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Effect of nitrogen fertilisation rates on the content of fatty acids, sterols, tocopherols and phenolic compounds, and on the oxidative stability of walnuts

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ABSTRACT

The influence of nitrogen (N) fertilisation on the content of lipids and phenolic compounds in walnut kernels (cv. Chandler) has studied for three consecutive growing years. Moreover, a new technique (OXITEST) was set up to analyse the oxidative stability of the kernel directly from the whole sample. Significant differences in the fatty acid composition were observed, and linoleic acid was the main fatty acid present. N fertilisation reduced the oleic acid content relative to the control. High amounts of N increased the linoleic acid content and reduced the linolenic acid content. On the other hand, the control and the lower N fertilised samples had the highest levels of n-3 fatty acids. Comparing control and fertilised samples, there were no statistical differences in the sterol and tocopherol compositions (with the exception of α-tocopherol). With regard to phenolic content, N fertilisation had a significant negative effect on the phenolic compounds in walnut kernel samples. The OXITEST technique confirmed that the oxidative stability of kernels was related to the fatty acid composition and the PUFA (polyunsaturated fatty acid) content.

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1. Introduction

Nuts have received some attention in the search for bioactive components present in foods that reduce the risk of cardiovascular disease (Kris-Etherton et al., 1999). The favourable fatty acid profile of nut kernels provides a beneficial effect on plasma lipids and lipoproteins (López-Uriarte, Bulló, Casas-Agustench, Babio, & Salas-Salvadó, 2009). Walnuts, in particular, have a unique profile: they are rich in polyunsaturated fatty acids, which may improve blood lipids and have beneficial effects, such as helping to reduce the risk of cardiovascular disease. Overall, walnut-enriched diets significantly decrease total and LDL cholesterol for the duration of the short-term trials (Banel & Hu, 2009). Willis, Shukitt-Hale, Cheng, and Joseph (2009) have been examined the effects of walnut supplements on motor and cognitive ability in aged rats. The results demonstrated improved working memory: the 2% walnut diet improved performance on rod walking while the 6% walnut diet improved performance on the medium plank walk. The results of recent studies (Carvalho et al., 2010; Yang, Hai Liu, & Halim, 2009) have demonstrated that walnut green husks and leaf methanolic extracts possess effective antihemolytic and antiproliferative activities on human renal cancer cells. These properties are likely related to the phenolic constituents of walnuts.

The oil content in walnut kernels is determined by the genotype but may be influenced by environmental conditions and irrigation rate (Prasad, 2003). Some varieties, such as Chandler, Franquette, Hartley, Lara, Mayette, Serr, Sorrento and Tulare, have oil contents exceeding 700 g kg^{-1} kernel when grown in Argentine soil and climate. Some of these same varieties have lower oil contents when grown in Portugal (Amaral, Casal, Pereira, Seabra, & Oliveira, 2003).

Some bioactive compounds, including phytosterols and tocopherols, are present in the oil fraction (Savage, Dutta, & McNeil, 1999). Phytosterols inhibit the intestinal absorption of cholesterol, thereby reducing total plasma cholesterol and low-density lipoprotein (LDL) levels (Ellegård, Andersson, Normen, & Andersson, 2007; Patel, 2008). In addition, phytosterol consumption has been associated with the inhibition of cancer cell growth (Tapiero, Townsend, & Tew, 2003). Spaccarotella et al. (2008) observed

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a significant decrease in the α-T/γ-T ratio with an increased amount of γ-T serum and an increased ratio of free prostate specific antigen (PSA)/total PSA in an 8-week walnut supplement study. Their findings suggested that walnuts can improve prostate biomarkers and vascular status. Several studies have reported that phenolic compounds represent 95% of the total antioxidant activity of whole walnut kernel (Arranz, Pérez-Jiménez, & Saura-Calixto, 2008). Gómez-Caravaca, Verardo, Segura-Carretero, Caboni, and Fernández-Gutiérrez (2008) showed the high abundance of different classes of phenolic substances in walnuts, with aglycone and glycosylated ellagic acid representing 64–75% of the total phenol content.

As reported in literature (Dag et al., 2009; Fernández-Escobar et al., 2006; Leser & Treutter, 2005; Zheljazkov, Vick, Wayne Ebelhar, Buehring, & Astatkie, 2012) fertilisation affects the plant product composition, therefore the aim of the present research was to determine the effect of nitrogen fertilisation on the content of fatty acids, tocopherols, sterols and phenolic compounds in Chandler walnuts. These compounds were assessed over a three-year experimental period. Moreover, a new method using the OXITEST reactor was carried out to evaluate oxidative stability directly from the whole kernels without prior extraction.

2. Materials and methods

2.1. Samples

The experimental irrigated walnut orchard, located in the province of Forlì in northern Italy (latitude 44.5° north), was established in 1998 with *Juglans regia* cv. Chandler scions (Pedro x UC-56-224) grafted onto seedling rootstock (*J. regia*).

The trials were set up using three nitrogen fertilisation levels (0 as a control, 100 and 200 kg/ha per year). Nitrogen was soil-applied as mineral source (prilled urea, CH_4N_2O, at 46% of N) in spring and the total amount was split in 2 applications: i) bud burst; ii) after 30 days the first application on 5 rows of trees with a total of 40 trees.

Trees were managed in terms of nutrition, pruning, irrigation as well as pest and disease following the regional advisories for Integrated Crop Management (ICM, 2012). In particular, the standard rate of N admitted in the area of cultivation for this crop is 90 kg N/ha year with the possibility to increase it up to 140 kg N/ha year in case of low soil fertility. Thus, rates of applied N were chosen in order to verify the effect of the standard N rate adopted by regional growers (100 kg N/ha); 200 kg/ha nitrogen fertilisation was applied to produce a surplus of nitrogen that increase the vegetative growth and fruit production (data not shown).

The interspace between plants and rows was 7 × 7 m, corresponding to 204 trees/ha. Each N treatment was applied to 5 individual lines, and each row was used as a replicate. Five kilograms of dry nuts were collected from each line. The walnuts from the five lines were mixed, and a sample of 5 kg was used for the analysis. The walnut orchard was under organic system management, and the samples were collected in October 2007, 2008 and 2009 (corresponding to commercial harvesting) using mechanical methods.

2.2. Fat extraction

For chemical analysis, walnuts were manually cracked and shelled before chopping. Oil was extracted as reported by Verardo et al. (2009). Each extraction was carried out three times ($n = 3$) for each treatment.

2.3. Fatty acid analysis

The fatty acid methyl ester (FAME) composition of walnut oil was determined by capillary gas chromatography after alkaline treatment, as described by Christie (1998). The chromatographic conditions were the same adopted by Verardo et al. (2009).

2.4. Sterol analysis

The oil fraction was analysed by GC after silylation. The trimethylsilyl derivatives (TMS) of sterols were analysed as reported by Iafelice, Verardo, Marconi, and Caboni (2009). The analysis was carried out in triplicate for each treatment.

2.5. Tocopherol analysis

One gram of oil sample was dissolved in 10 mL of *n*-hexane, and the tocopherols were determined as previously described by Gómez-Caravaca, Verardo, and Caboni (2010).

2.6. Extraction and determination of phenolic compounds

The phenolic compounds were extracted using the optimised protocol described by Verardo et al. (2009). Each extraction was carried out three times for each treatment ($n = 3$) for each year.

The MEKC method was used to analyse the phenolic fraction, as previously described (Verardo et al., 2009).

2.7. OXITEST analysis

An OXITEST reactor (Velp Scientifica, Usmate, Milan, Italy) fitted with two separate oxidation chambers was used (Fig. 1). The oxidation tests were performed at 90 °C with an initial oxygen pressure of 6 bar. The sample was mixed with liquid nitrogen then chopped using a laboratory mill (IKA A10-IKA-Werke GmbH & Co. KG, Staufen, Germany). A sample amount of 7.5 g was used for the analysis. Each sample was measured four times for each treatment for each year, and the IPs (induction periods), expressed in minutes, were obtained by using the two tangent method.

2.8. Statistical analysis

The results are the average of the data for the extractions from three samples, and each extraction was analysed in triplicate. The OXITEST data were the results of four analytical determinations for each treatment for each year.

A one-way analysis of variance (ANOVA), Tukey's honest significant difference multiple comparison procedure and Pearson's correlations were evaluated using Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA).

3. Results and discussion

3.1. Fatty acid, sterol and tocopherol composition

The oil content in walnuts varied from 51.2% to 52.6%. No statistical differences were reported among the samples with different nitrogen fertilisation rates, and these data are in agreement with previous studies (Amaral et al., 2003; Bada, Leon-Camacho, Prieto, Copovi, & Alonso, 2010; Verardo et al., 2009).

The fatty acid composition of the oil extracted from walnut samples is shown in Table 1. No qualitative differences in the fatty acid composition were observed among the extracted oil samples. According to the literature (Amaral et al., 2003; Bada et al., 2010; Maguire, O'Sullivan, Galvin, O'Connor, & O'Brien, 2004), the major

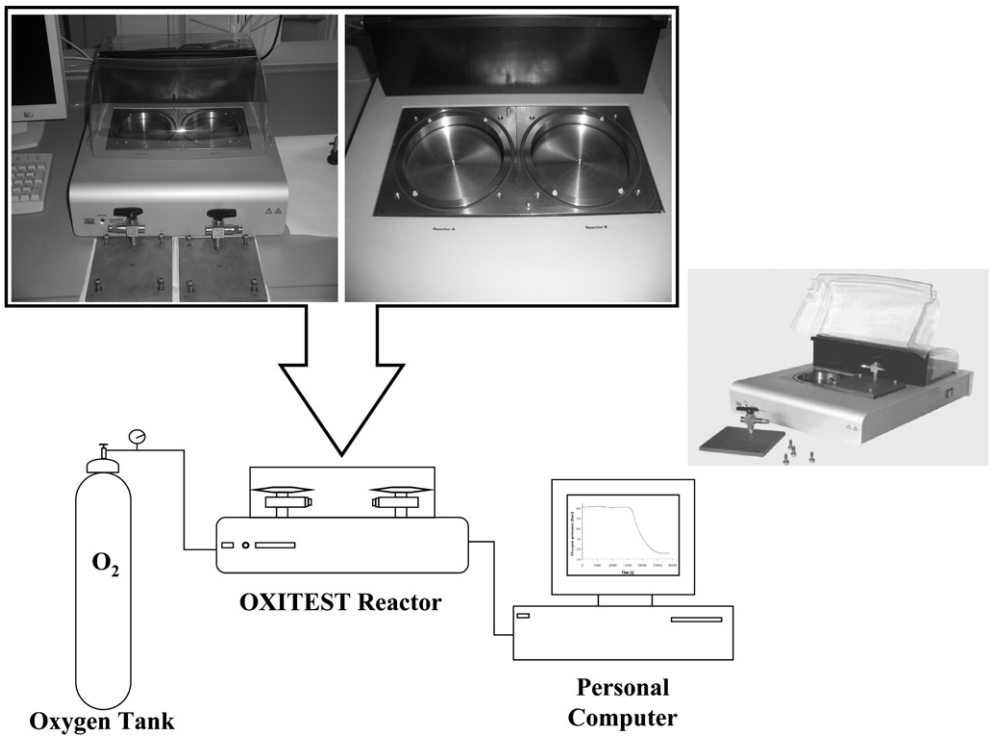

Fig. 1. OXITEST reactor equipment.

unsaturated fatty acids in walnut oil are C18:2 n-6, C18:3 n-3 and C18:1, and the major saturated fatty acids are C16:0 and C18:0. Low quantities of other fatty acids (C20:1 n-9, C20:0 and C16:1) have also been reported.

Linoleic acid (C18:2 n-6) was the most abundant fatty acid, ranging between 58.6 and 63.9 g/100 g of fat, followed by oleic acid (C18:1) and linolenic acid (C18:3 n-3), with contents of 13.9–16.8 g/100 g of fat and 12.1–15.6 g/100 g of fat, respectively. The amount of C16:0 and C18:0 were 5.9–6.5% and 1.9–2.9%, respectively. C20:1 accounted for 0.2–0.3%, while C16:1 and C20:0 combined accounted for approximately 0.1 g/100 g of fat of total fatty acids. These ranges were similar to those reported by Crews et al. (2005) for Italian walnuts and also confirm our previous work (Verardo et al., 2009).

Quantitative differences were observed between the control and fertilised walnuts, and statistical differences in the amounts of C18:1, C18:2 and C18:3 n-3 were also observed (Table 1).

Nitrogen fertilisation reduced the oleic acid content in treated samples compared with the untreated control. Similar results were reported by Dag et al. (2009) for the fatty acid composition of olive oil. High quantities of nitrogen (200 N kg/ha) led to an increase in the linoleic acid (C18:2 n-6) content and, on the other hand, a reduction in the synthesis of linolenic acid (C18:3 n-3). More specifically, the control and the samples fertilised with low quantities of nitrogen (100 N kg/ha) exhibited a higher content of linolenic acid. Eitenmiller and Pegg (2008) reported the same results in pecan samples.

PUFAs were the major group of fatty acids present in the walnut oils, ranging from 74.1% to 76.8%, followed by MUFAs, which ranged from 14.2 to 17.1%, and SFAs, which were the least abundant group (8.5–9.1%). According to the ANOVA, nitrogen fertilisation reduced MUFA content and increased PUFA content; these data confirm the results reported by Dag and colleagues for olive fruits (Dag et al., 2009). SFA content did not differ significantly ($p < 0.05$) between the control and fertilised samples. A significant linear trend was observed for the n-6/n-3 fatty acid ratio, which increased with the nitrogen fertilisation rate. Plant fat metabolism is quite a complex process, which mainly takes place in plastid and endoplasmic

Table 1
Fatty acid composition (%) of walnut samples. Different letters in the same line indicate significantly different values ($p < 0.05$, HSD Tukey test).

Fatty acid	Control			100 N kg/ha			200 N kg/ha		
	2007	2008	2009	2007	2008	2009	2007	2008	2009
C16:0	5.90 a	5.88 a	5.93 a	5.90 a	5.92 a	6.12 a	6.20 a	6.46 a	6.11 a
C16:1	0.06 a	0.07 a	0.06 a	0.06 a	0.07 a	0.06 a	0.07 a	0.08 a	0.06 a
C18:0	2.94 a	2.50 a	2.85 a	2.81 a	2.61 a	2.70 a	2.81 a	2.94 a	2.81 a
C18:1	16.59 a	16.80 a	16.82 a	14.71 b	14.26 b	15.12 b	13.89 b,c	14.74 b	15.04 b
C18:2 n-6	58.58 c	59.28 c	59.29 c	61.30 b	61.27 b	61.19 b	63.71 a	63.89 a	63.55 a
C18:3 n-3	15.59 a	15.19 a	14.77 a	14.91 a	15.57 a	14.52 a	12.94 b	12.55 b	12.13 b
C20:0	0.11 a	0.09 a	0.09 a	0.09 a	0.09 a	0.09 a	0.11 a	0.10 a	0.09 a
C20:1 n-9	0.23 a	0.19 a	0.19 a	0.22 a	0.21 a	0.20 a	0.17 a	0.24 a	0.21 a
∑SFA	8.95 a	8.47 a	8.87 a	8.80 a	8.62 a	8.91 a	9.12 a	8.50 a	9.01 a
∑MUFA	16.88 a	17.06 a	17.07 a	14.99 b	14.54 b	15.38 b	14.23 b	15.06 b	15.31 b
∑PUFA	74.17 b	74.47 b	74.06 b	76.21 a	76.84 a	75.71 a	76.65 a	76.44 a	75.68 a

∑SFA, sum of saturated fatty acids; ∑MUFA, sum of monounsaturated fatty acids; ∑PUFA, sum of polyunsaturated fatty acids. All chemical parameters are expressed in g per 100 g of total fat.

reticulum. The modifications in fatty acid composition showed in this work, might be explained by the enhancement or inhibition of oleate desaturase activity during triacylglycerol biosynthesis (Dag et al., 2009). This observation could be explained by the fact that nitrogen supply can materially assist in retaining leaves in active photosynthesis. High photosynthetic activity can accelerate the seed maturity. As the highest polyunsaturated fatty acid accumulation is expected in fully ripened seed, this might be a reason why nitrogen increases the content of linoleic acid (Ghasemnezhad & Honermeier, 2008).

The tocopherol composition of walnut oil is shown in Table 2. The total tocopherol content ranged from 24.9 to 26.2 mg/100 g of oil with an average of 25.5 mg/100 g of oil. Among tocopherols identified, γ-tocopherol was the most abundant and represented approximately 94–95% of the total tocopherol composition, which is equivalent to 23.5–24.8 mg/100 g of oil. Low amounts of α-tocopherol and δ-tocopherol were present and ranged from 0.8 to 1.0 and from 0.5 to 0.6 mg/100 g of oil, respectively. β-Tocopherol was not detected. Crews et al. (2005) reported similar findings, including the absence of β-tocopherol, in walnuts harvested in different countries. Lavedrine, Ravel, Poupard, and Alary (1997) presented some data on the vitamin E content of walnuts grown in France and in the USA. They identified α-, β- and γ-tocopherol in fresh and stored nuts and reported γ-tocopherol as the main tocopherol in walnut oil. The same results were obtained by Amaral, Alves, Seabra, and Oliveira (2005) and Martınez, Barrionuevo, Nepote, Grosso, and Maestri (2010). Taken together, these findings highlight the variability of tocopherol content in walnuts based on the cultivar and geographical origin.

Our results show that nitrogen fertilisation had no effect on tocopherol content, except for α-tocopherol, which was statistically reduced when nitrogen fertiliser was applied (Table 2).

The sterol composition of the walnut samples in the present study was in agreement with the literature (Amaral et al., 2003; Crews et al., 2005). Sitosterol dominated the sterol profile of walnut oil and was present in the range of 76.4–77.6%. Sitosterol was followed by Δ^5-avenasterol, cholesterol and campesterol, which were detected in the ranges of 11.4–12.4%, 6.0–7.0% and 3.8–4.5%, respectively. Low amounts (≈0.8%) of sitostanol were also detected in the walnut oils.

In Table 3, the sterol composition of the walnut oils is expressed in milligrams per 100 g of oil. The total levels ranged from 130.3 to 133.6 mg/100 g, and no statistical differences ($p < 0.05$) were observed between the control and fertilised walnuts.

The levels of sitosterol ranged from 101.1 to 102.9 mg/100 g of oil, and these data were in agreement with previous reports (Amaral et al., 2003; Crews et al., 2005; Martınez, Labuckas, Lamarque, & Maestri, 2010). The second major sterol was Δ^5-avenasterol, with levels between 15.1 and 16.2 mg/100 g of oil, followed by cholesterol (7.9–9.3 mg/100 g oil) and campesterol (5.0–5.9 mg/100 g oil). These results were also in agreement with previous reports (Amaral et al., 2003; Crews et al., 2005), with the exception of the cholesterol content, which was higher than the levels reported in the cited works. However, Miraliakbari and Shahidi (2008) reported cholesterol content values similar to those in the present study.

3.2. Determination of phenolic compounds

To identify the phenolic compounds present in the walnut oil, a capillary electrophoresis method was performed, as previously described (Verardo et al., 2009). The electropherogram obtained from the injection of ethanolic extracts was characterised by two separate peak zones. The first, from 1.4 to 5.5 min, represented phenolic acids, flavonoids and naphthoquinones (i.e., juglone), the second zone, according to Gómez-Caravaca et al. (2008) and considering the chemical structures and electrophoretic properties of walnut phenolic compounds, may represent tannins, ellagic derivatives and glansreginin derivatives.

The simple phenolic content (SPC), complex phenolic content (CPC) and total phenolic content (TPC) of the walnut samples are shown in Table 4.

The simple phenolic content (SPC) was 9.8–11.1% of the total phenolic fraction. The SPC varied between 125.1 and 151.3 mg ferulic acid/100 g d.w. Walnuts fertilised with 200 kg/ha of nitrogen had the lowest SPC content, compared to the control samples and walnuts fertilised with 100 kg/ha of nitrogen, which had similar simple phenolic contents in the range of 141.1–151.3 mg ferulic acid/100 g d.w. This finding underscored the negative effect of high quantities of nitrogen (200 kg/ha) on simple phenolic content. No statistically significant differences ($p < 0.05$) were observed between the control and the samples fertilised with 100 kg/ha nitrogen. The CPC of the walnut samples varied between 1155.9 and 1228.6 mg tannic acid/100 g d.w. and represented 88.9–90.2% of the total phenolic fraction of walnuts. These data were in agreement with the findings of Sze-Tao, Schrimpf, Teuber, Roux, and Sathe (2001).

As reported for the SPC, the CPC was also affected by high nitrogen fertilisation. As reported by some authors (Karamac, 2009; Landete, 2011), the CPC is involved in the *in vitro* and *in vivo* antioxidant activities.

The TPC ranged from 1281.0 to 1378.9 mg/100 g d.w., and these data are in the same order of magnitude as previously reported values (Salcedo, López de Mishima, & Nazareno, 2010; Samaranayaka, John, & Shahidi, 2008; Yang et al., 2009). Other authors have observed a reduction of phenolic content in other crops in response to higher doses of nitrogen (Dag et al., 2009; Fernández-Escobar et al., 2006; Leser & Treutter, 2005). According to Nguyen and Niemeyer (2008) and Fernández-Escobar et al. (2006) these results may be explained using the growth-differentiation balance (GDB) framework which is based on the principle that a "physiological trade-off" exists between plant growth and secondary metabolite production. When environmental conditions are good and nitrogen levels are adequate, the GDB theory states that plant growth will be favoured, with production of photosynthetic proteins receiving resource priority. However, when environmental conditions are poor and the availability of an essential nutrient such as nitrogen is limited, the GDB

Table 2
Tocopherol composition (mg/100 g oil) of walnut samples. Different letters in the same line indicate significantly different values ($p < 0.05$, HSD Tukey test).

Tocopherol	Control			100 N kg/ha			200 N kg/ha		
	2007	2008	2009	2007	2008	2009	2007	2008	2009
α	0.98 a	0.99 a	0.98 a	0.90 b	0.91 b	0.90 b	0.82 b	0.80 b	0.81 b
γ	24.72 a	24.19 a	23.59 a	23.98 a	23.46 a	23.69 a	24.77 a	24.61 a	23.92 a
δ	0.55 a	0.54 a	0.56 a	0.52 a	0.52 a	0.50 a	0.52 a	0.51 a	0.52 a
Sum	26.25 a	25.72 a	25.13 a	25.40 a	24.89 a	25.09 a	26.11 a	25.92 a	25.25 a

Table 3
Sterol composition (mg/100 g oil) of walnut samples. Different letters in the same line indicate significantly different values ($p < 0.05$, HSD Tukey test).

Sterol	Control			100 N kg/ha			200 N kg/ha		
	2007	2008	2009	2007	2008	2009	2007	2008	2009
Cholesterol	7.87 a	8.53 a	8.04 a	8.42 a	8.45 a	7.90 a,b	8.55 a	9.34 a	8.74 a
Campesterol	5.22 a	5.66 a	5.93 a	5.83 a	5.02 a	4.99 a	5.51 a	5.41 a	5.10 a
Sitosterol	101.91 a	102.06 a	102.12 a	101.78 a	102.95 a	100.12 a	100.18 a	101.98 a	101.28 a
Sitostanol	1.02 a	1.10 a	1.11 a	1.04 a	1.05 a	1.06 a	1.00 a	1.02 a	1.03 a
Δ^5-avenasterol	15.51 a	16.23 a	15.22 a	15.93 a	15.16 a	16.20 a	15.43 a	15.11 a	15.19 a
Sum	131.53 a	133.58 a	132.42 a	133.00 a	132.63 a	130.27 a,b	130.67 a,b	132.86 a	131.34 a

framework proposes that growth allocation for a plant will decrease while the production of secondary metabolites that may aid in storage and defence subsequently increase. Within the GDB framework, the carbon/nutrient balance (CNB) hypothesis more specifically addresses the effects of fertilisation on plant resource allocation. The CNB theory states that under limited nutrient conditions, plants increase their production of carbon-based compounds such as polyphenolic compounds.

3.3. OXITEST analysis

A Rancimat apparatus is typically used to evaluate the oxidative stability of walnuts after their oil has been extracted (Amaral et al., 2003; Martınez et al., 2011; Rabrenovic, Dimic, Maksimovic, Sobajic, & Gajic-Krstajic, 2011).

In the present study, however, an OXITEST instrument was used to evaluate the oxidative stability of walnut samples. One advantage of this technique is that food stability against rancidity can be measured directly on whole foods (solid, liquid, dough) without any fat separation. Moreover, the information obtained from the instrument pertains not only to the induction period (IP) of the autoxidation process but also to the rate and acceleration of the autoxidation process and, finally, to the amount of oxygen consumed by the product (under specific accelerated conditions).

In our previous work (Comandini, Verardo, Maiocchi, & Caboni, 2009), a new method was used to evaluate the oxidative stability of different vegetables oils, and the results were compared with an established technique known as the OSI test. The results obtained were correlated with those achieved using OSI technology. Given this previous setting up, the aim of the present work was to validate the OXITEST parameters and to investigate the oxidative stability of walnut as a solid sample.

The first step was the evaluation of the sample quantity to be loaded into the oxidation chamber. Four sample amounts were considered (2.5, 5, 7.5 and 10 g), and the analysis was carried out six times ($n = 6$) for each quantity. According to Verleyen, Van Dyck, and Adams (2005, chap. 9), when 2.5 and 5 g of samples were used, the IP values were highly variable (RSD % > 20%). As reported by Verleyen et al. (2005, chap. 9), a small sample size will oxidise with a much greater variability than a larger sample size. The best results were observed using 7.5 and 10 g of sample, with the RSD % between 7.4 and 9.7%. The 7.5 g quantity was selected as the optimal quantity and used to establish the optimal temperature for the oxidation test. Two different temperatures, 90 and 110 °C, were considered. The choice of these temperatures was according to Verleyen and co-workers. They reported that the oxidative stability of oil and fat is usually determined at 100–110 °C, and accelerated tests of saturated fats may be carried out at 120–130 °C. At temperature > 120 °C, volatilisation of antioxidants might occur, leading to an underestimation of the oxidative stability.

The best results were obtained using 90 °C, which resulted in a lower RSD % (5.3%) compared with the RSD % at 110 °C (8.6%). These results indicated that the optimal conditions for the analysis of walnut samples are 90 °C temperature and 7.5 g of sample.

The control, 100 N kg/ha and 200 N kg/ha walnut samples were analysed, and the IPs for these samples are shown in Table 5.

The control sample exhibited the highest IP value (average 1905.6 min), followed by the 200 N kg/ha sample (average 1573.7 min) and the 100 kg/ha sample (average 1453.2 min) (Fig. 2). Fernández-Escobar et al. (2006) showed that nitrogen fertilisation of olive trees causes a significant decrease of oil stability. The positive correlation between the OXITEST results and the MUFA content ($r^2 = 0.8921$, $p < 0.001$) and the negative correlation between the OXITEST results and the PUFA content ($r^2 = -0.8865$, $p < 0.001$) confirmed the influence of fatty acid composition on the oxidative stability of walnut samples. Effectively, the control sample had a higher MUFA content, oleic acid in particular, and higher oxidative stability. Fertilised samples had a higher PUFA content and, consequently, lower oxidative stability than the control sample. The higher oxidative stability of the 200 N kg/ha sample over the 100 N kg/ha sample can be explained by the higher linoleic acid content and lower linolenic acid content of the 200 N kg/ha sample. Min and Boff (2002, chap. 11) reported that the reaction rate of triplet oxygen with linolenic acid is about twice as fast as the reaction with linoleic acid. As reported by Min and Boff (2002, chap. 11), oils that are more unsaturated are oxidised more quickly than less unsaturated oils. As the degree of unsaturation increases, both the rate of formation and the amount of primary oxidation compounds accumulated at the end of the induction period increase; this confirmed the trend of oxidation stability reported in walnut samples (control > 200 N kg/ha > 100 N kg/ha). Briefly, control samples fatty acids contain a lower unsaturation

Table 4
Phenolic content of walnut samples. Different letters in the same line indicate significantly different values ($p < 0.05$, HSD Tukey).

Phenolic compounds	Control			100 N kg/ha			200 N kg/ha		
	2007	2008	2009	2007	2008	2009	2007	2008	2009
SPC[a]	151.29 a	150.35 a	149.93 a	142.29 a,b	141.10 a,b	143.47 a,b	126.04 c	125.12 c	125.66 c
CPC[b]	1216.06 a	1228.58 a	1221.23 a	1199.35 a,b	1197.71 a,b	1201.56 a,b	1164.49 c	1155.9 c	1161.29 c
TPC	1367.35 a	1378.93 a	1371.16 a	1341.64 a,b	1338.81 a,b	1345.03 a,b	1290.53 c	1281.02 c	1286.95 c

SPC, simple phenolic compounds; CPC, complex phenolic compounds; TPC, total phenolic compounds.
[a] mg Ferulic acid/100 g of d.w. walnut.
[b] mg Tannic acid/100 g of d.w. walnut.

Table 5
Oxidative stability of walnut samples measured by OXITEST instrument. Different letters in the same column indicate significantly different values ($p < 0.05$, HSD Tukey).

Sample	Year	Oxidative stability (min)
Control	2007	1887.0 a
	2008	1910.4 a
	2009	1919.4 a
100 N kg/ha	2007	1452.6 c
	2008	1458.8 c
	2009	1448.2 c
200 N kg/ha	2007	1589.0 b
	2008	1573.4 b
	2009	1558.8 b

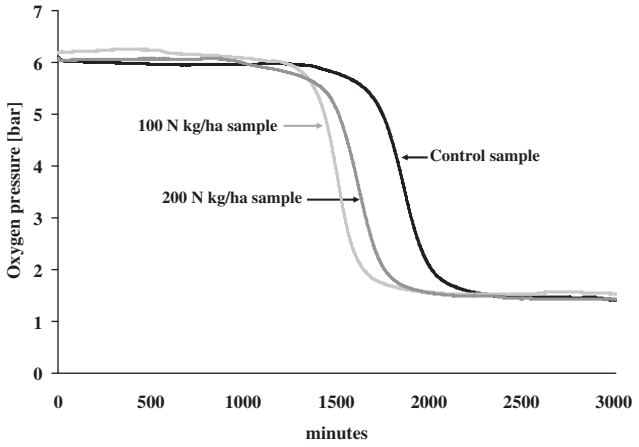

Fig. 2. Oxidation curves of walnut samples obtained using the OXITEST reactor.

degree compared to fertilised samples (more MUFA and lower PUFA); 200 N kg/ha samples showed lower amounts of linolenic acid and highest quantity of linoleic acid compared to 100 N kg/ha fertilised walnut, therefore 100 N kg/ha samples reported a higher unsaturation degree compared to 200 N kg/ha and this demonstrated its lower lipid stability.

No correlation was observed between phenolic content and oxidative stability. This result may be explained by the localisation of the phenolic fraction in the pellicle of the walnut, where it is not in contact with the oil present in the fruit.

4. Conclusions

This study confirmed that walnuts are a good source of n-3 and n-6 fatty acids, tocopherols, sterols and phenolic compounds. The lipid content, phenolic composition and oxidative stability of walnuts were studied in response to nitrogen fertilisation; moreover, a new technique to evaluate the oxidative stability in whole walnut was established.

Our results suggest that nitrogen levels significantly affect fatty acid content, phenolic composition and oxidative stability of walnut kernels. In particular, PUFA levels increased while MUFA levels decreased, and this behaviour suggests a contradictory effect on quality: decreased oil stability coupled with increased nutritional benefits. Higher linoleic acid n-6 content was observed when nutrient availability was limited at the highest nitrogen fertiliser level; on the other hand, the control and low nitrogen fertiliser treatments resulted in an increased content of linolenic acid n-3. The tocopherol analysis indicated no significant effect of N fertilisation, except on α-tocopherol, which was negatively influenced by the use of nitrogen fertiliser. Walnuts from the highest nitrogen treatment level exhibited significantly lower phenolic content than control samples and walnuts grown under low nitrogen fertiliser conditions.

To our knowledge, the present study is the first accelerated oxidative study on walnuts without any prior extraction of fats. The results showed that the oxidative stability of walnuts was strictly related to fatty acid composition and negatively influenced by nitrogen fertilisation.

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