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Analytical Methods

Use of an enzyme-assisted method to improve protein extraction from olive leaves

M. Vergara-Barberán, M.J. Lerma-García, J.M. Herrero-Martínez, E.F. Simó-Alfonso *

Department of Analytical Chemistry, University of Valencia, C. Doctor Moliner 50, E-46100 Burjassot, Valencia, Spain

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ABSTRACT

The improvement of protein extraction from olive leaves using an enzyme-assisted protocol has been investigated. Using a cellulase enzyme (Celluclast® 1.5L), different parameters that affect the extraction process, such as the influence and amount of organic solvent, enzyme amount, pH and extraction temperature and time, were optimised. The influence of these factors was examined using the standard Bradford assay and the extracted proteins were characterised by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). The optimum extraction parameters were: 30% acetonitrile, 5% (v/v) Celluclast® 1.5L at pH 5.0 and 55 °C for 15 min. Under these conditions, several protein extracts from olive leaves of different genetic variety (with a total protein amount comprised between 1.87 and 6.64 mg g^{-1}) were analysed and compared by SDS–PAGE, showing differences in their electrophoretic protein profiles. The developed enzyme-assisted extraction method has shown a faster extraction, higher recovery and reduced solvent usage with respect to the use of the non-enzymatic methods described in literature.

1. Introduction

Olive tree (*Olea europeaea*, Oleaceae) is an important crop in the Mediterranean area, which produces 98% of the world total amount of olive oil. The olive tree and its products (leaves, olive fruit and its beneficial oil) have a rich history of nutritional, medicinal and commercial purposes (Soni, Burdock, Christian, Bitler, & Crea, 2006). In particular, olive leaves are one of the harvest by-products which can be found in high amounts in olive oil industries (5% of the total weight of the olives) since they accumulate during the pruning of the olive trees (Molina-Alcaide & Yáñez-Ruiz, 2008). Olive leaves are considered as a cheap raw material which can be used as a useful source of high-value added products, such as phenolic compounds (Briante, Patumi, Terenziani, Bismuto, & Febbraio, 2002). Recently, the interest in the chemical composition of olive leaves has been increased since they are capable to prevent certain diseases and present a large number of health benefits such as anti-oxidative, anti-inflammatory and antimicrobial properties (Pereira et al., 2007; Sudjana et al., 2009). The olive leaf composition varies depending on its origin, proportion of branches, storage and climatic conditions, moisture content, and degree of contamination with soil and oils (Molina-Alcaide & Yáñez-Ruiz, 2008). The analysis of proteins in leaves has received less attention (García, Avidan, Troncoso, Sarmiento, & Lavee, 2000; Malik & Bradford, 2005; Wang et al., 2003) than those of olive pulp (Esteve, Del Río, Marina, & García, 2011; Hidalgo, Alaiz, & Zamora, 2001; Montealegre, García, Del Río, Marina, & García-Ruiz, 2012; Salas & Sánchez, 1998; Salas, Williams, Harwood, & Sanchez, 1999; Zamora, Alaiz, & Hidalgo, 2001), seed (Alché, Jiménez-López, Wang, Castro-López, & Rodríguez-García, 2006;Montealegre et al., 2012; Ross, Sanchez, Millan, & Murphy, 1993; Wang, Alché, Castro, & Rodríguez-García, 2001; Wang, Alché, & Rodríguez-García, 2007) and oil (Georgalaki, Sotiroudis, & Xenakis, 1998; Martín-Hernández, Bénet, & Obert, 2008; Montealegre, Marina, & García-Ruiz, 2010; Zamora et al., 2001). Some of these works have employed the protein profiles to understand its accumulation during the fruit maturation (Wang et al., 2007), its role in oil stability (Georgalaki et al., 1998) and to differentiate between different genetic varieties (Montealegre et al., 2010, 2012; Wang et al., 2007).

The analysis of olive leaf proteins is fairly difficult due to the low crude protein content present (70–129 g/kg dry matter) (Molina-Alcaide & Yáñez-Ruiz, 2008) and the high levels of interfering components (such as pigments, polyphenols, etc. (Granier, 1988)) during its extraction procedure and subsequent electrophoretic separation. Several extraction protocols have been proposed (García et al., 2000; Malik & Bradford, 2005; Wang et al., 2003) to overcome this troublesome. Thus, García et al. (2000) employed an aqueous sodium borate buffer, followed by cold methanolic

* Corresponding author. Tel.: +34 963543176; fax: +34 963544436.
E-mail address: ernesto.simo@uv.es (E.F. Simó-Alfonso).

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ammonium acetate isolation of juvenile-related proteins in olive leaves; however, the protein profiles in SDS–PAGE showed a background due to the presence of interfering compounds. To improve protein extraction, Wang et al. (2003) developed a procedure based on phenol extraction of proteins from olive leaves in presence of SDS for their determination by two-dimensional electrophoresis. Slight modifications of this protocol were introduced by Malik and Bradford (2005) to achieve a higher protein yield. However, these latter extraction methods employed several cleanup steps using acetone and/or trichloroacetic acid, being laborious, time-consuming and non-environmentally friendly protocols. Enzyme-assisted protein extraction can be an alternative method due to its mild extraction condition and lower environmental impact (Sari, Bruins, & Sanders, 2013; Shen, Wang, Wang, Wu, & Chen, 2008). Thus, several specific enzymes have been employed in protein extraction in tea leaves (Shen et al., 2008), in *Leguminosae* gums (Sebastián-Francisco, Simó-Alfonso, Mongay-Fernández, & Ramis-Ramos, 2004) and in different oilseed meals (Sari et al., 2013), providing improved protein extraction yields compared to alkaline or acidic extractions. However, to our knowledge, this methodology has been not applied to the olive leaves protein extraction.

The aim of this work was to investigate the possible use of cellulase enzyme (Celluclast® 1.5L) in assisting the extraction of proteins from olive leaves. The protein extraction was optimised in terms of several experimental conditions such as organic solvent and enzyme amounts, pH and extraction temperature and time. To monitor the extraction, the total protein amount was measured using the standard Bradford assay, and protein profiles were characterised by SDS–PAGE. Additionally, a comparison of the resulting protein profiles from olive leaves belonging to different genetic varieties was performed.

2. Materials and methods

2.1. Chemicals

Acetonitrile (ACN), acetone, methanol (MeOH), 2-propanol and glycerine were purchased from Scharlau (Barcelona, Spain). Ammonium persulfate (PSA), tetramethylethylenediamine (TEMED), tris(hydroxymethyl)aminomethane (Tris), acrylamide, bisacrylamide, SDS, Coomassie Brilliant Blue R and bovine serum albumin (BSA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). 2-Mercaptoethanol and hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany) and bromophenol blue from Riedel-de-Haën (Hannover, Germany). Two molecular weight size protein standards (6.5–66 kDa and 36–200 kDa) were also provided by Sigma–Aldrich. A Protein Quantification Kit-Rapid from Fluka (Steinheim, Germany) was used for Bradford protein assay. A cellulase enzyme (Celluclast® 1.5L) was donated by Novozymes (Bagsvaerd, Denmark). This enzyme is produced by submerged fermentation of a selected strain of the fungus *Trichoderma reesei* (ATCC26921) and catalyses the breakdown of cellulose into glucose, cellobiose and higher glucose polymers. Celluclast® 1.5L activity is 1500 NCU/g (NCU = Novo Cellulase Unit). For practical applications, the optimal working conditions are about pH 4.5–6.0 and 50–60 °C. Deionized water (Barnstead deionizer, Sybron, Boston, MA) was also employed.

2.2. Samples

The olive leaves employed in this study (Table 1) were kindly donated by different olive oil manufacturers. To assure a correct sampling, olive leaves were collected at the same period (end of November 2011) directly from trees located in different Spanish regions. The genetic variety of samples was guaranteed by the suppliers. The leaves were previously selected to assume the absence of mould or other microorganisms, washed with water to remove dust or airborne particles settled on the leaf surface and then stored at −20 °C prior their use.

Table 1
Genetic variety, geographical origin and total protein amount (mg g^{-1} fresh leaf) of the olive leaves used in this study.

Genetic variety	Geographical origin	Protein content[a] (mg g^{-1} fresh leaf) (n = 3)
Solà	Castellón	3.67 ± 0.18
Cornicabra	Castellón	1.87 ± 0.10
Hojiblanca	Córdoba	4.84 ± 0.22
Picual	Murcia	6.64 ± 0.24
Arbequina	Murcia	5.68 ± 0.23
Manzanilla	Valencia	5.06 ± 0.26
Blanqueta	Valencia	6.09 ± 0.23
Grosol	Valencia	5.87 ± 0.29

[a] Obtained by standard Bradford assay, and expressed as the mean value ± standard deviation.

2.3. Instrumentation

SDS–PAGE experiments were performed using a vertical mini-gel Hoefer SE260 Mighty Small system (Hoefer, MA, USA). Protein extraction was carried out with a Sigma 2–15 centrifuge (Sigma Laborzentrifugen, Osterode am Harz, Germany) and a D-78224 ultrasonic bath (Elma, Germany). To measure the absorbance at 595 nm for the Bradford protein assay, a Model 8453 diode-array UV–vis spectrophotometer (Agilent Technologies, Waldbronn, Germany), provided with a 1-cm optical path quartz cell (Hellma, Müllheim, Germany), was used.

2.4. Protein extraction

Protein extraction was performed as follows: 10 g of fresh leaves were frozen in liquid nitrogen and ground to a fine powder in a pre-cooled mortar and pestle, and next lyophilized. The lyophilized powder was then homogenised, and 0.3 g were treated with 3 mL of a water:ACN mixture (7:3, v/v) containing a 5% (v/v) Celluclast® 1.5L enzyme at pH 5.0, sonicated at 55 °C for 15 min using an ultrasound power of 0.5 W/mL, and centrifuged at 10,000×g for 10 min. An aliquot of the supernatant was taken and used for Bradford's assay, and the rest of solution was stored at −20 °C until their use.

For SDS–PAGE analysis, 200 μL of protein extract were precipitated by adding 800 μL of ice-cold acetone for 20 min. The proteins were collected by centrifugation at 10,000×g for 10 min at 4 °C, and the resulting pellet was dissolved in 100 μL of SDS sample buffer (0.0625 M Tris–HCl pH 6.8, 10% (v/v) glycerol, 5% (w/v) SDS, 0.004% (w/v) bromophenol blue and 5% (v/v) 2-mercaptoethanol) (Laemmli, 1970). Then, the sample was heated for 5 min at 95 °C and an aliquot was loaded on the gel.

For Bradford's assay (Bradford, 1976), a calibration curve up to 1 mg mL^{-1} of BSA was prepared in the extraction solvent (water:ACN mixture (7:3, v/v). Sample protein amount was measured according to the protocol described in the Bradford assay Protein Quantitation Kit-Rapid (Fluka). A sample blank containing 5% (v/v) Celluclast® 1.5L enzyme was also made in order to remove its contribution to the final sample absorbance.

2.5. SDS–PAGE separation

In order to determine the molecular weight of the extracted proteins, their separation was carried out on 10 cm long gels under reducing conditions. The running gels were composed by 30%

(w/w) total acrylamide and 8% (w/w) bisacrylamide. Electrophoresis was performed for 1.5 h at constant voltage (180 V) using a running buffer containing 3.03 g L^{-1} Tris, 14.4 g L^{-1} glycine and 1 g L^{-1} SDS. Then, gels were stained overnight in a solution of 0.2% (w/v) Coomassie Brilliant Blue R. Gels were washed with a solution composed of 40% (v/v) MeOH and 10% (v/v) acetic acid. Then, the gels were stored using an aqueous solution containing 5% (v/v) glycerol.

3. Results and discussion

3.1. Influence of organic solvent amount on protein extraction

First, the optimisation of a method for the extraction of proteins was performed to minimise interfering compounds previous to its posterior analysis. Based on the results previously published by Sebastián-Francisco et al. (2004), the use of an organic solvent extractant (such as ACN) in combination with an enzyme was investigated. In this work, a cellulase enzyme (Celluclast® 1.5L) was selected for the protein extraction, since its function is to hydrolyse the mesocarp tissue structure, which is a lipid deposition in fruits and leaves, and thereby helps to liberate the leaf components.

Thus, the influence of ACN amount on the protein extraction was firstly studied, keeping constant the Celluclast® 1.5L concentration at 3.5% (v/v) at pH 5.0, sonicated at 25 °C for 20 min. Villalonga variety leaves were selected to carry out the optimisation studies. For this purpose, the standard Bradford assay was first used. As shown in Fig. 1A, an increase in the absorbance was obtained when ACN percentage was increased up to 30%. However, when the ACN percentage was further increased, a signal decrease was observed. This behaviour could be explained taking into account that protein solubility decreased when high ACN percentages were used, which provided a high hydrophobicity in the medium.

The influence of the ACN percentage in the separation of olive leaf protein extracts was also tested by SDS–PAGE. For this purpose, eight molecule markers were used to establish the molecular weight band (Fig. 1B). As observed in this figure, when a 30% ACN percentage was used, clear bands were identifiable with higher intensity than those obtained at 20% and 40% ACN. Three prominent protein bands, located at 44, 55 and 110 kDa were distinguished. The band located at 44 kDa was identified as Celluclast® 1.5L enzyme, whereas the 55 kDa protein was assigned as ribulose-1,5-bisphosphate carboxylase (Rubisco), which was characteristic of leaf protein fractions of many plant species (García et al., 2000; Wang et al., 2003). However, the protein located at 110 kDa has not been previously reported in literature. As a result of both Bradford assay and SDS–PAGE results, a 30% ACN was selected for further studies.

3.2. Influence of enzyme amount on protein extraction

Next, the effect of enzyme amount on the protein extraction was examined by keeping constant the optimum percentage of organic modifier (30% ACN) at pH 5.0, sonicated at 25 °C for 20 min. For this purpose, enzyme concentrations were varied from 0% to 8% (v/v). In all cases, and as mentioned above, a reagent blank was also employed. As observed in Fig. 2A (standard Bradford assay), an increase in the total protein amount was obtained when the Celluclast® 1.5L percentage was increased up to a 5% (v/v). However, when the enzyme amount was further increased, a decrease in the signal was observed. This behaviour could be explained taking into account two effects: (i) the appearance of turbidity in the protein extracts by using enzyme percentages higher than 5% (v/v) and (ii) the so-called competitive inhibition, in which the extracted protein molecules are supposed to act as inhibitors combining with the enzyme to form a complex and thereby preventing its activity, although there is no evidence of this mechanism (Laidler & Bunting, 1973, chap. 3). This fact was in agreement with other studies reported (Shen et al., 2008). In addition to this, an increase in enzyme concentration would normally enhance the solubility of protein into the solvent and, therefore, increase the extraction yield. However, the existence of free enzyme in the solvent could interact with the protein and cause degradation of protein molecules, which could also explain signal decrease. This fact was in agreement with previously published studies (Shen et al., 2008).

Also in this case, the influence of the enzyme percentage was tested by SDS–PAGE. As shown in Fig. 2B, similar molecular weight distributions than those reported in Fig. 1B were observed.

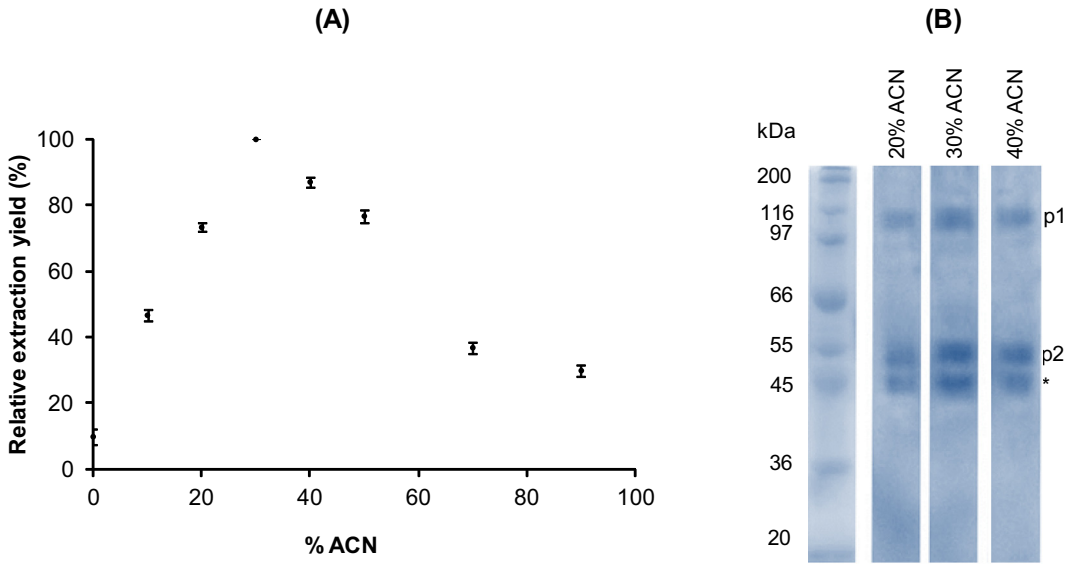

Fig. 1. Influence of ACN percentage on the olive leaf protein extraction monitored by (A) Bradford assay and (B) SDS–PAGE. Band identification: asterisk, 44 kDa (Celluclast® 1.5L); p1, 110 kDa; p2, 55 kDa. The proteins were extracted at 25 °C for 20 min using a Celluclast® 1.5L percentage of 3.5% (v/v) at pH 5.0.

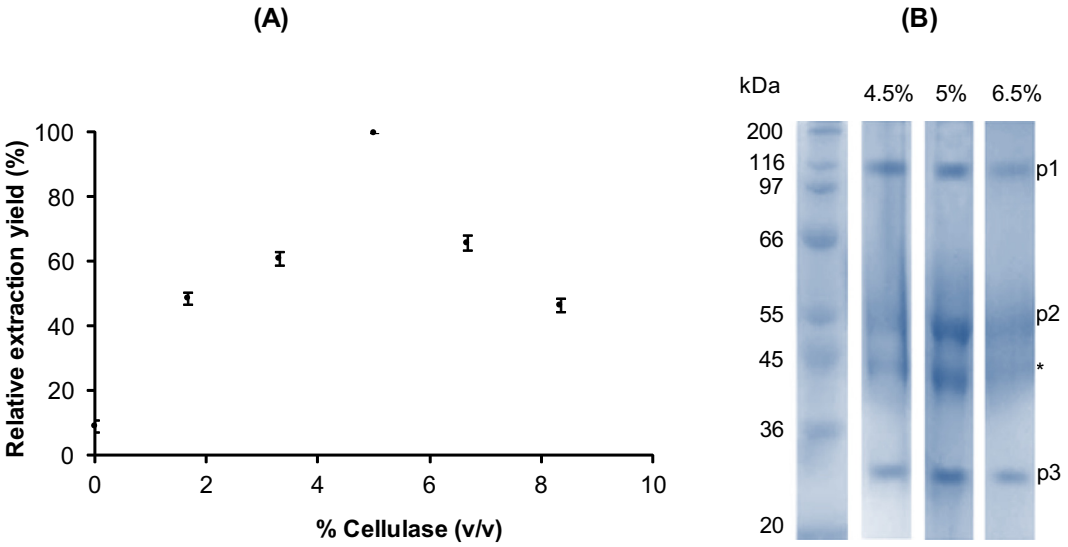

Fig. 2. Influence of Celluclast® 1.5L percentage on the olive leaf protein extraction monitored by (A) Bradford assay and (B) SDS–PAGE. Band identification: p3, 29 kDa; other bands as in Fig. 1. The proteins were extracted at 25 °C for 20 min using 30% ACN at pH 5.0.

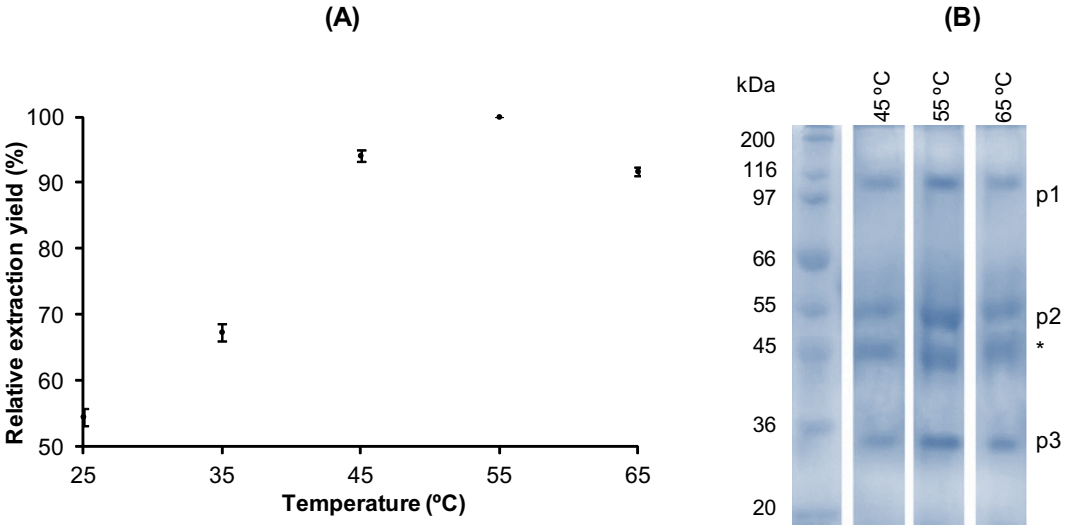

Fig. 3. Influence of temperature on the olive leaf protein extraction monitored by (A) Bradford assay and (B) SDS–PAGE. Band identification as in Fig. 2. The proteins were extracted for 20 min using 30% ACN and 5% (v/v) Celluclast® 1.5L at pH 5.0.

Nevertheless, the bands provided higher intensity and the presence of a protein at 29 kDa was clearly evidenced, especially at 5% (v/v). Consequently, the optimum enzyme concentration was set at 5% (v/v) for further studies.

3.3. Influence of pH, temperature and extraction time on protein extraction

The effect of pH, temperature and extraction time on protein extraction in the selected medium was also investigated. Thus, pH was varied from 4.5 to 6.0, as recommended by the enzyme supplier. Since small differences in the total protein amount were observed along the pH range studied, a pH 5.0 was selected for the following studies (data not shown).

Next, the influence of temperature was tested by varying it from 25 to 65 °C under the previously optimised conditions (30% ACN, 5% (v/v) Celluclast® 1.5L at pH 5.0). As shown in Fig. 3A, temperature had a significant effect on the enzyme's capability in

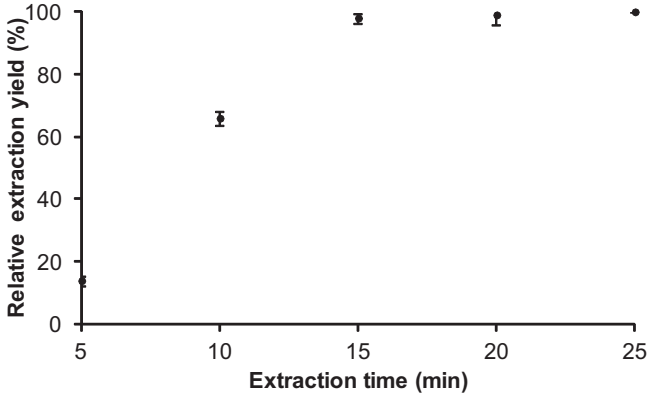

Fig. 4. Influence of extraction time on the olive leaf protein extraction monitored by Bradford assay. The proteins were extracted at 55 °C using 30% ACN and 5% (v/v) Celluclast® 1.5L at pH 5.0.

extracting proteins up to 55 °C, however, higher temperatures led to a decrease in protein extraction, which could be explained by a possible degradation of proteins. These results were consistent with those observed by SDS–PAGE (see Fig. 3B). Thus, 55 °C was selected as the optimum extraction temperature.

Finally, the extraction time was also tested by varying it from 5 to 25 min. As observed in Fig. 4, an increase in the total protein amount was obtained when extraction time was increased up to 15 min, while at higher times (20 and 25 min), the total protein amount obtained remained practically the same. Thus, an extraction time of 15 min was selected as the optimal one for further studies.

3.4. Analysis of olive leaves from different genetic variety

Under the optimal extraction conditions (55 °C for 15 min using 30% ACN and 5% (v/v) Celluclast® 1.5L at pH 5.0), the protein amount of the olive leaf tissues from the different genetic varieties was determined using the standard Bradford assay (see Table 1). The total amount of protein mass on fresh weight basis was comprised between 1.87 mg g^{-1} (Cornicabra variety) and 6.63 mg g^{-1} (Picual variety). A study of repeatability of the recommended extraction protocol from the same tissue was also performed. In all cases, satisfactory RSD values (below 5.2%) were obtained. In addition, these contents were higher (*ca.* 2–3-folds) than those found by other extraction protocols reported for the same genetic varieties (Wang et al., 2003).

Thus, the protein distribution of the different genetic varieties was studied by SDS–PAGE. From the examination of the electrophoretic patterns (Fig. 5), a new protein band at 63 kDa, present in Solà, Hojiblanca and Picual varieties, was identified. This protein was assigned to oleuropein β-glucosidase (Hatzopoulos et al., 2002). The band observed at 110 kDa was observed in Solà, Cornicabra, Hojiblanca and Grosol, being less intense in Grosol variety. On the other hand, the band observed at 29 kDa was only observed in Picual, Arbequina, Manzanilla, Blanqueta and Grosol varieties, being in this case the intensity observed very similar. Finally, the band at 55 kDa was the only one present in all the genetic varieties considered in this study. Thus, the differences observed could be helpful to distinguish the olive leaf tissues according to their genetic variety.

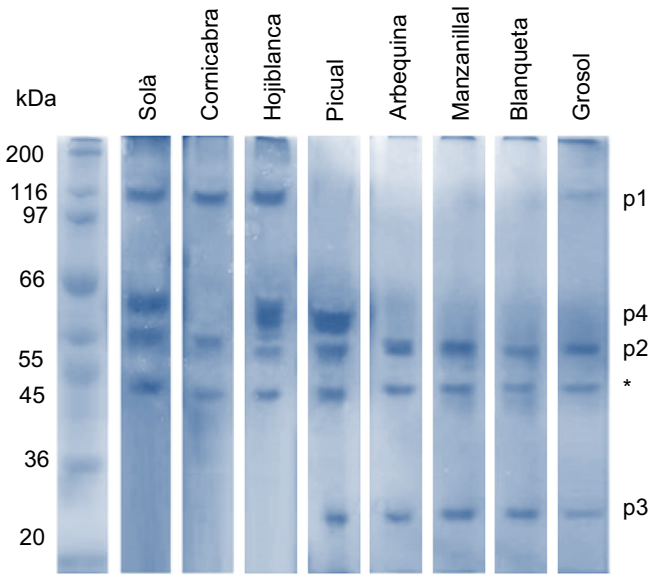

Fig. 5. SDS–PAGE electrophoretic protein profiles obtained from the different olive leaf genetic varieties considered in this study. Band identification: p4, 63 kDa; other bands as in Fig. 2. The proteins were extracted at 55 °C for 15 min using 30% ACN and 5% (v/v) Celluclast® 1.5L at pH 5.0.

4. Conclusions

In conclusion, this work demonstrated that the employment of a Celluclast® 1.5L enzyme is a fast, feasible and effective method to extract proteins from olive leaves. Several parameters affecting Celluclast® 1.5L assisted extraction of proteins were optimised, e.g., organic solvent percentage, Celluclast® 1.5L concentration, pH, and extraction temperature and time. It was found that the use of Celluclast® 1.5L improve the protein extraction, which is due to the breakdown of the cell wall rendering the intracellular materials more accessible for extraction. The mild extraction conditions were environmentally sustainable and faster than other protocols described in the literature. In addition, electrophoretic protein profiles from diverse genetic varieties were evaluated finding differences, which could be useful to classify the olive leaves according to their genetic variety.

Conflict of interest

The authors have declared no conflict of interest.

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