

EVALUATION OF OMEGA-3 FATTY ACIDS CONTENT AND ANTIOXIDANT ACTIVITY IN WHEAT (*Triticum aestivum* L.) LEAVES

Avaliação do teor de ácidos graxos ômega-3 e atividade antioxidante em folhas de trigo (*Triticum aestivum* L.)

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ABSTRACT

The objective of this study was to quantify alpha-linolenic acid (LNA, 18:3n-3) of dried wheat leaves, harvested at different development stages (20, 40 and 80 days), to determine the antioxidant potential and the total phenolic compounds of leaves harvested at 80 days, as well as to perform mineral analysis (Na, K, Fe, Ca, Mg, P, Zn, Cu and Mn). It was observed a predominance of polyunsaturated fatty acids (PUFA) compared to saturated fatty acids (SFA) in the lipid fraction of leaves. Leaves collected in all periods presented ratios of PUFA/SFA, omega-6 and omega-3 fatty acids (n-6/n-3) considered suitable for food. The highest content of LNA was found in leaves harvested at 60 days, corresponding to 2.146,72 mg 100 g⁻¹ dried matter. The range time between 60 and 80 days showed a decline of nearly 30% in the concentration of LNA. The acetate fraction was the most effective over DPPH radical assay, showing IC₅₀ value of 154 µg mL⁻¹, and the phenolic compounds content was 615.4 ± 36.4 mg EAG L⁻¹, both analyses carried out in leaves harvested at 80 days. In this development stage, wheat leaves showed significant levels of the minerals P, Zn, Cu and Mn. These results reinforce the potential of using wheat leaves in foods, according to its antioxidant content and significant levels of LNA.

Index terms: Alpha-linolenic acid, phenolic compounds, antioxidant activity.

RESUMO

Conduziu-se este estudo, com o objetivo de quantificar o ácido alfa-linolênico (LNA, 18:3n-3) em folhas de trigo secas e colhidas em diferentes estádios de desenvolvimento (20, 40 e 80 dias), determinar o potencial antioxidante e o conteúdo de fenólicos totais em folhas colhidas aos 80 dias, assim como realizar a análise de minerais (Na, K, Fe, Ca, Mg, P, Zn, Cu e Mn). Foi verificada uma predominância de ácidos graxos poli-insaturados (AGPI) comparado com os ácidos graxos saturados (AGS) na fração lipídica das folhas. As folhas colhidas em todos os períodos apresentaram razões de AGPI/AGS e de ácidos graxos ômega-6 e ômega-3 (n-6/n-3), dentro dos valores considerados adequados para a alimentação. O maior teor de LNA foi encontrado nas folhas colhidas aos 60 dias, correspondendo a 2.146,72 mg 100 g⁻¹ de matéria seca. Entre 60 e 80 dias, observou-se uma queda de cerca de 30% na concentração de LNA. A fração acetato foi eficiente na inibição do radical DPPH, apresentando valor de IC₅₀ de 154 µg mL⁻¹, e o teor de compostos fenólicos foi de 615,4 ± 36,4 mg EAG L⁻¹, ambas as análises para as folhas colhidas aos 80 dias. Nesse estágio de crescimento, as folhas apresentaram consideráveis teores dos minerais P, Zn, Cu e Mn. Tais resultados reforçam a importância do uso de folhas de trigo para alimentação humana, em função de seu potencial antioxidante e consideráveis teores de LNA.

Termos para indexação: Ácido alfa-linolênico, fenólicos totais, atividade antioxidante.

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INTRODUCTION

In recent decades, many studies have been performed regarding the beneficial effects of wheatgrass intake. Its consumption has been associated with the fortification of the immune and cardiovascular systems, in addition to detoxifying action in the human body (MARWAHA et al., 2004; TRIANTAFILLIDIS, 2008). Wheatgrass is considered wheat leaves harvested at the first eight days of growth. The wheatgrass has showed

antioxidant activity (MOTOMURA et al., 2008), and phenolic content of 1000 mg chlorogenic acid equivalents 100 g⁻¹ dried (CEVALLOS-CASALS; CISNEROS-ZEVALLOS, 2010). Wheatgrass presented also lipid composition rich in linolenic fatty acid. This fatty acid are produced by ω-3 fatty acid desaturases located in the endoplasmic reticulum and plastid membranes (HORIGUCHI et al., 1996a) and increases with maturation of leaf cells (HORIGUCHI et al., 1996b). Besides these great

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nutritional proprieties, wheatgrass is considered an effective source of minerals (K, Mn, Zn, Fe and Na) (KULKARNI et al., 2007).

However, despite the extensive literature on chemical and biological effects of wheatgrass intake, few studies are found for wheat leaves harvested at different development.

In general, green leaves have the alpha-linolenic acid (LNA, 18:3 n-3) in their lipid composition, which is an important component of the lipid membrane of chloroplasts (SIMOPOULOS, 2002). Other nutritionally important compounds that are present in leaf tissue are antioxidants such as carotenoids, vitamins, phenols and flavonoids (WANG; LIN, 2000).

The LNA is a polyunsaturated fatty acid of the n-3 series (PUFA n-3), precursor of long chain fatty acids in this series, such as docosahexaenoic acid (DHA, 22:6 n-3) and eicosapentaenoic acid (EPA, 20:5n-3), considered essential to maintaining a healthy body (Martin et al., 2006). Several studies have showed that they are involved in metabolic processes, cardiovascular activities (PSOTA et al., 2006; SCHACKY; HARRIS, 2007), prevention of chronic neurodegenerative diseases such as Alzheimer's disease (MAZZA et al., 2007), among other effects reported.

A countless variety of foods derived from plants is a source of compounds that have antioxidant activity (MOURE et al., 2001), including herbs (ZHEN; WANG, 2001), mushroom (*Agaricus blazei*) (SILVA et al., 2009) and some vegetables and fruits (PIENIZ et al., 2009). Antioxidants can be defined as species that when present in low concentrations compared to the oxidizable substrate prevent or retard the oxidation of lipids and other molecules (ZHEN; WANG, 2001). The compounds responsible for this trait, such as alpha-tocopherol, carotenoids, flavonoids and some enzymes, offer protection for plant against stress caused by reactive oxygen species (MISHRA et al., 1993).

Many studies have investigated the importance of consuming foods containing substances with antioxidant properties in its composition, preventing chronic diseases such as cardiovascular diseases, cancer and degenerative brain disorders associated with aging (CHU et al., 2000; LIYANA-PATHIRANA; SHAHIDI, 2006).

Thus, the purpose of this study was to quantify the LNA of wheat leaves harvested at different development stages (20, 40 and 80 days), and to estimate the antioxidant potential against DPPH radical assay, the total phenolic content, and minerals (Na, K, Fe, Ca, Mg, P, Zn, Cu and Mn) of leaves harvested at 80 days.

MATERIAL AND METHODS

The experiment was conducted in the countryside of Maringá - PR (23°25' south and 51°57' west) from March to May 2007. The average altitude is 596 m and the climate is mesothermal humid, Cfa type (Köppen).

The plot consisted of six rows of 4 m long, spaced 0.50 m. Plants were harvested manually at 20, 40 and 80 days, washed in water, dried in an oven with circulating air for 48 hours at 40° C, milled in a grinder of knives, stored in polyethylene bags at -18° C and then subjected to analysis.

Extraction of total lipids

The total lipids were extracted according to the methodology of Bligh e Dyer (1959) with modification in the solvents volumes. Samples (1.00 g) was added into a mixture of 30 mL chloroform-methanol (1:2 v/v) and shaken vigorously for 5 minutes, following by addition of 10 mL of chloroform, kept under stirring for 2 minutes, and 18 mL of distilled water, agitated for 5 minutes. The mixture was filtered under vacuum on Büchner funnel. The organic solvent phase containing chloroform and lipids was drained into flask and the solvent removed in rotary evaporator under vacuum at 40° C. Lipid content was determined gravimetrically. All chemical tests were performed in triplicate.

Transesterification and analysis of methyl esters of fatty acids

The fatty acid methyl esters (FAME) were prepared by the method of Hartman e Lago (1973), modified by Maia e Rodrigues-Amaya (1993). The FAME were analyzed in a Shimadzu 14-A (Kyoto, Japan) gas chromatography equipped with a flame ionization detector (FID) and a fused silica capillary column CP-Select CB-FAME (100 m x 0.25 mm id., 0.25 µm film thickness, Varian, EUA). The operational parameters were as follows: detector temperature, 230° C; injection temperature, 220° C; column temperature, from 150 to 185° C at 2° C min⁻¹ and to 225° C at 10° C min⁻¹, final holding time of 20 min; carrier gas, hydrogen at 1.2 mL min⁻¹; make-up, nitrogen gas at 30 mL min⁻¹; split injection at 1:100 ratio. Sample (2.0 µL) was injected into the gas chromatograph. Peak areas were determined in CG-300 Computing integrator (CG Instruments, Brazil) and FAME were identified by comparison with known retention times of standards from Sigma (USA).

Quantification of alpha-linolenic acid

Quantification (in mg fatty acid g⁻¹ of total lipids) was made against tricosanoic acid methyl ester as an internal standard (23:0), as described by Joseph e Ackman (1992). Theoretical FID (flame ionization detector) correction

factor (VISENTAINER; FRANCO, 2006) values were used to obtain concentration values. Fatty acid contents were calculated in mg g⁻¹ of total lipids by using equation 1.

$$FA = \frac{A_x W_{IS} CF_X}{A_{IS} W_X CF_{AE}} \quad (1)$$

where FA is mg of fatty acids per g of total lipids, A_x is the peak area (fatty acids), A_{IS} is the peak area of internal standard (IS) methyl ester of tricosanoic acid (23:0), W_{IS} is the IS weight (mg) added to the sample (in mg), W_x is the sample weight (in mg), CF_x is the theoretical correction factor, and CF_{AE} is the conversion factor necessary to express results as mg of fatty acids rather than as methyl esters.

The results were converted from mg g⁻¹ of total lipids to mg fatty acid 100 g⁻¹ dried leaves.

Mineral content

The minerals (Na, K, Fe, Ca, Mg, P and Zn) of leaves harvested at 80 days were determined by atomic absorption spectrometry as described in AOAC (CUNNIFF, 1998).

Antioxidant activity

Methanol extract was prepared by the 48 h percolation of 70 mL through 10.0 g of dried leaves harvested at 80 days. New rates of methanol was added every 24 h. After being filtered, the extracts were concentrated under reduced pressure at 40° C. A fraction of methanol extract was performed using solvents of increasing polarity: hexane, ethyl acetate, 1-butanol and water. The fractions were collected separately and evaporated under reduced pressure.

The antioxidant capacity of 80-days dried leaves were studied against 1,1-diphenyl-2-picrylhydrazyl (Sigma) according to El-Massary et al. (2002). Different aliquots of extract solutions prepared by addition of 20.0 mg of methanolic extract and 10 mL methanol were mixed with 2.0 mL of DPPH methanolic solution. The mixture was thoroughly vortex-mixed and kept in the dark for 30 min. Next, absorbance was measured at 517 nm against a methanol blank without DPPH using spectrophotometer (Cary Win UV 50, Varian). The results were expressed as percent inhibition of the DPPH radical (% inhibition DPPH[•]), which was calculated according to the following equation 2.

$$\% \text{Inhibition DPPH}^{\bullet} = \frac{(Abs_{DPPH^{\bullet}} - Abs_{sample})}{Abs_{DPPH^{\bullet}}} \times 100 \quad (2)$$

where Abs_{DPPH[•]} is the absorbance of the DPPH[•] solution without extracts, and Abs_{sample} is the absorbance of the sample solution.

The analyses were carried out in triplicate and the extract concentration providing 50% inhibition (IC₅₀) was obtained by plotting the concentrations of the extract solutions versus percent inhibition.

Total phenolics were determined according to the Shahidi e Naczk (1995) method with slight modifications. A 0.25-mL aliquot of extract solution (2.50 mg mL⁻¹ in methanol) was mixed with 0.25 mL of Folin-Ciocalteu reagent previously diluted with water 1:1 v/v, 0.50 mL of a saturated sodium carbonate solution and 4.0 mL of water. The mixture was rested at room temperature for 25 min and then centrifuged at 5000 rpm for 10 min. The supernatant absorbance was measured at 725 nm using a spectrophotometer (Cary Win UV 50, Varian). The results were expressed as milligrams gallic acid equivalents per L (mg GAE L⁻¹).

Statistical analysis

The results were submitted to variance (ANOVA) analysis and Tukey's test (5% probability) using the software Statistica 5.0 (STATISTICA, 1995).

RESULTS AND DISCUSSION

The lipid contents were 3.21, 3.29, 3.91 and 2.73 g 100⁻¹ g of dried matter for 20, 40, 60 and 80 days respectively. It was found saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in wheat leaves. Among the SFA, were identified myristic (14:0), palmitic (16:0), stearic (18:0), behenic (22:0) and lignoceric (24:0) acids. A predominance of palmitic acid, ranged from 309.92 to 509.95 mg 100 g⁻¹ of dried leaves, was observed in this category of fatty acids. The MUFA consisted in palmytoleic (16:1 n-7) and oleic acid (18:1 n-9). The PUFA were the largest fatty acid portion found in wheat leaves, and linoleic acid (LA, 18:2n-6) and LNA were detected. The LNA was the most abundant in the lipid fraction extracted from wheat leaves (Table 1), with mass percentage ranging from 63.4 to 75.0%.

The LNA values for wheat leaves harvested at 20, 40 and 60 days did not differ significantly (P<0.05). There was a significant decrease (P<0.05) in the LNA concentration in the wheat leaves between 60 and 80 days.

Almeida et al. (2006) reviewed different types of grasses, and found levels of 238.46 and 230.34 mg 100 g⁻¹ of dried matter of *Brachiaria decumbens* and *Rynchospora speciosa*, respectively, both values lower than those found for wheat leaves at different growth times.

Table 1 – Fatty acids concentration (mg 100 g⁻¹ dried material) in wheat leaves at different growth stages.

	20 days	40 days	60 days	80 days
14:0	11.52 ^b ±10.42	17.90 ^b ±3.08	41.08 ^a ±5.78	20.56 ^b ±7.08
16:0	317.42 ^{ab} ±133.86	309.92 ^b ±38.08	509.95 ^a ±106.11	377.89 ^{ab} ±121.23
16:1 n-7	26.78 ^{ab} ±8.25	31.36 ^{ab} ±3.34	33.45 ^a ±5.06	21.82 ^b ±7.55
18:0	57.66 ^a ±8.96	27.35 ^b ±7.70	61.39 ^a ±7.09	28.68 ^b ±8.54
18:1 n-9	40.49 ^b ±6.59	51.31 ^b ±13.16	96.11 ^a ±16.92	36.99 ^b ±12.91
18:2n-6	226.71 ^b ±25.09	238.98 ^b ±23.61	338.57 ^a ±36.09	285.87 ^{ab} ±88.33
18:3n-3	1,979.63 ^{ab} ±79.55	2,034.31 ^a ±137.21	2,146.72 ^a ±258.07	1,438.98 ^b ±467.90
22:0	13.98 ^a ±1.71	19.17 ^a ±6.06	17.00 ^a ±6.56	26.56 ^a ±7.52
24:0	3.94 ^b ±0.13	9.31 ^{bc} ±0.87	17.87 ^c ±2.85	34.15 ^a ±10.25

Values are mean ± standard deviation of triplicate analyses. Different letters in the same line indicate significant differences (P<0.05) by Tukey test.

In wheat leaves, the ratios obtained of polyunsaturated fatty acids and saturated fatty acids (PUFA/SFA) ranged from 3.54 (80 days) to 5.93 (40 days). According to the recommendation of the Department of Health (HER MAJESTY'S STATIONERY OFFICE - HMSO, 1994), this ratio in the diet should be higher than 0.45 in relation to cardiovascular disease.

For the ratio of n-6 and n-3 fatty acids was found the mean value of 0.15. In most Western countries, there is a growing concern in decreasing dietary n-6/n-3 ratio to values between 10 and 5:1 (DAVIS; KRIS-ETHERTON, 2003). This condition can be favored by the insertion of wheat leaves in the diet.

Evidence from epidemiological studies suggests that diets rich in n-3 PUFA may be associated with reduced cancer risk (BARACOS et al., 2004). Although the lipid content in plant tissues is not high, the presence of nutritional fatty acids, such n-3 fatty acids, provides increasing intake of these on the diet (MARTIN et al., 2006). According to both ratios presented below, these leaves can be considered nutraceutical food.

It was established that the recommended daily intake (RDI) of LNA for men and women aged between 19 and 70 years is 1.1 and 1.6 g, respectively (INSTITUTE OF MEDICINE OF THE NATIONAL ACADEMIES, 2002). The incorporation of about 20 g of dried wheat leaves in the diet of individuals in this age group provides around 28% of the RDI for this fatty acid.

The changes on fatty acid composition during growth are related to alterations in lipid membranes (WANG; LIN, 2006). Temperature and other environmental factors (CHAPMAN et al., 1983), the salinity levels

(TAARIT et al., 2010) and the geomagnetic field (NOVITSKAYA et al., 2006) are factors affecting fatty acid composition of plant growth in Nature.

The antioxidant activity of wheat leaves harvested at 80 days showed satisfactory values, especially for the acetate fraction, which inhibited 50% of the absorbance of DPPH radical (IC₅₀) after the addition of 154 µg mL⁻¹ of acetate extract (Table 2). In this analysis, the higher the IC₅₀ value, the less effective is the antioxidant activity. For the aqueous fraction, this value was higher than 500 µL, indicating that the compounds that act as antioxidants in wheat leaves might have intermediate polarity, such as ethyl acetate and 1-butanol alcohol. These compounds range from simple phenolic acids to complex aromatic structures (CHUN et al., 2005).

Cavaliere et al. (2005) detected flavonoid compounds such as apigenin, luteolin, chrysoeriol, tricetin, in their glycoside forms, which might be responsible for antioxidant activity in the leaf tissue of wheat.

Polar solvent, such as methanol (CEVALLOS-CASALS; CISNEROS-ZEVALLOS, 2010), water and ethanol (Motomura et al., 2008), have commonly been used to optimize the phenolic compound extraction in wheat leaves. The average of phenolic compound in wheat leaves harvested at 80 days was 615.4 ± 36.4 mg GAE L⁻¹. Cevallos-Casals e Cisneros-Zevallos (2010) found a changing in phenolic content over different development stages, and noted an increase of more than 500% after 7 days of germination. Table 3 shows the concentration of minerals found in the wheat leaves harvested at 80 days.

There are minerals, such as calcium, phosphorus, magnesium and potassium which are considered

Table 2 – Antioxidant activity of 80-days wheat leaves extract.

Fraction	IC ₅₀ (µg mL ⁻¹)
Hexanic	241.9 ^d ±2.3
Acetate	153.7 ^e ±1.0
Butanolic	228.7 ^c ±0.9
Methanolic	387.0 ^b ±3.8
Water	> 500 ^a

Values are mean ± standard deviation of triplicate analyses. Different letters in the same column indicate significant differences (P <0.05) by Tukey test.

Table 3 – Minerals concentration (mg 100 g⁻¹) of dried wheat leaves harvested at 80 days.

Mineral	Concentration	Mineral	Concentration
Sodium (Na)	5.79±0.14	Total phosphorus (P)	46.25±1.16
Potassium (K)	6.12±0.26	Zinc (Zn)	9.07±0.04
Iron (Fe)	18.84±0.61	Copper (Cu)	1.25±0.02
Calcium (Ca)	27.70±1.25	Cobalt (Co)	Nd ¹
Magnesium (Mg):	11.02±0.11	Manganese (Mn)	10.03±0.86

¹Below the limit of detection (0.0001 mg kg⁻¹).

macronutrients, and are necessary in larger quantities. Moreover, the micronutrients are needed in very small quantities, but with equal importance, such as iron, manganese, copper and zinc (KRAUSE; MAHAN, 1991).

Regarding mineral considered macronutrients, the total phosphorus and calcium showed higher concentrations in leaves and the ingestion of 100 g of dried leaves would supply about 6.61% (P) and 2.77% (Ca) of the RDI for adults, in accordance with current legislation (Brasil, 2005). For minerals considered micronutrients, the intake of 100 grams of wheat leaves would provide 129% of the RDI for Zn, 139% for Cu and 426% for Mn, however it must be considered the bioavailability of these nutrients.

CONCLUSION

The results indicated that the wheat leaves are abundant in alpha-linolenic acid, especially during the first 60 days of development, and offer considerable antioxidant activity, total phenolics and minerals. This represents an alternative way to feed animals and to increase the LNA levels in the human diet.

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