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Characterization Of Pesticide Residues And Quantitative Study Of Phenolic Compounds Of Two Grapefruit Species: Citrus Paradisi Yellow And Citrus Paradisi Blood

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Abstract

Citrus fruits and their juices are an important source of bioactive compounds; called secondary metabolites, including phenolic and non-phenolic compounds. Several studies have demonstrated the antioxidant properties of these citrus fruits. Grapefruit, scientifically called citrus paradisi, is one of those citrus fruits consumed primarily for its medicinal and antioxidant properties due to the presence of flavonoids, vitamin C and tannins. A characterization in phenolic compounds of two species of grapefruit, namely; citrus paradisi yellow and blood, was carried out by a quantitative study preceded by an assay of pesticide residues in order to enhance the value of the samples tested.

The results obtained showed the presence of a single pesticide; AZOXYSTROBINE with a content that does not exceed the required European standard (15 mg / kg of dry matter). In contrast, the characterization study of polyphenols demonstrated very potent antioxidant activity due to the presence of important phenolic compounds which may be the source of highly sought-after therapeutic properties and successful use in the medical and cosmetic fields.

Key words: Citrus paradisi, phenolic compounds, pesticide residues, antioxidant activity

Introduction

A diet rich in fruits and vegetables provides a multitude of essential vitamins, antioxidants (vitamin C, carotenoids, and flavonoids), minerals, fiber and water. Therefore, nutritionists recommend eating at least five servings of fruits and vegetables per day in order to protect oneself as much as possible against the appearance of various chronic pathologies in which oxidative stress is potentially involved (Ross & Kasum, 2002). Antioxidant bioactive compounds; including polyphenols; are a class of nutrients known to reduce the incidence of these conditions.

Citrus fruits are one of those foods very rich in vitamin C (ascorbic acid) and other bioactive compounds, including flavonoids, coumarins, carotenoids and limonoids with important antioxidant properties (Sanz et al., 1994; Noroozi et al., 1998)

This is also cited at the work level of Fernandez Lopez et al (2005), Jayaprakasha and Patil (2007) and Ebrahimzadeh et al (2004).
Grapefruit; citrus belonging to the Rutaceae family is a very fruit famous for its richness in bioactive compounds such as vitamin C, polyphenols (Gardner et al., 2000) and carotenoids (Liu & Lee, 2000), therefore the interest of this study which relates firstly to a search for pesticide residues by liquid chromatography coupled to mass (lc /ms/ms) and secondly to a quantitative study of polyphenols, total flavonoids and condensed tannins, a study of the antioxidant activity by DPPH completed the characterization of the two species of grapefruit.

Materials and Methods

Extraction, purification and pesticides analysis from the two grapefruit species

The extraction, purification and analysis of pesticides from the two grapefruit species was carried out according to the Qu ECh ERS method described in standard NF EN 15662.
Validation of the method and confirmation of contamination or harmlessness of the samples analyzed were based on the concept of MRLs (maximum residue limit). MRLs are set by the European Food Safety Authority.

Extraction

Precisely weigh 10 g of sample to ± 0.1g in a 50ml tube, add 100 µl of the internal standard solution to a 20 µg / ml solution.

Add 10 ml of Acetonitrile. Close the bottle and shake with a vortex for 1 minute and add the salts: 4 g Magnesium sulfate ± 0.2g; 1 g Sodium chloride at ± 0.05g; 1 g Trisodium citrate dihydrate at ± 0.05g;0.5g Disodium hydrogen citrate sesquihydrate at ± 0.05g. Then shake with a vortex for 15 minutes and Centrifuge at 3500 rpm for 5 minutes.

Purification

The volume of the extracted aliquot is transferred to a centrifuge tube containing 25 mg of PSA and 150 mg of magnesium sulfate per ml of extract.

Purification using amino adsorbent:
A 5 ml aliquot of acetonitrile phase obtained after extraction into a disposable polypropylene centrifuge tube containing: 150 mg of PSA; 900 mg of Magnesium sulfate.

Shake with a vortex for 30 seconds and centrifuge at 3500 rpm for 5 minutes.

The pesticides chromatographic analysis of was carried out using a liquid chromatography system coupled to an API3200 brand mass spectrometer (MS / MS) equipped with a C18 column, the mobile phase is a gradient of two phases (phase A: water + 5ml of ammonium formate; phase B: methanol + 5ml of ammonium formate.

Samples Preparation for quantification

The fruits were peeled using a manual peeler, to separate the rind from the pulp which were cut into small pieces and used for the quantification of phenolics.

Extracts preparation

Two extraction methods were used; maceration called cold extraction, and soxhlet; hot extraction, while using two solvents with increasing polarity; dichloromethane and ethanol.
In practice, 15g of the zest of the fresh fruit was macerated in 100 ml of dichloromethane for 24 hours at room temperature and in the dark. The filtrate obtained was then evaporated to dryness using a rotary steamer and at boiling temperature (40 ° C). The extracts thus obtained were adjusted to 2 ml each as final volume.
The dry marc was macerated in 100 ml of ethanol for 24 hours at room temperature and in the dark, apart from the boiling point used (78.4 ° C).
The same process of dry evaporation was carried out in order to obtain the extracts of the fruit pulp, except that the latter is completely exhausted following the first maceration, so 15 g of the pulp are taken for the obtaining ethanolic extract.
The hot soxhlet extraction of the fresh fruit pulp allowed us to repeat the extraction cycle with the two solvents; namely dichloromethane and ethanol until the pulp is completely depleted. 25 g of the latter were extracted into 500 ml of the solvent and placed in the soxhlet for 1h30min. The same procedure is developed for the zest but the extraction for the latter is successive.
The yield of the extracts was calculated according to the following formula:

$$R\ (\%) = \frac{Mext}{Méch} \times 100$$ (Fallah Huseini et al., 2008)

Where R is the yield in%; Mext is the mass of the extract after evaporation of the solvent in mg; MS is the dry mass of the sample in mg.

The extracts were stored in the refrigerator until use for the determination of total polyphenols, total flavonoids, and condensed tannins and for evaluation of antioxidant power.

Total polyphenols dosage

The total polyphenols dosage of the zest and pulps of the two grapefruit species was carried out according to the Folin-Ciocalteu method (Mansouri et al., 2005). Gallic acid was used as a standard.
A stock solution of gallic acid at 0.1 mg / ml, the Folin-Ciocalteu reagent diluted in distilled water at 1/10, and sodium carbonate (7.5%) were previously prepared. The preparations and incubation are carried out at room temperature
A range of 9 gallic acid concentrations from 0.025 to 0.6 mg / ml was prepared from a 0.6 mg / ml solution. The calibration curve is produced according to the following (Figure 1).

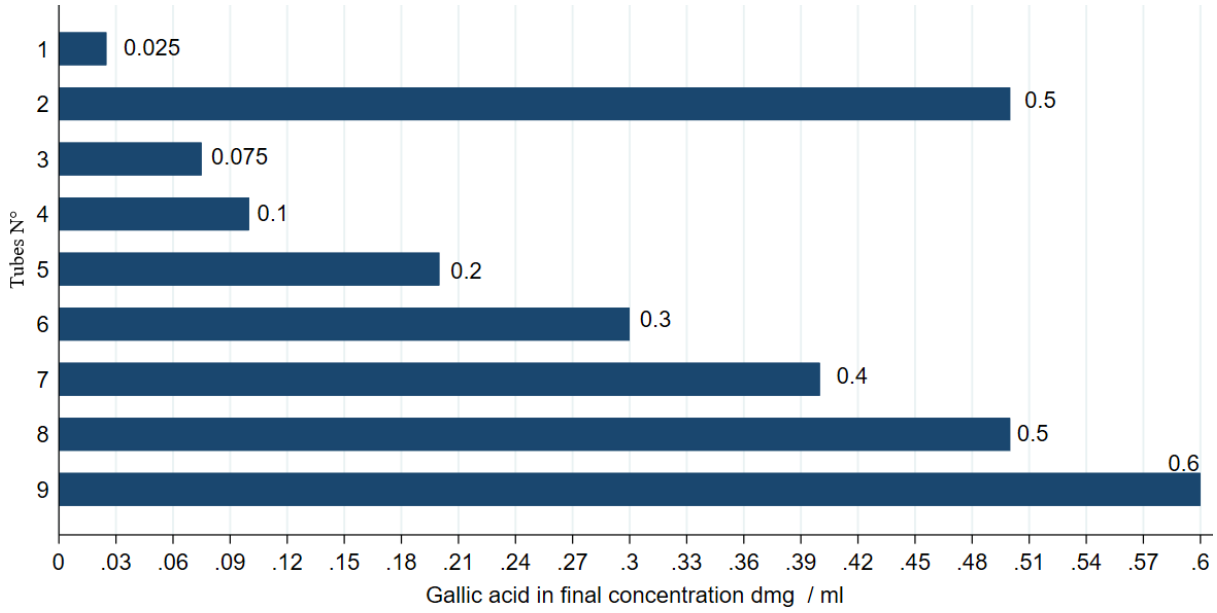

Figure 1: Gallic acid calibration range

300 µl of the gallic acid solution were introduced at different consentrations into the tubes of a first series. A second series of 300 µl of each sample to be analyzed was placed in tubes. 1500 µl of the reagent and then 1200 µl of the sodium carbonate solution were added to each tube. All preparations were shaken and then incubated in the dark for 1 hour. The absorbances were read at 760 nm (Lamaison., 1991).

The blank was therefore represented by 300 µl of methanol, added to 1.5 ml of the Folin-Ciocalteu reagent and 1.2 ml of 7.5% sodium carbonate.

The values obtained were used to deduce from the calibration curve the concentrations of total phenols.

The results were expressed in milligrams equivalent of gallic acid per gram of the dry weight of the powdered plant by applying the following formula:

$$C = \frac{(c \; x \; V)}{m}$$

With;

C: Total phenol content (mg gallic acid / g dry matter)

c: Gallic acid concentration established from the calibration curve (mg / ml)

V: Volume of E-MeOH or E-DCM

m: Dry matter weight (g)

Total flavonoids dosage

The method used to estimate the total flavonoid content of the two grapefruit species is that described by Lamaison and Carnat and cited by Bahorun (Lamaison., 1991; Bahorun., 1998).

The reagent used for this assay was prepared by adding 2 g of Aluminum Chloride (AlCl3) to 100 ml of absolute methanol;

A range of 9 concentrations of quercetin from 2.5 to 40 µg / ml was prepared from a stock solution of 40 µg / ml (400 µg of quercetin dissolved in 10 ml of methanol).
0,2 to 3.0 ml aliquots of the quercetin stock solution were placed in a series of test tubes. The final volume in each tube was made up to 3 ml by the addition of absolute methanol. Then, 1 ml was taken from each tube and transferred to another, to which is added 1 ml of the 2% methanolic solution of aluminum chloride. After 10 min incubation at room temperature and in the dark, the absorbance was read at 430 nm. The optical densities thus obtained are used to establish a calibration curve representing the concentration of quercetin (µg / ml) as a function of the absorbance (Figure 2).

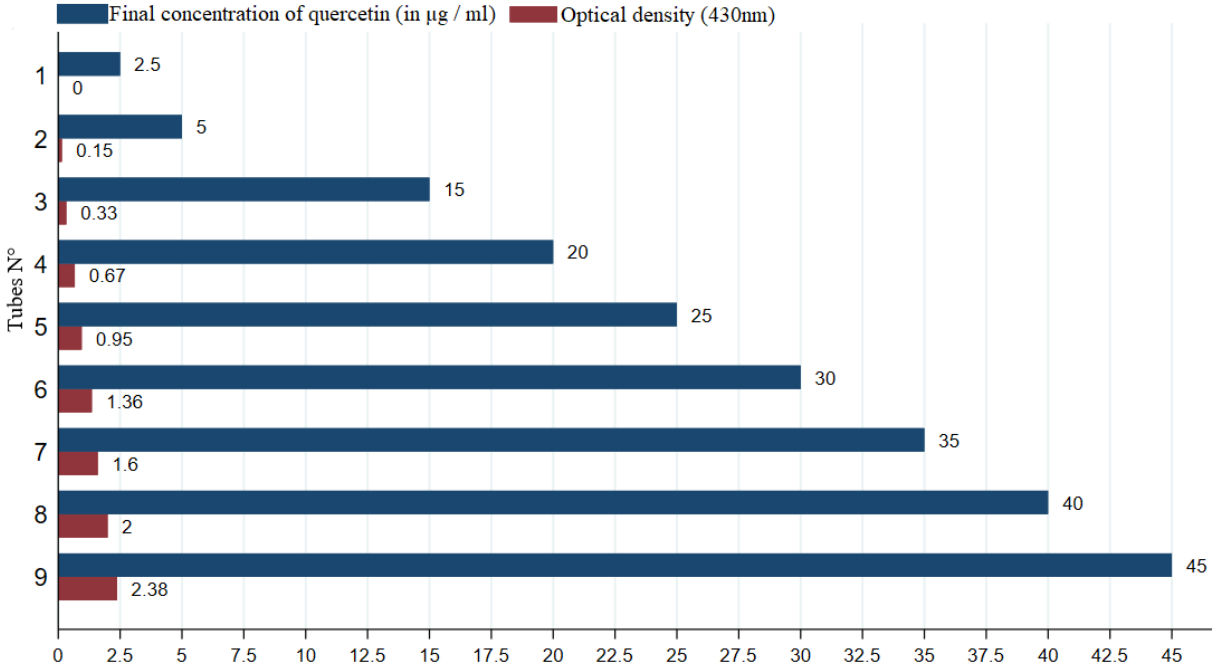

Figure 2: Quercetin calibration range

In order to analyze the extracts, two sets of test tubes were prepared. 1 ml of each extract was introduced into a tube of each of the two series. 1 ml of the 2% methanolic aluminum chloride solution was added to each of the tubes of the first series and 1 ml of the absolute methanol was added to each of the tubes of the second series serving as blank. After 10 min, the absorbance was read at 430 nm.
The absorbance of the extracts from the tubes of the 2nd series were subtracted from those of the 1st series to avoid possible interference from the pigments.
The concentration of flavonoids was determined by referring to the calibration curve obtained using quercetin as a standard.

Dosage of condensed tannins

The condensed tannins dosage was carried out by the vanillin method described by Hagerman (2002). The assay reagent was prepared by mixing the 1% vanillin solution (in methanol) and the 8% methanolic HCl solution in equal parts. The Standard used is catechin at 0.3 mg / ml.
A range of 6 catechin concentrations ranging from 0 to 0.3 mg / ml was prepared from the stock solution.
Aliquots of 0,2 to 1,0 ml of the stock catechin solution were placed in a series of test tubes, the final volume in each tube was made up to 1 ml by the addition of absolute methanol. 5 ml of the assay reagent were subsequently added at 1 min intervals to each tube in the series and placed in a water bath set at 30 ° C for 20 min. The absorbance is read at 500 nm (Figure 3).

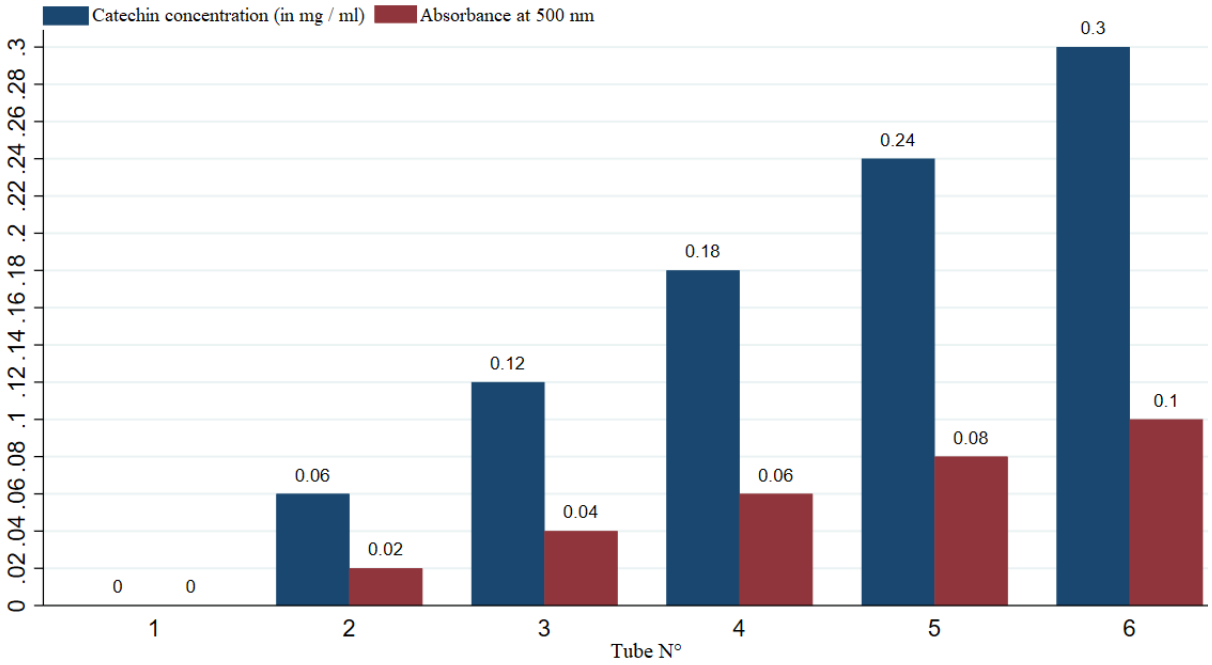

Figure 3: Catechin Calibration Range

In order to analyze the extracts; two sets of test tubes were prepared. A 1st series consists of tubes containing 1 ml of each extract and 5 ml of the analysis reagent added at 1 minute intervals to each of the tubes. The second set consists of 1 ml of each extract added to 5 ml of the 4% methanolic solution of HCl at 1 minute intervals. All the tubes were then placed in a water bath at 30 ° C. for 20 min. The absorbance reading at 500 nm was taken with the interval of one minute.

The absorbance of the tubes of the second series (the blanks) is subtracted from that of the corresponding tubes of the first series (the blank is considerable for fabrics that contain a large amount of pigments).

The values obtained are used to deduce the concentrations of the condensed tannin extracts from the calibration curve.

The antioxidant power DPPH

The scanning activity of the DPPH radical was measured according to the protocol described by Lopes-Lutz et al (2008).

The methanolic solution of DPPH was prepared by dissolving 5,5 mg of DPPH in 100 ml of methanol followed by sonication for 3 min. 2,5 ml of each extract and 1 ml of the methanolic solution of DPPH (55 µg / ml) were introduced into test tubes. After vortexing, the tubes are placed in the dark at room temperature for 30 min. The optical density is read by measuring the absorbance at 517 nm.

The results are expressed as anti-free radical activity where the inhibition of free radicals is calculated as a percentage (I%) using the following formula:

$$\textbf{Inhibition } \% = [1 - \left(\frac{\textbf{Abs e}}{\textbf{Abs c}} \right)] \times 100$$

With;

Abs c: Control absorbance

Abs e: Absorbance of the tested sample

For each extract, the IC_{50} value; Also called EC_{50} (Efficient concentration), representing the concentration of the substrate producing the 50% loss of DPPH activity (Samarth et al., 2008), was determined graphically from the curve of the percentages of inhibition as a function of different concentrations of the extracts tested (Torres et al., 2006).

The results can also be expressed in anti-free radical power (ARP) (Brand-Williams et al., 1995).

$$ARP = \frac{1}{IC_{50}}$$

Results and Discussion

Characterization of pesticide residues in grapefruits

Several types of pesticides used in Morocco to fight against citrus fruit attack were researched (insecticides, fungicides, acaricide and nematicide). The qualification and quantification of pesticides in the two grapefruit species was carried out by CL / SM / SM. The following chromatograph represents the mixture of active materials used as standard

Figure 4: interne standard of TPP 0.5 ppm

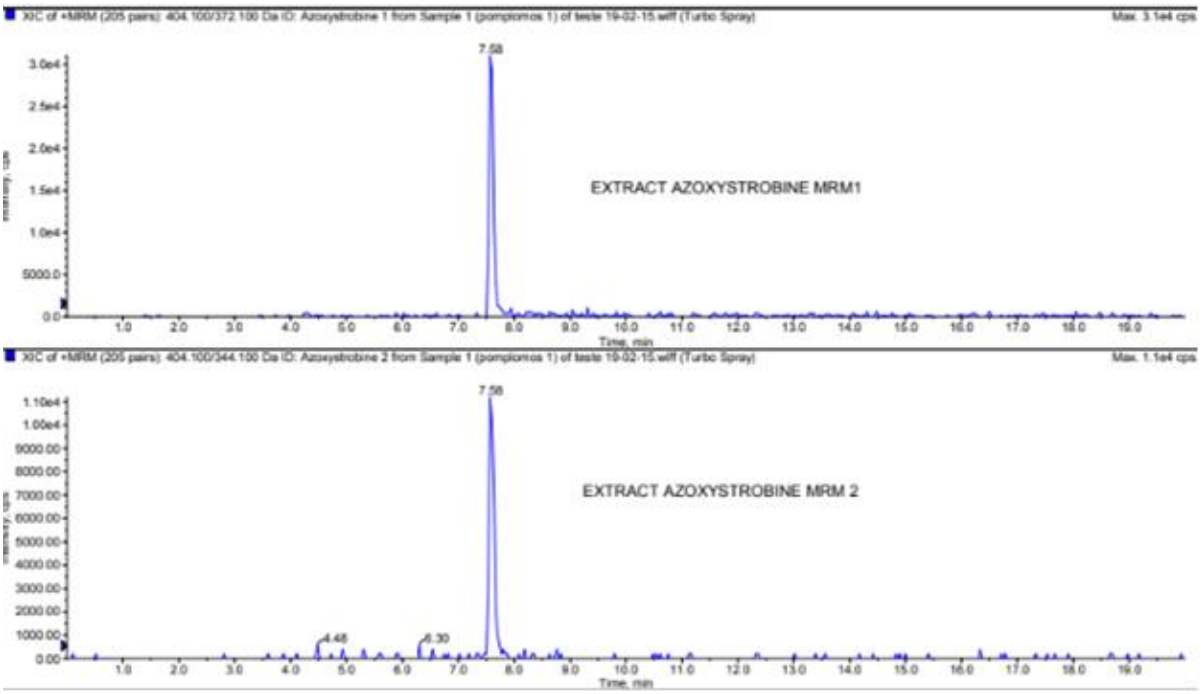

Figure 5: Azoxystrobin's chromatogram of citrus paradisi

The results obtained show that the two grapefruit species contain only one pesticide "azoxystrobin" which is a fungicide used to reduce the damage caused by phytopathogenic fungi. The content of Azoxystrobin found in both yellow and blood grapefruit species is 0.005mg / kg. This value is lower than the MRL set by the EU.

Pesticides are widely applied in fields in the post-harvest phase for some fruits and vegetables against weeds, insects, and some diseases. Although the correct use of pesticides does not cause problems of public health and environmental concern, the potential risk to consumers resulting from chronic dietary exposure is increasing (Abou-Arab., 1999; Claeys et al., 2011).

Several studies have shown that food processing, including washing, peeling and juicing can greatly reduce the content of pesticide residues in agricultural products (Bartnick et al., 2006). In our study, the treatment with pesticides was done several days before harvest, washing the fruits before analysis would have contributed to the elimination of pesticide residues. In view of the results obtained, the two grapefruit species harvested in the Ghrab region are free of pesticides and therefore can be used for food consumption and for various non-food applications (cosmetic and pharmaceutical) without any risk of toxicity.

Extraction yield

The extraction yield obtained for each of the extracts of the zest and pulp of the two grapefruit species was calculated and presented in the following (Table 1):

Table 1: yield of extraction methods for the two grapefruit species

	Excerpts (%)	Solvent used	Yield
MACERATION	PRP	Dichloromethane	13,3
	PRZ		8
	PJP		16

	PJZ		6,7
	PRP	Ethanol	18,2
	PRZ		25,1
	PJP		16
	PJZ		21,2
SOXHLET	PRP	Dichloromethane	2
	PRZ		3,2
	PJP		1,6
	PJZ		3,6
	PRP	Ethanol	21
	PRZ		5,3
	PJP		18,9
	PJZ		7,4

Legend PRP: pulp of red grapefruit PJP: pulp of yellow grapefruit
PRZ: zest of red grapefruit PJZ: zest of yellow grapefruit

The comparative results of the yield for the maceration show that ethanol proves to be a better extraction solvent for red grapefruit (25,1% obtained for the peel and 18,2% for the pulp) and yellow grapefruit (21, 2% for the bark and 16% for the pulp) compared to dichloromethane. Soxhlet extraction showed the same result; namely 21% for the pulp of red grapefruit, 5,3% for the peel and 18,9% for the pulp of yellow grapefruit and 7.4% for the peel.
Because of its efficiency and safety, ethanol is the most recommended solvent for the extraction of phenolic compounds from citrus peels for food and cosmetic applications. Indeed, most authors have found that methanol gives the highest yield. However, in industrial applications, methanol is not used due to its toxicity. It is often replaced by other non-toxic organic solvents with satisfactory extraction yields, such as ethanol, n-butanol, isopropanol or petroleum ether (Bartnick et al., 2006).

Table 4 also shows that the extraction by soxhlet gives better results in terms of yield compared to the extraction by maceration, for example for the pulp of red grapefruit extracted by soxhlet and by ethanol which gave a yield of 21.0%, versus 18.2% for the maceration. The same results were obtained by the work of… indicating that the extraction method significantly influences the yield and the levels of phenolic compounds in E. helioscopia and that
the extraction by sonication and by soxhlet allow an enrichment of the extracts compared to the maceration. Effect of the solvent and the extraction method on the content of phenolic compounds and the antioxidant potential of Euphorbia helioscopia

Total polyphenols dosage

The total polyphenol contents measured for the two grapefruit species are reported in the table below. A calibration curve (Annex 1) is produced using galic acid for the determination of the total polyphenols of two species of grapefruit

Table 2: Total polyphenol content of the two grapefruit species

	EXCERPTS	Total polyphenol content in mg EAG / 100 g of dry plant material

		Ethanol	Dichloromethane
MACERATION	PRP	71,169934	3,2588774
	PRZ	65,421945	2,6077662
	PJP	64,800984	1,8744329
	PJZ	33,447881	8,2099885
SOXHLET	PRP	30,044951	20,625154
	PRZ	60,339012	25,644414
	PJP	39,306079	8,2518211
	PJZ	40,263283	18,284414

Legend PRP: pulp of red grapefruit PJP: pulp of yellow grapefruit
PRZ: zest of red grapefruit PJZ: zest of yellow grapefruit

Roughly speaking, the majority of the ethanolic extracts showed better results than the dichloromethane extracts. Likewise, hot extraction (soxhlet) gave better results than those obtained by maceration.

Indeed, the contents obtained by soxhlet, they vary from 8,25 and 25,64 mg EAG / 100g MS and from 30,04 and 60,34 mg EAG / 100g MS for dichloromethane and ethanol respectively for the two types of grapefruit (Table 2). this confirms that the extraction method influences the content of polyphenols.

As indicated in the same table, we note that the values obtained by dichloromethane are less important than those obtained by ethanol for the two types of extraction and for all the extracts analyzed. These results show that the different extraction solvents differ in their ability to extract phenolic compounds from the two species of citrus paradisi.

A study conducted by Li and his colleagues (2006) on the effect of solvent concentration and extraction time on the phenolic content of several varieties of Citrus confirms the results obtained.

There is a variation in the polyphenol content relative to the different parts of the fruit. This was confirmed by the study by Boudries et al. on mandarin juice which effectively proves that the polyphenol concentrations vary according to the part of the plant used in the tests (Boudries et al., 2012).

Dosage of flavonoids

A calibration curve (Annex 2) is produced using quercetin for the determination of the total flavonoids of two species of grapefruit. Table 3 below illustrates the results obtained.

Table 3: Flavonoid content in the two grapefruit species

	EXCERPTS	Total flavonoid content in mg E quercetin / 100 g of dry plant material	
		Ethanol	Dichloromethane
MACERATION	PRP	277,30839	85,48035359
	PRZ	291,35854	92,95294618
	PJP	237,101814	115,524798
	PJZ	355,427727	119,7796128
SOXHLET	PRP	388,1947461	18,28179926
	PRZ	133,926324	73,18402148
	PJP	270,4594714	25,47291037

	PJZ	117,711359	15,65957704

Legend PRP: pulp of red grapefruit PJP: pulp of yellow grapefruit
PRZ: zest of red grapefruit PJZ: zest of yellow grapefruit

The measured total flavonoid contents obtained by maceration vary from 85.48 to 119.78 mg EQ / 100g DM and from 237.10 to 355.43 mg EQ / 100g DM for dichloromethane and ethanol respectively (Table6).
Concerning the contents obtained by soxhlet, they vary from 15,66 and 73,18 mg EQ / 100g MS to 117,71 and 388,19 mg EQ / 100g MS for dichloromethane and ethanol respectively (Table 3).

As indicated in the same table, we note that the values obtained by dichloromethane are less important than those obtained by ethanol for the two types of extraction and for all the extracts analyzed.
According to these results, we can see an uneven distribution of flavonoids in different parts of the plant. This variability in flavonoid content has been observed in lemon and orange by other authors (Ghasemi et al., 2009; Ramful et al., 2010). This can be explained by the influence of certain extrinsic factors such as the extraction method and the nature of the solvent used.

Condensed tannins dosage

The dosage of the condensed tannins was carried out using the catechin which was used to produce the calibration range (Annex 3). Analysis was performed spectrophotometrically at a wavelength of 500 nm.
Analysis of the condensed tannin contents reported in Table 4 reveals that maceration with ethanol gives much more effective results than that carried out with dichloromethane.

Table 4: condensed tannins content in both species of grapefruit

	EXCERPTS	Content of condensed tannins in mg E catechin / 100 g of dry plant material	
		Ethanol	Dichloromethane
MACERATION	PRP	75,24	1,92
	PRZ	16,15	0,04
	PJP	4,36	3,52
	PJZ	--	7,04
SOXHLET	PRP	21,65	0,54
	PRZ	0,30	0,21
	PJP	--	--
	PJZ	1,51	0,39

Legend PRP: pulp of red grapefruit PJP: pulp of yellow grapefruit
PRZ: zest of red grapefruit PJZ: zest of yellow grapefruit

He measured condensed tannin contents obtained by maceration vary from 0,04 to 7,04 mg EC / 100g DM and from 4,35 to 75,24 mg EC / 100g DM for dichloromethane and ethanol respectively (Table 7).
Regarding the contents obtained by soxhlet, they vary from 0,21 and 0,54 mg EC / 100g MS and from 0,30 and 21,65 mg EC / 100g MS for dichloromethane and ethanol respectively (Table 4).

A complete absence of tannins was observed for the yellow grapefruit zest macerated in ethanol and for the pulp of the same grapefruit species extracted by soxhlet. The work of Arianna Ricci et al confirms the presence of tannins in grapefruit traditionally exploited in the wine industry as vectors in the production chain in order to precipitate excess protein material.

The antioxidant power DPPH

The antioxidant activity of the various ethanolic and dichloromethane extracts of the two species of grapefruit in relation to the DPPH radical was evaluated by spectrophotometry (517nm).

Figure 6 reports the results of the antioxidant power of the methanolic extracts of the two species of grapefruit as a function of different concentrations by the method of trapping the free radical DPPH.

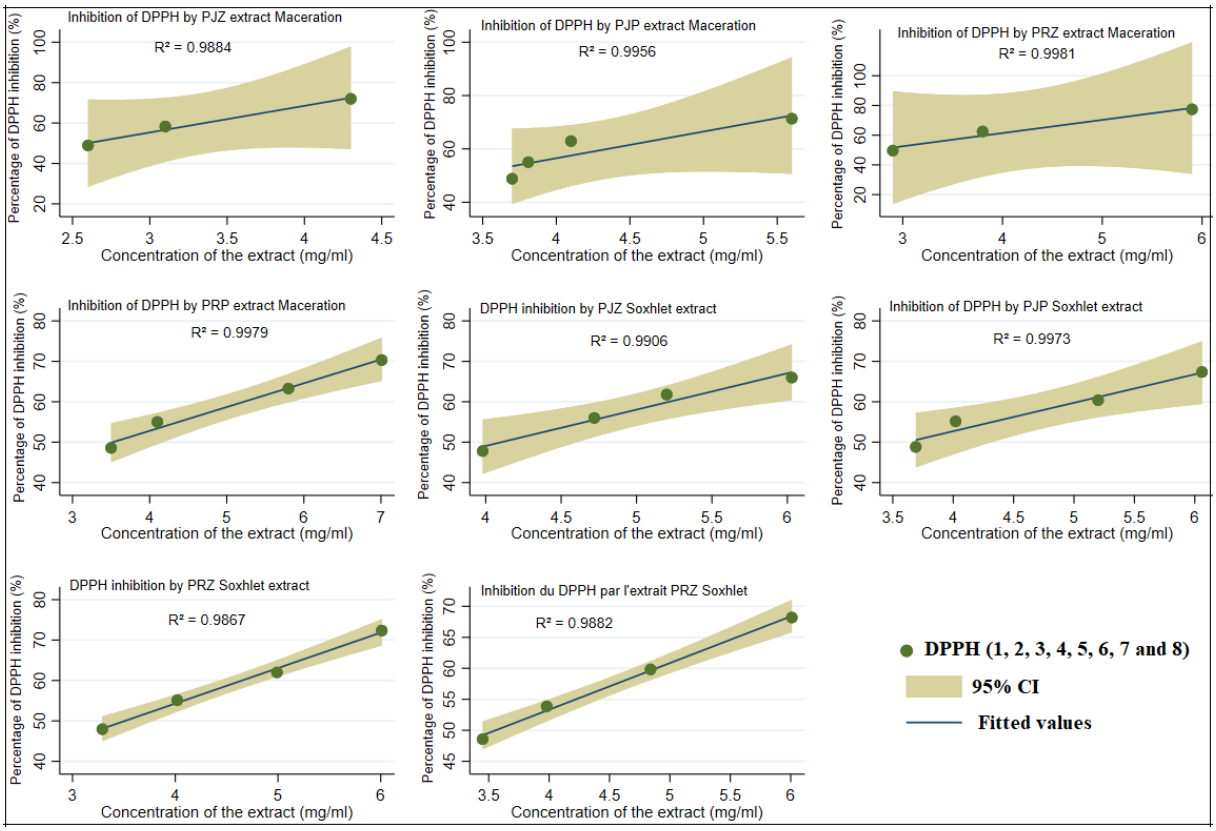

Figure 6: Percentage of inhibition of the free radical DPPH according to the concentrations of the ethanolic extracts of the Zests (PRZ and PJZ) and the pulps (PRP and PJP) of the two grapefruit species.

The DPPH percentage inhibition curves as a function of the two grapefruit species extract's concentrations generally and explicitly shows a remarkable decrease in the free radical DPPH, which gives the extracts tested a possible anti-free radical power.

The calculation of the IC_{50} defines the effective ethanolic extracts concentration which causes a 50% reduction of the DPPH in solution and makes it possible to calculate the anti-free radical power (ARP); inversely proportional to the IC_{50}.

The anti-radical power (ARP) values are presented in Figure 7.

Figure 7: the anti-free radical power (APR) of extracts from the two grapefruit species

The anti-free radical histograms showed a peak of 0.352 for the zest of yellow grapefruit extracted by maceration, while a peak of 0.28 was recorded for the zest of red grapefruit extracted by soxhlet.
For maceration, the anti-free radical power calculated at the level of the zest of the two red and yellow species of grapefruit is always greater than that of the pulps. Unlike the soxhlet extraction which shows a lower ARP value for the yellow grapefruit peel.
The difference in the anti-free radical activity between the extracts of the two citrus trees analyzed is probably due to their composition in different phenolic compounds. The reduction in DPPH is usually not due to a single compound but to interactions between several compounds. Indeed, studies have been able to demonstrate that this antioxidant activity against the free radical DPPH depends not only on the richness in polyphenols but also on the phenolic nature, the structure and the synergistic interactions (Djeridane et al., 2006). In addition, the phenolic compounds of an extract can act antagonistically or synergistically or even cumulatively, which influences the final antioxidant activity of the extract (Troszynska & Ciska, 2002; Subba Rao & Muralikrishna, 2002; Lim & Quah, 2007).

Our results are in agreement with those already published which have shown that citrus peels represent the fraction richest in polyphenols, which revealed a very powerful antioxidant potential (Bocco et al., 1998; Gorinstein et al., 2001).

Conclusion

The use of grapefruit is widespread in several areas other than food. This involves valuation by characterizing the samples as pesticide residues. The results showed that the samples comply with the European standard. The quantitative zest and pulp study of the two species has shown that they are an important source of phenolic compounds, such as flavonoids, total polyphenols and condenses tannins, hence the interest of their uses in traditional herbal medicine as well as the pharmacological and cosmetic field. Accordingly, the two species of grapefruit; citrus paradisi yellow and blood showed a very powerful antioxidant power.

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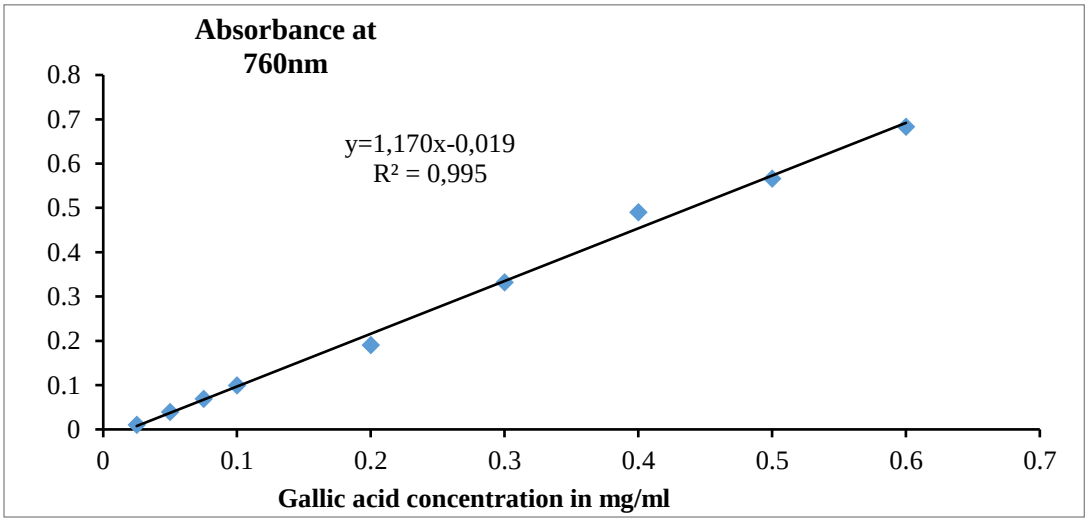
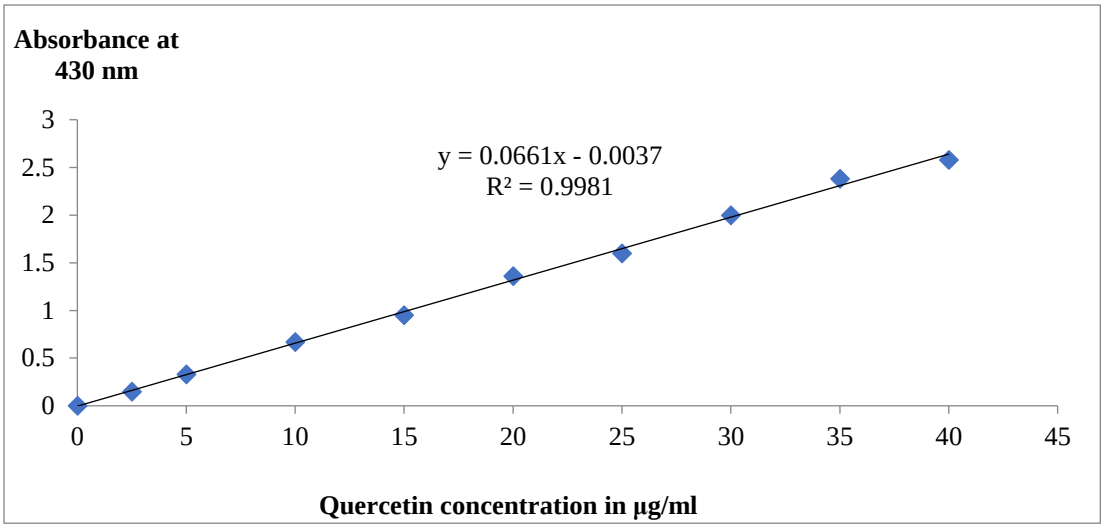
ANNEXES

Appendix 1. Gallic acid calibration curve for the determination of total phenols



Appendix 2. Quercetin calibration curve for the determination of flavonoids



Appendix 3. Catechin calibration curve for the determination of condensed tannins