PAPER

AN ANCIENT CROP REVISITED: CHEMICAL COMPOSITION OF MEDITERRANEAN PINE NUTS GROWN IN SIX COUNTRIES

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ABSTRACT

The aim of the study was to analyze the proximate composition of pine nuts harvested from 15 growing areas in Chile, Argentina, Italy, Spain, Turkey, and Israel. The main component was fat, followed by protein. Pine nuts from Chile and Argentina were similar, zones with the highest thermal oscillation and rainfall. Italian pine nuts had the highest fiber content, while Spanish nuts had the highest fat content. Israel presented the highest number of dry months, where pine nuts contained more protein and minerals, while nuts from Turkey showed an intermediate position. Minimum and maximum average temperatures, amount of dry months, and thermal oscillation affected the chemical composition.

Keywords: agro-climatic conditions, chemical composition, growing zone, pine nuts, *Pinus pinea*

1. INTRODUCTION

Pinus pinea L., also known as stone pine, is one of the nine most important tree nut species in the world. Pine nut is the most expensive nut worldwide, while stone pine is one of the oldest fruit trees, as demonstrated by archaeological remains that evidence its cultivation in the pre-Christian era (ROTTOLI and CASTIGLIONI, 2011). A risk for depletion and degradation of European stone pine forests has appeared, in relation to emerging threats such as climate change (MILANO *et al*., 2015) and demographic dynamics (UNITED NATIONS, 2015). Climate change has affected the production of pine nuts worldwide (MUTKE *et al*., 2005), and efforts are being made to improve the growth of valuable Mediterranean forests in non-traditional areas. The species is endemic to the Mediterranean basin, cultivated mainly in Spain, Portugal, Italy, Turkey, and Tunisia. Pine nuts are part of the traditional Mediterranean diet, which is well recognized for reducing cardiovascular risk factors (REES *et al*., 2014; ROS, 2015).

As part of the Mediterranean diet, pine nuts contribute to reducing risk factors of cardiovascular disease (CVD), type-2 diabetes, and some types of cancer (ALASALVAR and BOLLING, 2006; SABATÉ and ANG, 2009; BAO *et al*., 2013; ESTRUCH *et al*., 2013; SORLÍ *et al*., 2013). In general, nuts are energy-dense foods, since they contain 4.4 to 7.4 g kg^{-1} fat (ROS and MATAIX, 2006; RYAN *et al*., 2006; KORNSTEINER-KRENN *et al*., 2013; USDA, 2016). However, nut consumption improves blood lipid levels (SABATÉ *et al*., 2010) and reduces risk factors of CVD (KRIS-ETHERTON *et al*., 2008). The cardio-protective constituents of pine nut oil include unsaturated fatty acids, phytosterols, tocopherols, and squalene, among other bioactives (WOLFF and BAYARD, 1995; MAGUIRE *et al*., 2004; ALASALVAR and BOLLING, 2006; BOLLING *et al*., 2011). Moreover, although nuts are high fat and energy dense foods, their consumption has been associated with reduced body mass index (BMI) (BES-RASTROLLO *et al*., 2009; IBARROLA-JURADO *et al*., 2013; LUTZ and LUNA, 2016). Pine nuts are not only a good source of fat, but they also contain high levels of proteins, they supply various vitamins (E, B_6, niacin, folic acid), minerals (potassium, phosphorus, magnesium, zinc, iron, copper), and a variety of phytochemicals, including phenolic compounds (NERGIZ and DÖNMEZ, 2004; EVARISTO *et al*., 2010; BOLLING *et al*., 2011; LUTZ *et al*., 2016). EVARISTO *et al.* (2010) reported significant differences in the mineral profile and other chemical components of pine nuts grown in different regions, suggesting that environment and soil types have an important influence. VANHANEN and SAVAGE (2013) also reported differences in minerals probably due to soil conditions, climate and growing practices.

Pine nuts supply has been extremely affected by *Leptoglossus occidentalis* Heideman, an insect spread in all the major producing countries, which represents an elevated economic impact in global markets (BLOOMBERG BUSINESS, 2013). On the other hand, given the high nutritional and outstanding organoleptic quality of pine nuts, its demand is increasing worldwide, reaching high prices (INTERNATIONAL NUT AND DRIED FRUITS, 2016), which represents an opportunity to produce the seeds in non-conventional growing areas, including the southern hemisphere.

Models at different scales have been developed to establish relationships between stone pine productivity and several variables, including climatic ones (CALAMA *et al*., 2011), but no reference has been found on their impact on pine nut quality.

The chemical characterization of pine nuts grown in different areas is important due to health, commercial, and genetic concerns. Taking into account that most available composition data have been obtained from the main productive countries, the aim of the study was to assess the current pine nut proximate composition across six countries: three located in the traditional growing areas (Italy, Spain, and Turkey), and three in areas where there is no current commercialization of pine nuts (Israel, Argentina, and Chile), accessible to authors for seed collection. The study focuses on the chemical composition, and the impact of climate variables on the composition of pine nuts harvested in these countries.

2. MATERIALS AND METHODS

2.1. Study area

Fifteen areas were selected for collecting pine nuts in Italy, Turkey, Spain, Israel, Argentina, and Chile. Average climatic variables of locations from where pine nuts were grown are summarized in Table 1, and their distribution is shown in Fig. 1.

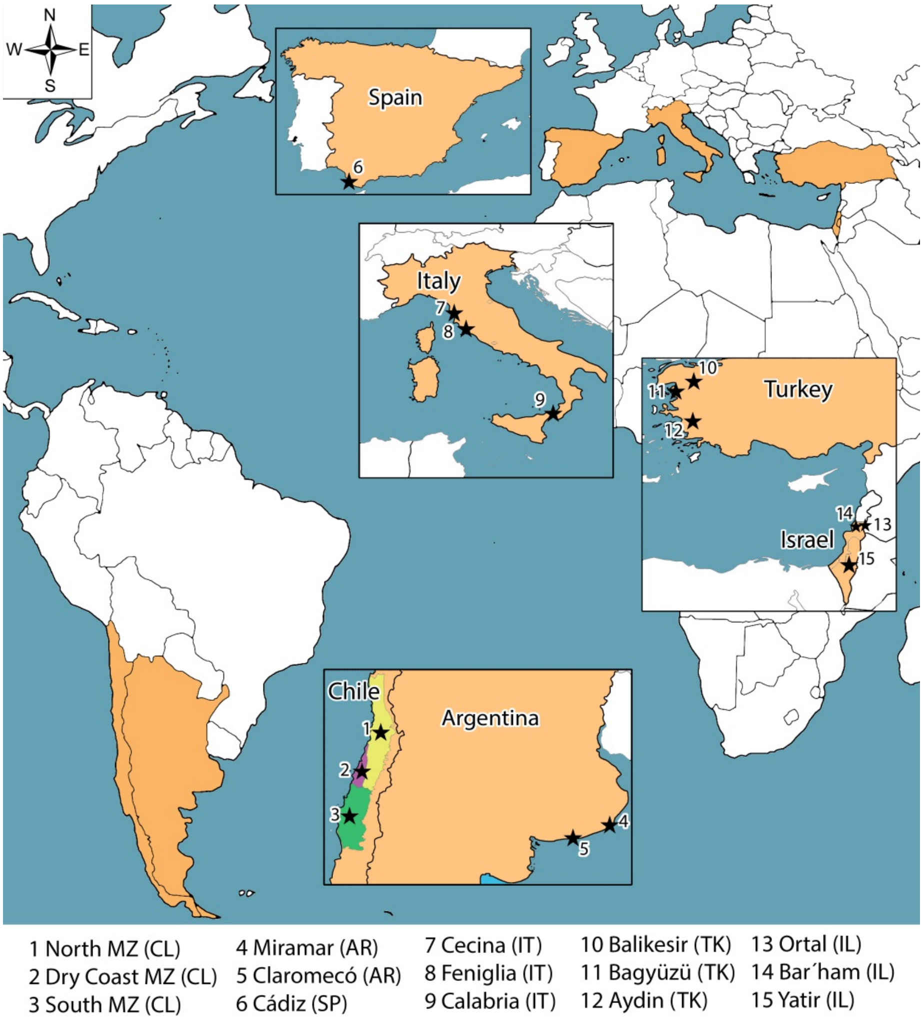


Figure 1. Distribution of locations in six countries where pine nuts were collected.

Table 1. Location and mean climatic variables of harvest sites of *Pinus pinea* L. nuts.

Location	Country	Annual rainfall (mm)	Annual average temperature (°C)	Dry months† (N°)	Maximum average temperature (°C)	Minimum average temperature (°C)	Thermal oscillation (°C)	Latitude	Longitude	Soil type
Miramar		660.7	15.1	5	22.1	8.0	14.1	38°16'S	57°50'W	Dunes afforested with conifers, poor in organic matter. Entisol (FREDES *et al*., 2009)
Claromecó		758.4	14.5	4	21.2	7.9	13.3	38°51'S	60°04'W	Clayey granular soils with 3-5% of organic matter, moderately acids. Molisols. Imperfect drainage (CARBONE and PICCOLO, 2002).
Mean	**Argentina**	**709.6**	**14.8**	**5**	**21.7**	**8.0**	**13.7**			
North MZ		383.7	14.1	9	21.9	7.5	14.3	33°26'S	71°04'W	Alluvial origin (alfisols, mollisol, entisols) (ALBERS, 2012).
Dry Coast MZ		648.3	13.6	8	21.0	7.0	14.0	35°14'S	72°12'W	Granitic origin (alfisols, inceptisols (ALBERS, 2012).
South MZ		1,047.0	13.2	7	19.8	7.5	12.3	37°54'S	72°40'W	Volcanic origin (altisols, red clay) (ALBERS, 2012).
Mean	**Chile**	**693.0**	**13.6**	**8**	**20.9**	**7.3**	**13.5**			
Ortal		830.0	14.7	6	20.6	8.9	11.7	33°05'N	35°50'E	Volcanic origin (ORENSTEIN *et al*., 2001). Basaltic brown Mediterranean soils and basaltic lithosols (DAN *et al*., 1975).
Bar'ham		682.1	16.2	7	20.4	12.0	8.4	33°04'N	35°26'E	Volcanic origin (ORENSTEIN et al., 2001). Pale rendzinas (DAN *et al*., 1975),
Yatir		275.0	17.6	8	22.4	12.8	9.6	31°18'N	35°01'E	Aeolian origin loess with a clay-loam texture, overlying chalk and limestone bedrock (NAAMA *et al*., 2012). Brown lithosols and loessial arid brown soil (DAN *et al*., 1975)
Mean	**Israel**	**595.7**	**16.2**	**7**	**21.1**	**11.2**	**9.9**			

Table 1. Continues.

Cecina (LI)		833.6	14.5	4	19.8	9.1	10.7	43°19'N	10°30'E	Alluvial soils, sometimes with shallow water table (calcaric cambisols, fluvisols and gleysols), clay accumulation along the profile (COSTANTINI *et al.*, 2004).
Feniglia (GR)		455.1	13.2	4	16.0	10.3	5.7	42°25'N	11°12'E	Sandy soils (PIRAINO *et al.*, 2012).
Calabria (RC)		546.8	18.3	6	22.5	14.1	8.3	39°19'N	16°21'E	Eroded soils (eutric and calcaric regosols) with accumulation of carbonates and soluble salts, rich in iron oxides and clay. Volcanic soils (umbric andosols) (COSTANTINI *et al.*, 2004).
Mean	**Italy**	**611.8**	**15.3**	**5**	**19.4**	**11.2**	**8.2**			
Cádiz	**Spain**	**524.0**	**18.7**	**5**	**21.7**	**15.6**	**6.1**	36°32'N	06°17'W	Litoral dunes (entisols). Sandy, poor soils (MUÑOZ and GRACÍA, 2009).
Balikesir		576.8	14.6	6	20.5	8.9	11.5	39°39'N	27°53'E	Sandy-loamy soil texture, neutral pH, non-calcareous or slightly calcareous, salt-free and organic matter weak (YILMAZ and SATIL, 2017).
Bagyuzu		743.2	13.1	5	18.9	8.8	10.1	39°18'N	26°58'E	Rough broken land (brown forest soil material) (OAEKS and ARIKOK, 1954).
Aydin		651.7	17.5	7	24.5	11.9	12.6	37°50'N	27°51'E	Alluvial and youthful soils (OAEKS and ARIKOK, 1954).
Mean	**Turkey**	**657.2**	**15.1**	**6**	**21.3**	**9.9**	**11.4**			

Climatic data sources: Argentine Meteorological Service (www.smn.gov.ar); Chilean Environmental Information System (www.inia.cl); The Israel Meteorological Service (www.ims.gov.il); Italian Army Aeronautic Meteorological Service (www.meteoam.it); State Meteorological Agency (www.aemet.es); Turkish Meteorological Service (www.mgm.gov.tr).

†Dry months were calculated as those with monthly rainfall/monthly potential evapotranspiration <0.5.

2.2. Materials

Stone pine seeds were collected from planted trees (none corresponds to cultivars) in 2013/14 in Turkey (n=3 zones), Israel (n=3 zones), Spain (n=1 zone), Italy (n=3 zones), Argentina (n=2 zones) and Chile (n=3 zones) in 2013; a minimum of 500 pine nuts from 10 trees were harvested in each collection area. Chilean stone pine seeds were collected from *Pinus pinea* L. planted trees distributed in three macrozones located between 30.82°N and 38.99°S (LOEWE *et al*., 2015). 167 trees were sampled taking into account the macrozone size and variability of the most variable chemical contents. From each tree, 500 pine nuts were harvested.

Samples were harvested during winter since it corresponds to the maturation season according to ABELLANAS and PARDOS (1989). Once obtained, in-shell pine nuts were kept in plastic nets individually tagged at room temperature until manually shelled. For sample preparation, shelled nuts were dried at 40°C until moisture reached 4 g kg^{-1}. All the seeds were ground with a kitchen processor (Moulinex®) and sieved to 0.5 mm, and then frozen in sealed plastic bags at -20°C until analyses.

2.3. Methods

All reagents and solvents were analytical grade chemicals from Merck (Darmstadt, Germany). Proximate analyses were performed using AOAC methodologies (AOAC, 2012). Protein content was determined by Kjeldahl assay (AOAC 920.54) using a nitrogen digestor DK6 (VELP®) and a nitrogen distiller UDK 129 (VELP®), applying factor of 5.3 to convert nitrogen to proteins (GREENFIELD and SOUTHGATE, 1972). Crude fat was assessed using the AOAC method 920.39, moisture was determined using the AOAC method 945.15, and ash was determined using the AOAC method 942.05. Total dietary fiber (TDF) was measured using the AOAC enzymatic-gravimetric method 991.43 using the MEGAZYME K-TDRF 05/12 kit supplied by Megazyme®.

2.4. Statistical analysis

Chemical analyses were done in triplicate; each replicate was quantified in duplicate, unless stated otherwise. All data given represent mean values ± standard error (SE). Data were compared using heteroscedastic ANOVA, and statistical significance was determined with an LSD test ($P<0.05$). The relative contribution of climatic variables to chemical components was estimated using CART (Classification and Regression Trees) algorithms (BREIMAN, 1999). As confirmatory analysis, the groups suggested by the identified climatic variables thresholds were also compared by ANOVA. Finally, a principal component analysis (PCA) was applied, generating a biplot for chemical composition of pine nuts and climate variables of different countries. Analyses were performed using the software Infostat® and its interface with the software R® (DI RIENZO *et al*., 2014).

3. RESULTS AND DISCUSSIONS

In this comparative study, we took into consideration geographic zones and agro-climatic conditions that may affect the chemical composition of pine nut seeds, considering that LOEWE *et al.* (2016a) reported marked differences on stone pine cone productivity along the climatic gradient in Chile, which could also be translated to chemical composition, as in fact has been determined by using a discriminant analysis by Near Infrared

Spectroscopy (NIRS) in stone pine nuts collected in different macrozones of Chile (LOEWE *et al.*, 2016b).
The chemical composition of pine nuts collected in six countries is shown in Table 2.

Table 2. Chemical composition of pine nuts by location and country (g/100 g)[1].

Location/Country	Moisture	Protein	Lipids	Ashes	Total Dietary Fiber
Miramar	4.6	33.1	42.0	4.2	10.5
Claromecó	3.0	31.2	41.6	4.6	9.1
Argentina	3.8±0.8ab	32.1±0.9b	41.8±0.2a	4.4±0.2ab	9.8±0.7c
North MZ	4.1	34.9	42.3	4.7	11.6
Dry Coast MZ	4.5	35.3	46.9	4.7	11.6
South MZ	4.3	32.1	43.6	4.6	11.8
Chile*	4.3±0.07a	34.1±0.5b	44.3±0.7a	4.7±0.03a	11.7±0.1b
Ortal	3.5	35.3	31.0	4.8	13.0
Bar'ham	3.7	37.2	30.1	4.7	12.3
Yatir	4.0	37.0	42.9	4.6	11.9
Israel	3.7±0.12b	36.6±0.5a	34.7±4.1a	4.7±0.06a	12.4±0.3b
Cecina	5.2	33.2	37.0	4.2	14.7
Feniglia	4.9	32.8	37.9	3.9	13.9
Calabria	5.2	30.3	36.4	4.7	15.2
Italy	5.1±1.0a	32.1±0.9b	37.1±0.4a	4.3±0.2ab	14.6±0.4a
Spain (Cádiz)	4.8	33.8	45.3	4.1	12.4
Balikesir	3.7	33.3	45.0	4.3	12.2
Bgyuzu	4.0	34.0	43.5	4.0	12.6
Aydin	4.0	37.0	40.1	4.1	14.4
Turkey	3.9±0.1ab	34.8±1.1ab	42.9±1.4a	4.1±0.1b	13.1±0.7ab

[1]Data are expressed as means±SE (n=3). Different letters in a column indicate statistically significant differences (P<0.05).
*LUTZ *et al.* (2016).

Significant differences were found among countries for protein (p=0.0053), TDF (p=0.0003), ash (p=0.0008), and moisture (p=0.0001). The main chemical component in pine nut seeds is lipids. In the analyzed seeds, fats ranged from 34.7% (Israel) to 45.3% (Spain) (p<0.05). These values are in agreement with NERGIZ and DÖNMEZ (2004) and RYAN *et al*. (2006), but lower than the fat content reported for this nut by KORNSTEINER-KRENN *et al*. (2013) and ESCHE *et al*. (2013), including the USDA Database (USDA, 2016), which reports a mean value of 68.4%. According to the principal components analysis, the lipid content of the pine nuts was enhanced in Chile by low minimum temperature, and across countries by high maximum temperatures. The lipid quality is relevant to the energetic and nutritive values of pine nuts, while the fatty acid profile, as well as phytosterols, phytostanols, tocopherols and other lipid bioactives contents play major roles in their healthy properties (KORNSTEINER *et al*., 2006; BOLLING *et al*., 2010; ESCHE *et al*., 2013).

Pine nuts are recognized as a good dietary source of proteins, and the average protein content ranges from 13% to 30% dry matter, depending on the *Pinus* species (EVARISTO *et al*., 2010; USDA, 2016). The protein content observed in the seeds collected from six countries ranged from 32.1% (Italy and Argentina) to 36.6% (Israel), which are above the reported averages. In three Chilean macrozones across 1,300 km with varying climatic conditions, proteins ranged from 32.1% to 35.3% (LUTZ *et al*., 2016). These results demonstrate that protein content can be significantly affected by the agro-climatic conditions in which the species grow. Moisture was highest in Italian pine nuts (5.1%), while it was lowest in the Israeli samples (3.7%); ashes varied from 4.1% (Spain and Turkey) to 4.7% (Israel and Chile), and TDF varied between 9.8% (Argentina) and 14.6% (Italy).

Fig. 2 represents the biplot of the two principal components by country, which explained 66% of the variability. Differences were observed between pine nuts grown in Chile and Argentina, and between Turkey and Israel, presenting a chemical composition that differs from Italy and from Spain. South American pine nuts, which grew in areas with the highest thermal oscillation and rainfall, showed similar chemical composition. Italian pine nuts exhibited the highest TDF and moisture contents. Spanish pine nuts showed a different composition, which would be related to the minimum average temperature and average temperature. Israeli and Turkish pine nuts showed a similar composition, exhibiting high protein content. Mineral content related to the number of dry months, and lipids to the maximum average temperature. Lipids content was superior when maximum average temperature was high.

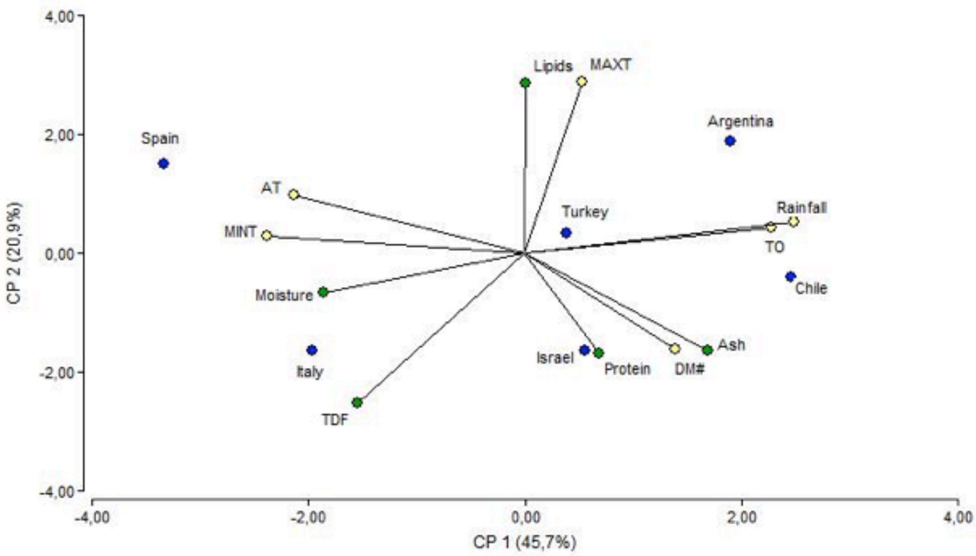


Figure 2. Biplot for chemical composition of pine nuts and climate variables according to country.
MAXT: maximum average temperature, AT: average temperature, MINT: minimum average temperature, DM#: dry month number, TO: thermal oscillation, TDF: total dietary fiber.

Fig. 3 shows the biplot of the two principal components, explaining 55.6% of the variability. It also shows that the Italian samples hold a high content of TDF and moisture, with Calabria (IT) and Cádiz (SP) being characterized by a high minimum average temperature. South American pine nut samples grown in areas with high thermal oscillation –especially those from northern and central zones in Chile – and rainfall –

especially those from the southern zone of Chile - showed a similar composition. Turkish and Israeli pine nuts showed some differences in the second component, being Yatir (IL) and Aydin (TK) characterized by a high maximum average temperature and dry month number, and high protein content. Bagyuzu (TK), an area with a high rainfall, holds a similar lipid content to the ones from southern Chile.

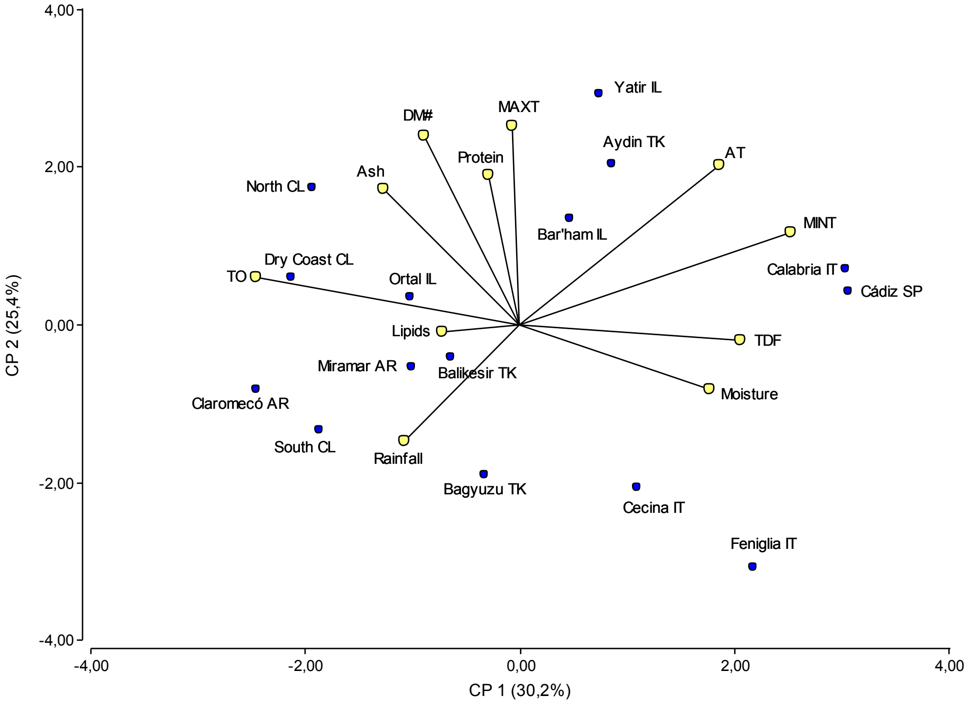


Figure 3. Biplot for chemical composition of pine nuts and climate variables by location.
MAXT: maximum average temperature, AT: average temperature, MINT: minimum average temperature, DM#: dry month number, TO: thermal oscillation, TDF: total dietary fiber.

In the CART analyses for each component (Table 3), data were first split into two subsets based on the predictor variable (MINT for TDF; TO for Moisture and DM# for Ash) and its thresholds (8.4°C, 8.4°C, and 5.5 months, respectively). Each subset, or node, for TDF was then analyzed independently using the same procedure (MAXT, 22.4°C). Top nodes are the most important to explain the chemical composition.

Interestingly, the influence of climate on some chemical components was observed. A significant negative influence of thermal oscillation on moisture was detected, as well as significant positive effects of dry months on minerals, and of minimum and maximum temperatures on TDF. In particular, the TDF content in the seeds of the six countries varied from 9.8% (Argentina) to 14.6% (Italy) ($p<0.05$). It was affected by climatic variables such as the minimum average temperature and maximum average temperature ($p<0.05$), increasing with minimal temperatures above 8.4°C and maximum temperatures above 22.4°C. A different situation was observed in the ash content, which increased in presence of longer dry periods (over 5.5 months) by 9.5%.

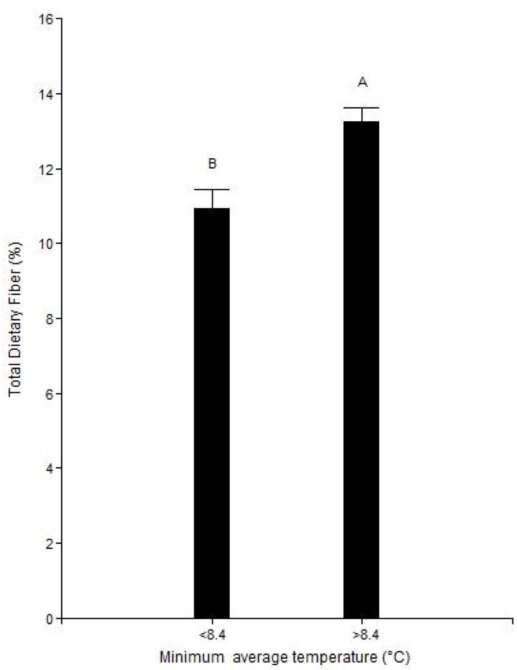
Fig. 4 depicts the effect of climatic variables on pine nut composition. Across locations, TDF was significantly influenced by climatic variables, with an increase of 21.1% at a minimum average temperature above 8.4°C (13.2% vs 10.9%, $p=0.0032$), and an increase of 22.3% at a maximum average temperature above 22.4°C (14.8% vs 12.1%, $p=0.0203$). Minerals were influenced by the number of dry months ($p=0.0063$), with an increase of

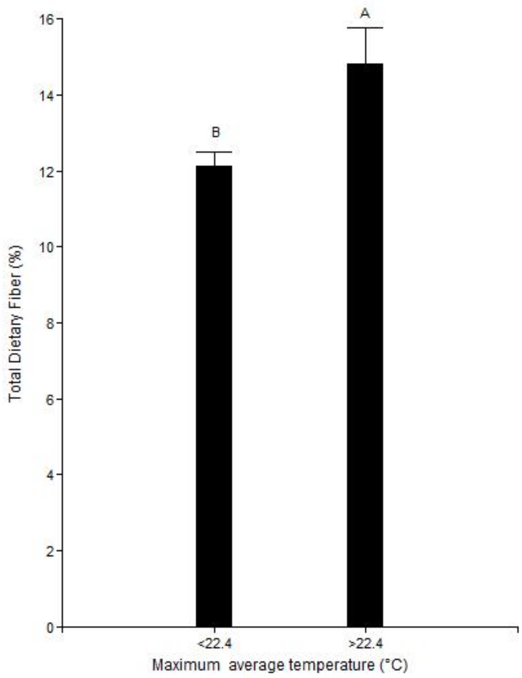
9.5% when dry months exceeded 5.5 (4.6% vs 4.2%). Moisture was influenced by thermal oscillation, being 22.5% higher when thermal oscillation was below 8.4°C (4.9% vs 4.0%, p=0.0101).

Table 3. Climatic variables that best explain Total Dietary Fiber (TDF), Moisture and Ash determined by CART analyses.

Node	Predictor variable	Average content (g/100 g)	n	Standard Error
		Total Dietary Fiber		
1	MINT≤8.4°C	10.9	5	1.33
2	MINT>8.4°C	13.2	10	1.42
2.1	MAXT≤22.4°C	12.1	8	0.90
2.2	MAXT>22.4°C	14.8	2	0.33
		Moisture		
1	TO≤8.4°C	4.9	3	0.05
2	TO>8.4°C	4.0	12	0.31
		Ash		
1	DM#≤5.5	4.2	6	0.05
2	DM#>5.5	4.6	9	0.05

MINT: annual average minimum temperature; MAXT: annual average maximum temperature; TO: thermal oscillation (annual average maximum absolute temperature minus annual average minimum absolute temperature); DM#: dry month number.





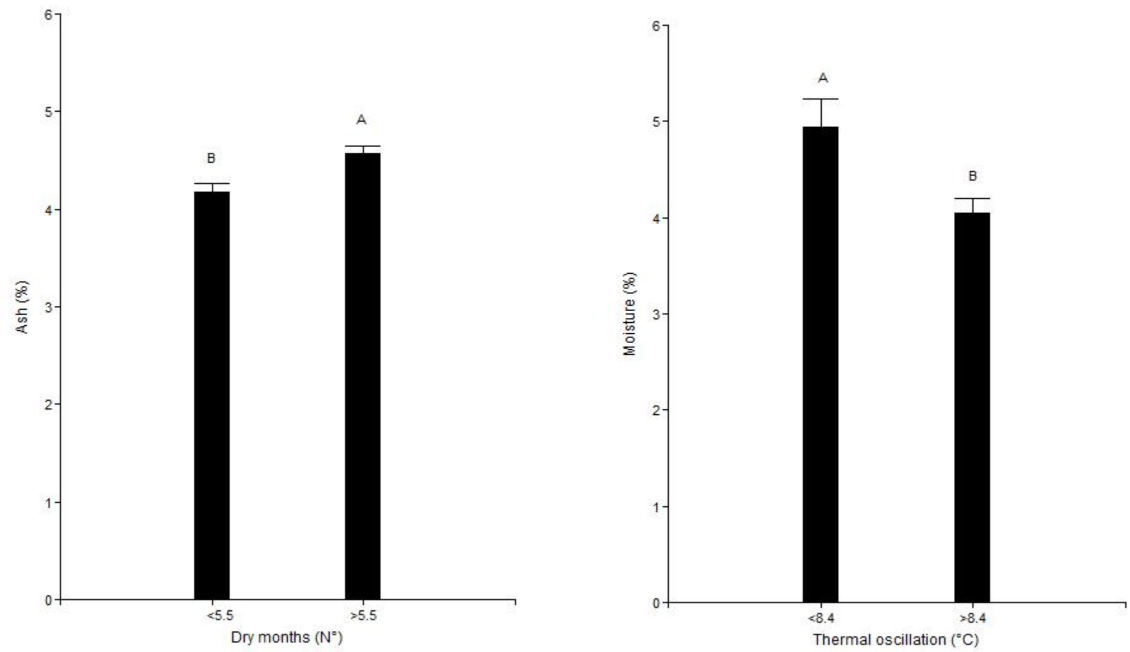


Figure 4. Climatic variables that influence chemical composition across fifteen localities distributed in six countries.
Each threshold was detected by CART analysis. Different letters indicate statistically significant differences ($p<0.05$).
Thermal oscillation: annual average maximum absolute temperature minus annual average minimum absolute temperature.

The soil nutrient content effect has been limitedly studied. In fact, in Turkey, a positive correlation between nitrogen, phosphorus, calcium and manganese depletion was detected in needles and cone loss (KILCI, 2013). In Israel, MALCHI and SHENKER (2011) found that iron deficiency decreased root growth and induced a reduction in chlorophyll concentration on needles in soils with high concentration of calcium carbonate, being a high soil pH the cause for reduced iron absorption. However, no studies have been performed on the relationship among pine nut composition and soil contents. In our study, we observed a similar pine nut protein content when grown in sandy soils, around 33%. Thus, the chemical composition of pine nuts grown in different regions could be explained, at least in part, by the environment and soil type variability between regions, which is in agreement with several authors (GÓMEZ-ARIZA *et al*., 2006; EVARISTO *et al*., 2013; LUTZ *et al*., 2016).
Future studies should also address the use of cultural practices such as fertilization on pine nut quality, considering that BORRERO (2004) reported an increase in pine nuts concentrations of fat, copper, magnesium and sodium in fertilized plots.

4. CONCLUSIONS

The study describes the proximate chemical composition of pine nuts harvested in six countries. The results obtained indicate that from a nutritional quality standpoint, all the analyzed seeds exhibited good nutritional properties, independently of the geographic zone where they were grown, justifying their inclusion in a healthy diet. The major components of pine nuts are lipids, protein and dietary fiber, while their carbohydrates

content is low, which make them a good choice in the prevention of diabetes, metabolic syndrome and other common non-transmissible diseases.
The effect of the climatic conditions, soil quality and other environmental variables are usually not taken into consideration when average values are used in food composition databases.
The results obtained in this study indicate significant differences among countries for protein, TDF, ash and moisture, variability probably related to climate and environmental conditions of the growing areas. Relevant climatic variables were thermal oscillation for moisture, dry months for minerals, and minimum and maximum average temperature for TDF.
The study reveals that *Pinus pinea* L., a traditional ancient tree grown in the Mediterranean basin, may also be successfully grown in South America, contributing to diversify agriculture, as pine nuts represent an opportunity for the global food industry as well. Finally, the composition of the seeds collected from different countries, in various climatic conditions, constitutes relevant information that should be considered when food composition data are included in tables and reference data, shown as mean values.

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