 ± 0.06^{d}	0.35 ± 0.07^{a}	0.33 ± 0.04^{a}	0.23 ± 0.06^{bc}
$C_{20:1}$	0.16 ± 0.03^{a}	0.12 ± 0.04^{b}	0.16 ± 0.02^{a}	0.14 ± 0.01^{ab}	0.11 ± 0.03^{b}	0.17 ± 0.05^{a}	0.15 ± 0.03^{a}

Values are means ± SD for three sunflower seed oils analysed individually in triplicate

Mean values in the same row followed by the same superscript letters are not significantly different ($P > 0.05$)

operational temperature during conventional hexane oilseed extraction might affect the oil quality, particularly, the oxidation state of the oils. No previous data were available on the oxidation parameters of EAAE sunflower oil for comparison. As expected no significant ($P > 0.05$) variation was observed in the fatty acid composition of the oils extracted by different means (Table 4).

The concentration of tocopherols (α-, γ-, and δ) of the EAAE sunflower oils ranged from 516–582, 259–268, and 0–6 mg kg^{-1}, respectively (Table 5). The α-tocopherol was found to be significantly ($P < 0.05$) higher in enzyme- and solvent-extracted oil (579 mg kg^{-1}) as against the control (537 mg kg^{-1}). The concentration of α-tocopherol was found to be highest in Alcalase 2.4L (582 mg kg^{-1}), followed by Protex 7L, Natuzyme, Kemzyme and Viscozyme extracted oils, 524, 523, 517 and 516 mg kg^{-1} respectively. However, no significant variation was observed for the level of δ-tocopherol in the oils extracted by different means. γ-tocopherol was found to be significantly ($P < 0.05$) higher in the enzyme-extracted oils (254–268 mg kg^{-1}) than those of control (258 mg kg^{-1}) and solvent-extracted (217 mg kg^{-1}) oils. It is reported that α-tocopherol has the stronger vitamin E activity, whereas the δ-tocopherol has better antioxidant efficacy than either γ-, β- or α-tocopherols [35].

The EAAE sunflower oils in the present analysis were significantly ($P < 0.05$) richer in total tocopherols (833–842 mg kg^{-1}) as against the control (778 mg kg^{-1}), showing an enhancement of *ca.* 7 to 9% in the total tocopherols, which may be attributed to the enzymatic pretreatment [36]. The hydrolysis of seed cell wall by the enzymatic preparations during oil extraction may cause release of greater amounts of tocopherols and phenolics resulting in higher availability of such bioactive components into the oil [36–38]. This may also be due to a reduced complexation of such compounds with the seed polysaccharides and consequent enhancement of partitioning into the oil phase [39]. No previously reported data on the tocopherol contents of EAAE sunflower oils are available in the literature to compare the results of our present analysis.

Antioxidant Activity of Extracted Oils

The TPC, DPPH scavenging capacity and inhibition of linoleic acid peroxidation of the oils extracted by different methods are shown in Table 6. TPC in the EAAE oils (1.3–1.5 mg GAE/100 g) were found to be significantly ($P < 0.05$) higher than that of control (0.9 mg GAE/100 g) and the solvent-extracted oils (0.8 mg GAE/

Table 5 Comparison of tocopherols (mg kg^{-1}) in sunflower seed oils

Tocopherols	Solvent-extracted	Enzymes-assisted					Control
		Protex 7L	Kemzyme	Alcalase 2.4L	Viscozyme L	Natuzyme	
α	579 ± 8.7^{a}	524 ± 13.2^{bc}	517 ± 8.1^{c}	582 ± 7.4^{a}	516 ± 9.3^{c}	523 ± 7.8^{bc}	537 ± 11.1^{b}
γ	217 ± 5.4^{d}	268 ± 3.3^{a}	266 ± 4.5^{a}	259 ± 3.9^{ab}	254 ± 2.8^{b}	261 ± 5.6^{ab}	238 ± 5.2^{c}
δ	3 ± 0.4^{b}	0	6 ± 0.9^{a}	4 ± 0.7^{b}	3 ± 0.5^{b}	5 ± 0.6^{a}	3 ± 0.4^{b}
Total	799	842	849	845	833	849	778

Values are means ± SD for three sunflower seed oils analysed individually in triplicate

Mean values in the same row followed by the same superscript letters are not significantly different ($P > 0.05$)

Table 6 Antioxidant activity of sunflower seed oils

Parameter	Solvent-extracted	Enzymes-assisted					Control
		Protex 7L	Kemzyme	Alcalase 2.4L	Viscozyme L	Natuzyme	
TPC[d] (mg GAE/100 g)	0.8 ± 0.1^{c}	1.3 ± 0.2^{c}	1.4 ± 0.2^{b}	1.3 ± 0.1^{c}	1.5 ± 0.3^{a}	1.3 ± 0.2^{c}	0.9 ± 0.1^{b}
DPPH[e], IC_{50} (μg/mL of oil)	73.4 ± 0.67^{a}	65.6 ± 1.25^{ab}	64.6 ± 1.19^{b}	65.8 ± 0.91^{ab}	63.8 ± 0.79^{b}	65.7 ± 0.86^{ab}	68.5 ± 1.28^{a}
Inhibition in linoleic acid system (%) after 175 h incubation at 40°C	42.5 ± 1.23^{bc}	51.8 ± 0.94^{b}	53.4 ± 1.39^{ab}	50.2 ± 1.44^{b}	55.2 ± 1.33^{a}	49.8 ± 1.28^{c}	48.5 ± 1.32^{bc}

Values are means ± SD for three sunflower seed oils analysed individually in triplicate

Mean values in the same row followed by the same superscript letters are not significantly different ($P > 0.05$)

[d] Total phenolic contents

[e] 2,2′-diphenyl-1-picrylhydrazyl (DPPH)

100 g). The improved TPC (relative to the control and solvent extracted oil) of the tested oil might be due to reduced complexation of phenolics with the seed polysaccharides and consequent enhanced partitioning into the oil phase. In the DPPH assay, the extracts from EAAE oil exhibited a greater ability to scavenge DPPH in terms of lower IC_{50} (63.8–65.8 μg/mL) as against solvent-extracted oil extracts (73.4 μg/mL). The level of % inhibition of linoleic acid oxidation of the EAAE oil extracts (49.8%–55.2%) was observed to be significantly ($P < 0.05$) higher as against the solvent extracted oil extracts (42.5%). It has been investigated that the enzyme treatment offered an enhanced release of minor components (phenols, tocopherols, volatiles, carotenes, xanthophylls and chlorophylls) into the oil phase consequently improving the analytical parameters related to flavour and shelf-life [38].

Conclusion

The aqueous enzyme-assisted extraction method, relative to the control was found to be an effective technique for obtaining a higher oil recovery from sunflower seeds. In the present work we simultaneously extracted high quality oil and food grade protein. The mild operational conditions during the current process ensure the retention of the nutritionally important and antioxidant components resulting in better oxidative stability of the extracted oils. This process may prove to be an environment-friendly alternative to solvent extraction. The two major drawbacks in the proposed process are the cost of the enzyme and the low oil yield. The authors suggest further work with modified mixtures of enzymes, which can efficiently break the sunflower seed cell wall thus facilitating the enhanced liberation of oil. The use of commercial enzymes (Kemzyme, and Natuzyme) as used in the present study may cover the cost of analytical enzymes.

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