Z Lebensm Unters Forsch (1996) 203: 374–378

ORIGINAL PAPER

C. Ben Nasr · N. Ayed · M. Metche

Quantitative determination of the polyphenolic content of pomegranate peel

Received: 22 September 1995/Revised version: 28 December 1995

Abstract The quantitative determination of total phenols, ellagic tannins and gallic and ellagic acids in the peel of the Tunisian pomegranate variety Chelfi, has been carried out. The ellagic tannin content is prominently less than the amount of total phenols, which led us to look for the presence of the condensed tannins. The determination of the content of catechic tannins in eight Tunisian varieties of the pomegranate was carried out using weekly samples over a period of 2 months.

Key words Pomegranate (*Punica granatum*) · Proanthocyanidins · Gallic acid · Ellagic acid · HPLC · Total phenols · Ellagic tannins

Introduction

The Tunisian patrimony contains a great number of clues indicating the frequent use of the pomegranate (and particularly its peel) in tinctures [1], cosmetic and therapeutic formulae [2] and food recipes.

In order to valorise the peel of the pomegranate, we projected to carry out an analytical study of its main components in relation to the tinctorial and tanning effects opposite proteinaceous supports.

The natural tannins can be used as markers which provide the best information about the polyphenolic plant substances of the pomegranate. This is only possible because of the huge number of metabolites (gallic acid, ellagic acid, proanthocyanidins, etc.).

However, we have not taken a census of the great number of varieties of the pomegranate in Tunisia. We have, however, collected pomegranate samples, at different stages of maturation, and from different regions of Tunisia, and analysed the peel for its proanthocyanidin content, which is well known for its natural capture of free radicals [3].

Materials and methods

Analysed plant material

The pomegranate peel studied was taken from eight varieties of pomegranate cultivated in Tunisia, which were collected at different stages of maturation and from several areas. The separation of the peel from the fruit was done manually. The peel was dried in the open air and in the shade for 9 weeks. Then, the peel samples were placed in a stove at 40°C, for 48 h, to submit them to an identical drying process without affecting their components. The peel samples were then pulverized and analysed so that the evolution of their condensed tannins, i.e. from the beginning of fruit formation to its maturation, could be followed. For the other quantitative analyses i.e. of total phenols, ellagic tannins, gallic and ellagic acid as well as metals (iron, magnesium and copper), only the peel of the Chelfi variety was studied.

Extraction methods

The sample (250 mg of pulverized pomegranate peel) was extracted with 10 ml MeOH/H_2O (4:1 v/v) for 2 h (×3) [4, 5]. The mixture was filtered through Buchner funnel and the MeOH was evaporated in a rotary evaporator. The residual aqueous phase was acidified to pH 2 by addition of some drops of HCl (3 N), and the volume was adjusted to 10 ml with distilled water. In order to isolate the gallic acid and ellagic acid, another extraction by Et_2O (3 × 10 ml) using a decanting bulb was carried out. The Et_2O phase was dried using anhydrous sodium sulphate overnight then evaporated, and the residual product was dissolved in 5 ml MeOH.

C. Ben Nasr (✉)
Faculté des sciences de Bizerte, Laboratoire des tanins, Département de chimie, 7021 Zarzouna, Tunisia

N. Ayed
Institut supérieur de l'éducation et de la formation continue, Laboratoire des colorants, 43, rue de la liberté, 2019 le Bardo, Tunisia

M. Metche
Laboratoire de biochimie appliquée, E.N.S.A.I.A. 2, Avenue de la forêt de Haye, BP 172, Vandoeuvre-Lès Nancy Cédex, France

In order to solubilize the maximum amount of proanthocyanidins, we used a mixture of acetone and water (9:1 v/v) which works better for the extraction of oligomeric proanthocyanidins [6] than techniques using methanol [7] and hot water [8, 9].

Analytical methods

Analysis of total phenols. Total phenols were estimated by the Folin-Ciocalteu method. The aqueous solution was diluted to decrease the absorbance at 760 nm to 0.5 or less. Thus 0.5 ml of the aqueous solution of pomegranate peel extract was added to 2.5 ml of Folin-Ciocalteu (Prolabo) reagent according to the conditions defined previously [10]. Just after a time varying between 30 s and 8 min, we add 2 ml of a solution of sodium carbonate (0.7 mol/l). The tubes were laid for 5 min in a water bath at 50 ± 0.5°C and then put in a cold water bath. The reading of the absorbance was made at 760 nm. The amounts are expressed in milligrams of gallic acid equivalents (GAE) per gram of dry matter. The standard curve was prepared with aqueous solutions made using pure gallic acid.

Analysis of ellagic tannins. Within a screw-capped tube, we mixed 1 ml of aqueous extract, 1 ml of the mixture MeOH in H_2O (9:1 v/v) and 0.16 ml of acetic acid/H_2O (6% by volume). A stream of nitrogen was bubbled through the mixture for 5 min before the addition of 0.16 ml of an aqueous solution of sodium nitrate (6%). Nitrogen was bubbled through again for a few seconds, then the tube was closed and placed for 10 min in a water bath at 25 ± 0.5°C. The absorbance was measured to its maximum at 590 nm by means of a spectrometer UV-Visible Shimadzu (UV. 260). The results are expressed in milligrams of 4,6-hexahydroxydiphenoyl-glucose equivalents (EHHDP-G) per gram of dry matter according to the following formula:

$$\mathrm{Te} = \frac{A \cdot V \cdot M \cdot 10^3 \cdot V'}{m \cdot v \cdot \varepsilon_{\mathrm{mol}}} \quad (1)$$

where, Te = mg EHHDP-G/g of dry peel, A = absorbance at 590 nm, V = volume of the extract, M = molecular weight of 4,6-HHDP-G (482 g/mol), V' = volume of the reactional medium, m = weight of the peel (g), v = volume of the sample, $\varepsilon_{\mathrm{mol}} = 2196$ molecular absorbance of 4,6-HHDP-G [11].

HPLC analysis. HPLC analysis of gallic and ellagic acid in Et_2O extracts were run on a Lichrospher RP 18 E 5 µm. 10 cm Lichrocart (Merck) with the following elution conditions: isocratic system for solvent H_2O–CH_3OH–H_3PO_4 in different proportions for gallic acid (Sigma) (49.5/949.5/1 v/v/v) and ellagic acid (Sigma) (449.5/449.5/1 v/v/v); flow rate 1 ml/min; UV detection 280 nm: integration on a chromatopac C-RIB Shimadzu.

Proanthocyanidin analysis. The method is based on the condensation of the vanillin onto the phloroglucinol nucleus catalysed by H_2SO_4 [12]. Of the extract of the pomegranate peel, 1 ml was added to 2 ml of a freshly prepared solution of vanillin (0.01 g/l) in H_2SO_4/H_2O (70% by volume). After exactly 15 min of reaction in a water bath at 35°C, the maximum absorbance was read (towards 505 nm in our case).

Mineral analysis by atomic absorption spectroscopy. To a furnace flask were added: 250 mg of pulverized pomegranate peel and 5 ml of perhydrol solution at 30% (v/v). They were left overnight at ambient temperature, then 2.5 ml of pure $HClO_4$ was added. Then the flask was heated on a plate, then 2 or 3 h later, the plant material was fully solubilized. The measurement of the mineral content (after cooling) was carried out using an atomic absorption spectrometer Perkin Elmer type HGA 700.

Results and discussion

Among the polyphenols we distinguished hydrolysable tannins and condensed tannins. Through hydrolysis, the former liberate gallic acid, ellagic acid, chebulinic acid and spaltic acid, and, at the same time, glucose and quinic acid. The condensed tannins are degraded in acidic media with the formation of anthocyanins, catechins and phlobaphens.

The analysis of polyphenolic derivatives indicates the presence of total phenols, ellagic tannins, gallic and ellagic acids as well as certain metals (iron, copper and magnesium) in the peel of the Chelfi variety of the pomegranate (Tables 1 and 2).

Figures 1–3 group the results showing the evolution of the content of condensed tannins in the peel of eight

Table 1 Analysis of polyphenols of pomegranate peel of the Tunisian Chelfi variety. (*GAE* Gallic acid equivalent, *EHHDP-G* hexahydroxydiphenoyl glucose)

Polyphenol			
Total phenols (mg GAE/g)	Ellagic tannins (mg EHHDP -G/g)	Ellagic acid (mg/g)	Gallic acid (mg/g)
216.9 ± 7.3	0.310 ± 0.045	0.117 ± 0.001	0.030 ± 0.001

Table 2 Analysis of metals (Fe, Cu and Mg) in pomegranate peel of the Tunisian Chelfi variety

Element		
Fe (mg/g)	Cu (mg/g)	Mg (mg/g)
47.46 ± 4.11	4.69 ± 0.27	32.33 ± 2.05

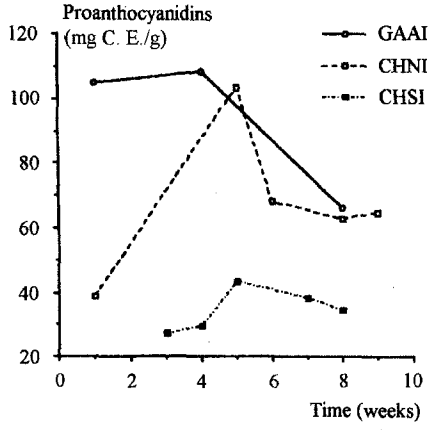

Fig. 1 Evolution of proanthocyanidin content as a function of time in different varieties of Tunisian pomegranate (*GAAI* Gabsi, *CHNI* Chelfi cultivated on irrigated land, *CHSI* Chelfi cultivated on non-irrigated land)

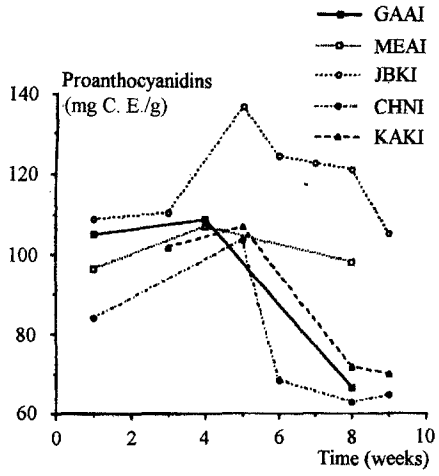

Fig. 2 Evolution of proanthocyanidin content as a function of time in different varieties of Tunisian pomegranate cultivated on irrigated land. (*GAAI* Gabsi, *MEAI* Mekki, *JBKI* Jebali, *CHNI* Chelfi, *KAKI* Khalladi)

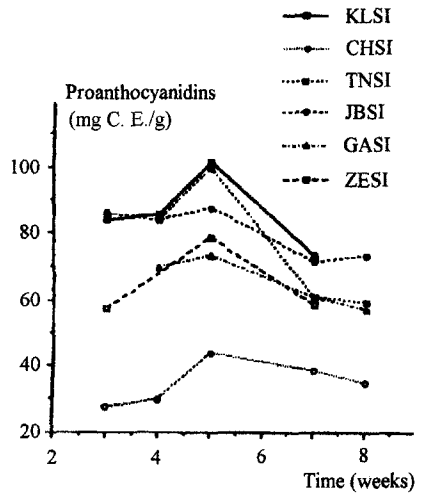

Fig. 3 Evolution of proanthocyanidin content as a function of time in different varieties of Tunisian pomegranate cultivated on non-irrigated land. (*KLSI* Kalai, *CHSI* Chelfi, *TNSI* Tounsi, *JBSI* Jebali, *GASI* Gabsi, *ZESI* Zehri)

Tunisian pomegranate varieties, which were collected between the 27 August and 27 October 1991.

The average content of total phenols over five trials was 0.217 g GAE/g dry peel and we noted that this concentration is almost twice as much as that obtained from the wood oak (*Quercus robur*) [13].

The standard curve of the total phenol determination using a standard solution of pure gallic acid follows the equation:

$$y = 1.1032\,10^{-2}x + 4.628\,10^{-3} \quad (2)$$

for a gallic acid concentration x varying between 0.010 and 0.080 g/l; y is the absorbance measured at 760 nm. The correlation coefficient of this equation, determined by the software Cricket Graph version 1.3.2 adapted to

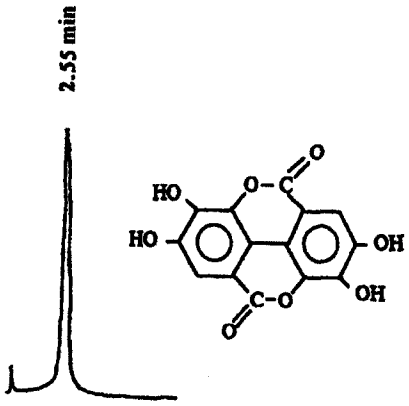

Fig. 4 HPLC chromatogram of ellagic acid of pomegranate peel

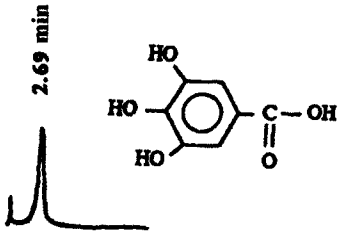

Fig. 5 HPLC chromatogram of gallic acid of pomegranate peel

Macintosh SE, is equal to 0.998. The ellagic tannins were analysed quantitatively using 4,6-EHHDP-G as a standard. The obtained result of 0.31 mg 4,6-EHHDP-G/g dry peel is quite surprising when compared to the significant quantity of total phenols. For this reason, we analysed for the content of free ellagic and gallic acids so that we could determine the polyphenolic substances responsible for the tanning power of the pomegranate peel. As for ellagic acid, analysed by HPLC (Fig. 4), its content is only 0.115 mg/g of dry matter. Gallic acid is present only in small quantities equal to 0.030 mg/g of dry peel according to the HPLC chromatograms (Fig. 5). These concentrations are equivalent to 15% and 10% respectively of the concentrations of ellagic acid and gallic acid measured in wood oak (*Q. robur*) (i.e. ellagic acid 0.75 mg/g and gallic acid 0.28 mg/g) [13]. The standard curves follow an $y = ax + b$ equation.

For gallic acid,

$$y = 1.1032\,10^{-2}x + 4.6281\,10^{-3} \quad (3)$$

for a concentration of reference compound x varying between 2 and 10 mg/l. The correlation coefficient of this equation, calculated by the above-mentioned software, is equal to 0.998.

For ellagic acid,

$$y = 3462.3x - 1105.4 \quad (4)$$

for a concentration of reference compound x varying from 10 to 60 mg/l. The correlation coefficient of this

equation, calculated by the previously used software, is equal to 0.997.

In the previous equations, y designates the area of the peak expressed in the arbitrary unit of Shumadzu integrator.

The low content of hydrolysable tannins, i.e. gallic [14] and ellagic [15–17] acids, as compared to the high concentration of polyphenolic components may be explained by the presence of condensed tannins. This reflection led us to try to prove the existence of this kind of tannin in the pomegranate peel, as traditionally used for tanning and tincture. In addition, the condensed tannins are responsible for the astringent properties found in tea, wine, cider, etc. [18].

For proanthocyanidin analysis, the standard curve was calculated using an aqueous solution of (+) catechin as reference. It follows the equation,

$$y = 0.5700x + 0.2715 \quad (5)$$

for a catechin concentration x varying between 0.25 and 1.25 g/l; y is the absorbance measured at 505 nm. The correlation coefficient of this equation is equal to 0.995.

An optimization of the operative conditions allowed us to avoid the problems caused by the use of a relatively high concentration of the extract of pomegranate peel.

The choice of the time reactional is essential concerning the dosage of proanthocyanidins. A kinetic study of the condensation reaction of proanthocyanidins with vanillin shows that the initial rate of reaction is fast, but that it decreases with time.

Temperature is also an important factor for this kind of reaction. Indeed, the results obtained indicate that the efficiency of this reaction increases with temperature for the time interval chosen for study.

The results obtained, i.e. 64.6 mg of catechin equivalent (CE) per gram of peel show that the amount of proanthocyanidins represents about 30% of the quantity of total phenols, and that, therefore, the pomegranate peel is quantitatively very rich in this type of tannin.

To estimate the complexation reaction of pomegranate peel tannins with iron, magnesium and copper, we proceeded to determinate the quantity of these elements.

The pomegranate contains 0.4 mg iron and 3 mg magnesium per 100 g [4]. As far as we know, no study has measured the metal content of pomegranate peel. Therefore, we analysed peel of the pomegranate variety Chelfi (Table 2). Iron and magnesium are present in large quantities in the peel as compared to the comestible part; as for iron, the average quantity of five trials was 47.5 mg/g of peel and for magnesium, the average was 32.1 mg/g of peel. Copper, the presence of which in the comestible part of the pomegranate has not been demonstrated, was measured at 4.7 mg/g within the pomegranate, the standard curves follow the $y = ax + b$ equation.

$$\text{Iron:} \quad y = 1.4657\,10^{-2}x - 4.7619.10^{-5} \quad (6)$$

$$\text{Magnesium:} \quad y = 0.2976x + 4.0400\,10^{-2} \quad (7)$$

$$\text{Copper:} \quad y = 2.6229\,10^{-2}x - 5.1275\,10^{-3} \quad (8)$$

The application interval and the correlation coefficient of these equations are grouped in Table 3.

The evolution of the proanthocyanidin content of pomegranate peel from the fruit formation to its maturation was followed for most of the varieties previously mentioned. At the start of the study (27 August), the tenor condensed tannin content of the pomegranate peel was significantly large corresponding to a value between 30 and 110 mg CE/g. It then increased progressively, reaching a maximum in the 5th week (24 of September 1994), then decreasing and stabilizing, for certain types, from the 7th week (7 October 1991). This fall in the proanthocyanidin content, noticed during the 5th week, is related to the increase of the condensed tannins polycondensation during the period of fruit maturation that lead an increase of insolubility [19].

The comparison of proanthocyanidin contents of the studied varieties led to the classification indicated in Table 4.

In this way, it should be possible to identify an unknown variety by comparing its proanthocyanidin profile with the one of the groups whose tenor is most similar.

The effect of irrigation of the land in which the pomegranate is cultivated is to favour an increase of the proanthocyanidin content of the fruit peel (Fig. 1).

Table 3 Application interval and correlation coefficient of standard curves of Fe, Mg and Cu analysis

Metal	Element		
	Fe	Mg	Cu
Application interval (mg/l)	2–10	2–8	5–15
Correlation coefficient	0.994	0.995	0.994

Table 4 Classification of Tunisian pomegranate varieties

Group	Variety	Proanthocyanidin content (mg CE/g)	
		Irrigated land	Non-irrigated land
I	Kalaï Jebali Tounsi	120	80
II	Zehri	105	65
III	Gabsi Khalladi Mekki	95	60
IV	Chelfi	75	35

Table 5 Effect of light on the polyphenol content of pomegranate peel

Substance	Total phenols (mg EHHDP-G/g)	Proanthocyanidins (mg CE/g)	Anthocyanins (mg/g)
Peel in shade	176.7 ± 1.2	55.4 ± 3.0	0.52 ± 0.02
Peel exposed to sun	85.8 ± 2.7	34.4 ± 2.7	1.03 ± 0.03

We noted that the peel of the fruits of the Chelfi variety that were collected from those parts of the tree that were most exposed to the sun presented a notably greater difference in their colour and proanthocyanidin content, as compared to those collected from shady parts.

The results obtained (Table 5) allows a conclusion in favour of differentiated biosynthesis of proanthocyanidins, anthocyanins and total phenols according to the lighting patterns to which the fruits are exposed.

While the proanthocyanidin contents in the peels exposed to the sun and those in the shade are practically the same (Table 5), the anthocyanin contents are clearly greater in the peels exposed to the sun. On the other hand, we noted variable increases, i.e. from small changes to a doubling of the value, in the total phenol content of those peels which had either been exposed to light or kept in the shade. This observation is very interesting. It poses the question as to the identity of the polyphenols formed, because the increase of the proanthocyanidin content is not dependent on the pattern of exposure to light. The identification of polyphenolic components other than the proanthocyanidins is in progress. This determination, it is hoped, will specify whether the evolution of the polyphenolic compounds has an effect on the tanning properties of pomegranate peel, in a manner which is dependent on the pattern of exposure to sunlight.

In conclusion, among the numerous varieties of pomegranate in Tunisia, eight of them have been analysed. We have been interested particularly in the pomegranate peel, that is a considerable source of polyphenolic substances (condensed tannins, hydrolysable tannins, ellagic and gallic acids, etc.).

The quantitative analysis of total phenols, condensed and ellagic tannins, gallic and ellagic acids has been carried out, using the peel of the Chelfi variety. The polyphenolic components include hydrolysable tannins, condensed tannins and free molecules of ellagic and gallic acids. The results obtained show the relative importance of the proanthocyanidin content, compared to those of the other polyphenolic constituents, particularly the ellagic derivatives, the most significant of which are punicallagin and punicallin [14].

From our results, we can disclaim the following conclusion; i.e. during the development of the pomegranate, the condensed tannin amount increases to its maximum which occurs at same time period (September) whatever the variety. Outside this optimum period, the tannin content diminishes according to solubility. The comparison of proanthocyanidin contents of pomegranate peel from the fruit formation to its maturation allows a classification of the studied pomegranate varieties into four groups, i.e. Kalai, Jebali and Tounsi; Zehri; Gabsi, Khalladi and Mekki; and Chelfi.

The effect of irrigation of the land in which the pomegranate is cultivated is in favour of a significant increase in the proanthocyanidin content of the fruit peel. Similarly, greater exposure of the fruits to light considerably the amount of total polyphenolic components of the peel of the mature pomegranate.

References

1. Gardon D, Du Chatenet G (1990) Guide des teintures naturelles Ed. Dellachaux et Niestlé Neuchâtel
2. Bruneton J (1987) Eléments de Phytochimie et de pharmacognose. Technique et documentation-Lavoisier Paris, pp 392–393
3. Apontine G, Fernandrez Y, Bourzeix M, Mitjavila S (1992) Journées Internationales d'Etudes sur les Polyphenols (Lisbonne). Groupe Polyphénols, Laboratoire de Pharmacognosie Université de Bordeaux II, Tome 1: 237–240
4. Davis AB, Hoseney RC (1979) Cereal Chem 56: 310–314
5. Flectcher AC, Porter LJ, Haslam E, Gupta R (1977) J Chem Soc Perkin Trans I 1628–1636
6. Shen Z, Haslam E, Falshaw CP, Bergly MJ (1986) Phytochemistry 25: 2629–2635
7. Thompson RS, Jacques D, Haslam E, Tanner RJN (1972) J Chem Soc Perkin Trans I 1387–1399
8. Yazaki Y, Hillis WE (1977) Holzforschung 31: 20–25
9. Yazaki Y (1983) Holzforshung 37: 87–90
10. Singleton VL, Rossi JRJA (1965) Am J Enol Vitic 16: 144–158
11. Bate-Smith EC (1972) Phytochemistry 11: 1153–1156
12. Scalbert A, Monties B, Janin G (1989) J Agric Food Chem 37: 1324
13. Charrier B, Marques M, Haluk JP (1992) Holzforschung 46: 87–89
14. Takashi T, Gen-Ichiro N, Itsuo N (1986) Chem Pharm Bull 34: 650–655
15. Takashi T, Gen-Ichiro N, Itsuo N (1986) Chem Pharm Bull 34: 656–663
16. Mayer W, Groener A, Andrae K (1976) Justus Liebigs Ann Chem 11–12: 1976–1986
17. Okuda T, Yoshida T, Mori K, Hatano T (1981) Heterocycles 15: 1323
18. Metche M, Girardin M (1980) In: Monties B (ed) Les tanins des végétaux in "les polyméres végétaux". Gauthier-Villars, Montrouge, pp 252–288
19. Golstein JL, Swain T (1981) Phytochemistry 2: 371–383