Determination of Total Phenolic Content in Almonds After Lipid Removal or After Deproteinization

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Abstract. The classical method for polyphenols extraction requires long time and large amounts of reagents used. Our objective was to compare the results obtained using this method with results obtained using a direct method. The research was made on almonds cultivated in Palma de Mallorca, Spain. The polyphenol content of almond kernels were examined in two different ways: polyphenols extraction after lipid removal or after sample deproteinization. Commune and selected kernels were provided from Spain. One gram of almonds with 10 mL water were homogenized. In the first case, 10 mL were collected and extracted two times with hexane to remove lipids, first extraction all night long, and second extraction, 4-5 h. Polyphenols were extracted from aqueous phase with HCl: H2O: methanol during 16 h at 4 °C. The methanolic extract was retained and evaporated under nitrogen and the residue was reconstituted in 10 mM phosphate buffer. In the second case, the homogenate was deproteinized with acetone. Total phenols were assayed colorimetrically with the Folin-Ciocalteu. The calibration curve was made using pyrogallol. The results showed an effect for obtaining method, but not for almond varieties. Using extraction to remove lipids may cause losses in total polyphenols content while deproteinization avoides these polyphenol losses and reduces time and reagents.

Keywords: polyphenols, almonds, extraction, deproteinization, Folin-Ciocalteu.

INTRODUCTION

Almonds are known to be a good source of proteins, fibers, and minerals. Experimental evidence suggests that almonds improve cholesterol status and serum lipid profile (Chen C.Y et al., 2005). Also, almonds have an anti-obesity effect (Geleijnse, J et al., 1999; Hughes L.A. et al., 2008; Keli S.O et al., 1996; Yochum L., 1999).

Almonds are low in saturated fats and contain a big amount of vitamin E, sterols and flavonoids, which has been suggested to play a good role in the promotion of health. Information about the intake of this components may be given by almond ployphenols quantification.

Polyphenols are a group of chemicals that have many health benefits, such as reducing risk factors against chronic inflammatory diseases and ageing disorders (Garrido et al., 2008; Milbury et al., 2006). It has also been shown their protective role against cancer and cardiovascular disease (Knekt et al., 2002; Liu, 2004) and their antimicrobial potential (Mandalari et al., 2010).

For extraction of polyphenols can be performed liquid liquid extraction or countercurrent chromatography. Solid phase extraction can also be made. Other techniques are ultrasonic extraction, heat reflux extraction, microwave-assisted extraction (Pan, X., 2003),
critical carbon dioxide (Palma, M. Taylor, L., 1999), pressurized liquid extraction (Alonsosalces, R. et al., 2001) or use of ethanol in an immersion extractor (Sineiro, J. et al., 1996). These methods are usually used when it is necessary to know the nature of those compounds, but when it is necessary to know only the total content of polyphenols other methods, more simple can be used.

One classical method to determine total polyphenols content consists in extraction of phenols after lipid removal (Milbury et al., 2006). These method involves lipid removal using hexane. Hexane extraction requires long time, and also a big amount of reagent.

The aim of this study was to develop a rapid method for determination of total phenols of almonds and compare the results obtained using this method with the classical method.

**MATERIALS AND METHODS**

Common and selected almonds have been provided from Palma de Mallorca, Spain.

For the first method used, polyphenols extraction after lipid removing, the next steps were followed: one gram of almonds kernel with 10 mL water were homogenized in a Omni-Mixer Homogenizer. 10 mL were collected and extracted with hexane (1:10, w/v) all night long in order to remove lipids. After first extraction the samples were centrifuged at 2000 r.p.m., 4°C for 10 min using a refrigerated centrifuge SIGMA 2-16 KC and the aqueous phase was transferred into plastic tubes. The residue was extracted again with 10 ml hexane during 4-5 h and centrifuged in the same conditions. The aqueous phases were mixed. From the aqueous phases the polyphenols were extracted with a 1:15 (w/v) with HCl:H2O:methanol (3.7:46.3:50, v/v/v). This mixture was kept into incubation for 16 h, at 4°C and in darkness. The methanolic extract was retained and evaporated under nitrogen. The residue was reconstituted in 10 mM. phosphat buffer.

In the second case, polyphenols extraction after deproteinization, the next steps were followed: 1g of almonds kernel with 10 mL water were homogenized in a Omni-Mixer Homogenizer. 10 mL were collected and deproteinized with acetone, (1:1,5, w/v) over night. After deproteinization, the samples were centrifuged 2000 r.p.m., 4°C for 10 min using a refrigerated centrifuge SIGMA 2-16 KC. The aqueous phase was transferred into tubes. From this phase phenols were colorimetrically assayed.

In both cases, total phenols were assayed colorimetrically with the Folin-Ciocalteu method (Milbury et al., 2006) except that instead of using gallic acid for calibration curve was used pyrogallol. Briefly, 2.5 mL of Folin-Ciocalteu reagent (diluted 10-fold), 2 mL of 7,5 % sodium carbonate and 0.5 mL of phenolic extract were mixed well. After the reaction mixture were incubated for 15 min at 45 °C, absorbance was measured at 765 nm on a Shimadzu-UV-2401 spectrophotometer. Ten millimolar phosphate buffer was employed as a blank. For calibration curve, the following concentration of pyrogalol were prepared: 0 ug/L; 500 ug/L; 2500 ug/L; 5000 ug/L; 100000 ug/L; 200000 ug/L.

**RESULTS AND DISCUSSION**

The concentration of pyrogallol was expressed in umol/L by dividing at 126,11(molar mass of pyrogallol). Table no.1 shows the absorbance values read at a wavelength of 765nm appropriate to the concentrations.
The absorbance appropriate to pyrogallol concentrations, read at 765nm

<table>
<thead>
<tr>
<th>Concentration of Pyrogallol [ug/L]</th>
<th>Concentration of Pyrogallol [umol/L]</th>
<th>Absorbance [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>500</td>
<td>3,965</td>
<td>0,010</td>
</tr>
<tr>
<td>2500</td>
<td>19,825</td>
<td>0,048</td>
</tr>
<tr>
<td>5000</td>
<td>39,651</td>
<td>0,093</td>
</tr>
<tr>
<td>10000</td>
<td>79,302</td>
<td>0,216</td>
</tr>
<tr>
<td>100000</td>
<td>793,021</td>
<td>1,034</td>
</tr>
<tr>
<td>200000</td>
<td>1586,042</td>
<td>2,158</td>
</tr>
</tbody>
</table>

The calibration curve and standard equation are shown in figure no.1.

![Calibration curve and standard equation of pyrogallol](image)

The statistical methods used for data analysis were: repeated measures analysis of variance (ANOVA) to test the effects of the two factors; obtaining method and varieties.

The total content of phenols found in common almonds ranged from 1.98 umol/g to 2.67 umol/g almond when extraction after lipid removal was used. In the same samples, when extraction after deproteinization was used the values ranged from 4.557 umol/g to 5.692 umol/g.

For selected almonds the values ranged from 1.99 umol/g to 2.65 umol/g when first method was used and 4.826 umol/l to 5.403 when the second method was used.

The research showed that using polyphenols extraction after lipid removal involves much time, at least 32 hours, meanwhile polyphenols extraction after deproteinization reduces this time at least half. Also, the amount of reagent used is less.

The results showed that the level of total phenols content obtained after sample deproteinization was higher than the level obtained by extraction after lipid removal.
The content of total phenols (mean in µmol/g) of commune and selected almonds

<table>
<thead>
<tr>
<th>Almond variety</th>
<th>Total phenols content obtained using extraction after lipid removal µmol/g almond</th>
<th>Total phenols content obtained using extraction after deproteinization µmol/g almendra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common</td>
<td>2,320</td>
<td>5,096</td>
</tr>
<tr>
<td>Selected</td>
<td>2,418</td>
<td>5,153</td>
</tr>
</tbody>
</table>

Note: Selected almonds used in research are: Ferragnes, Masbovera.

As it can be seen in table 2, there are no differences between almond varieties, so the effect was only for obtaining method.

Values of median, standard deviation, minim and maxim for common and selected almonds content of polyphenols obtained using extraction after lipid removal or deproteinization

<table>
<thead>
<tr>
<th>Almond variety</th>
<th>Median</th>
<th>Standard deviation</th>
<th>Minim</th>
<th>Maxim</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common 1</td>
<td>2,315</td>
<td>0,342</td>
<td>1,980</td>
<td>2,671</td>
</tr>
<tr>
<td>Common 2</td>
<td>5,067</td>
<td>0,472</td>
<td>4,557</td>
<td>5,692</td>
</tr>
<tr>
<td>Selected 1</td>
<td>2,433</td>
<td>0,381</td>
<td>1,993</td>
<td>2,812</td>
</tr>
<tr>
<td>Selected 2</td>
<td>5,192</td>
<td>0,263</td>
<td>4,826</td>
<td>5,403</td>
</tr>
</tbody>
</table>

Note: 1 and 2 from table are referring to the method used: 1 refers to extraction after lipid removal, 2 refers to deproteinization.

CONCLUSIONS

The results showed that using extraction to remove lipids from almonds may cause losses in total polyphenols content while deproteinization avoids these polyphenol losses.

Also, polyphenols extraction with deproteinization is a far more rapid and direct method.

Considering the results of total content of polyphenols, it may be said that an intake of almonds can be a good source of polyphenols. These constituents and other antioxidant constituents may contribute to the health effect of almonds.
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