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Antioxidant activity of butterhead lettuce: evaluation of significant factors affecting antioxidant extraction and quantification

Gabriela E. Viacava · Sara I. Roura · María V. Agüero

Abstract This work presents a standardized methodology for both the extraction of antioxidants from butterhead lettuce and the evaluation of the antioxidant capacity through DPPH methodology. The standardized extraction of antioxidants requires processing the fresh lettuce leaves (1 g) for 1 min, mixing with 10 mL of acidified ethanol (0.5 or 1 % citric acid) maintaining this mixture with continuous shaking during 1 h at 2 °C. After that, a centrifugation at 8,000 rpm for 15 min is necessary to obtain a fist supernatant (antioxidant source). A second extraction step with acetone:water (70:30 v/v) improves the extraction yield. Extracts can be stored for 14 days at −20 °C without changes in its antioxidant capacity. For quantification, a mixture 1:4 of lettuce extract and DPPH (100 μM) must be incubated for 60 min at 2 °C in dark before reading at 517 nm. Expression of results in terms of mg ascorbic acid equivalents/100 g fresh weight is recommended in order to compare results from different studies. A kinetic study of the reaction between lettuce antioxidants and DPPH was adjusted to a biphasic first-order kinetic model and showed evidence of the presence of antioxidants of slow and rapid antioxidants in lettuce extracts.

Keywords Lettuce · Antioxidants · Radical scavenging capacity · DPPH

Introduction

During recent years, consumers and professionals from various fields such as medicine, pharmacy, food science and technology have developed a growing interest in antioxidant properties of fruits and vegetables due to their demonstrated health benefits [1, 2]. This trend has been accompanied by the scientific community and this phenomenon is evidenced in the large number of research publications concerning the extraction and quantification of bioactive compounds from vegetable tissues [3–5].

In this context, several in vitro techniques have been developed in order to determine the antioxidant capacity of food matrices [6–8]. Among them, one of the most popular is the method employing the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), which is based on the colorimetric properties of the radical that bears a deep purple color at around 515 nm. When the DPPH reacts with a hydrogen/electron donor (an antioxidant), this radical loses its typical color and the measurement of this change, with an UV–Vis spectrophotometer, allows estimating the ability of a compound or a compound mixture to scavenge free radicals [6].

Although the DPPH method is technically simple and routinely applied in laboratories there are substantial variations in the literature regarding its procedure, including differences in the concentration of DPPH solution, vegetable extract to DPPH ratio, wavelength use for measurement, and expression of results [9, 10]. Moreover, studies using the

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DPPH assay to characterize the antioxidant capacity of extracts obtained from the same vegetable matrix give contradictory results because they use different operational variables such as food sample state (fresh, frozen, freeze-dried) and particle size, solid-to-solvent ratio, time considered as the end-point of reaction, and number of extractions steps, among others. Researches evaluating the antioxidant capacity of leafy vegetables are not an exception to this problem. Among these vegetables, lettuce is of particular interest due to its high consumption level around the world and its phytochemical content with antioxidant properties associated with specific compounds such as caffeic acid and its derivatives, flavonols, vitamins C and E, chlorophyll, and carotenoids [5, 11]. Several researchers have studied the antioxidant capacity of lettuce with the available DPPH methodology. However, they did not make a technical adaptation for this vegetable and used procedures that have been set-up for other vegetables, even fruits. Table 1 summarizes some of the most relevant publications characterizing antioxidant capacity of leafy vegetables, including lettuce, and it details variations in antioxidant extraction procedures as well as in the parameters employed in DPPH assay found in those works.

This study was therefore carried out with the aim of studying the most important operational parameters affecting the DPPH scavenging capacity of butterhead lettuce, with regards to both antioxidant extraction and measurement procedures; and, from these results, to select a protocol to be used in future studies. Additionally, the stability of the antioxidant capacity of lettuce extracts during storage at -20 °C was determined.

Materials and methods

Reagents

2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) was purchased from Sigma-Aldrich Co (St. Louis, Mo., USA). Glacial acetic acid, ethanol, and acetone were provided by Merck (Darmstadt, Germany). Ascorbic acid (AA) and citric acid were obtained from Cicarelli (Santa Fé, Argentina) and were of analytical quality.

DPPH assay parameters

In a first assay, several parameters related to the DPPH assay procedure were studied: wavelength used for measurement, DPPH calibration curve, and expression of results.

Selection of wavelength

An absorption spectrum from 400 to 700 nm of a 100 μM DPPH solution in ethanol was obtained using an UV1601 PC UV–Visible spectrophotometer (Shimadzu Corporation, Japan). The scans were run against ethanol. The wavelength was selected where the maximum absorbance was detected.

DPPH calibration curve

The absorbencies of ethanolic DPPH solutions with different concentrations (0, 33.3, 50, 66.7, 75, 80, and 100 μM) were measured, at the wavelength previously selected. The DPPH calibration curve was obtained by plotting absorbance against DPPH concentration ([DPPH]).

Expression of results

A standard curve with AA was plotted to allow the expression of results in terms of ascorbic acid equivalents (AAE) as suggested Kim et al. [4]. For this purpose, solutions with different AA concentrations (0, 1.5, 3, 4, 5, 6, 8, and 10 mg AA/100 mL) were prepared in ethanol. 0.25 mL of each AA solution or ethanol was mixed with 1 mL of 100 μM DPPH solution and the absorbance was determined at 517 nm when the reactions reached a plateau. The scavenged DPPH ([DPPH]$_{SCAV}$) was calculated as the difference between the initial and the remaining concentration of DPPH, and it was plotted against AA concentration.

Plant material

Heads of butterhead lettuce (*Lactuca sativa* var. Lores) were grown in greenhouses in Sierra de los Padres, Mar del Plata, Argentina. Lettuce heads were harvested after reaching a marketable size, immediately pre-cooled in refrigerated bags and transported to the laboratory. Plants were processed in the first hour after harvest for all experiments. Pulled leaves from three different lettuce heads were used in each experiment.

Kinetic study of the reaction between DPPH and lettuce antioxidants

In a second assay, the reaction kinetics between DPPH radical and lettuce antioxidants extract was studied in order to build a plausible kinetic model and determine its parameters (reaction order and constant). In this sense, a lettuce antioxidant extract was obtained using the methodology suggested by Lemoine et al. [12] with modifications. Briefly, lettuce leaves were cut transversely into 2 cm portions and immediately processed with a kitchen processor. Three samples of 3 g each were mixed with 9 mL of ethanol. Extraction of bioactive compounds was carried out at refrigeration temperature (2 °C) with

Table 1 Summary of some representative publications using the DPPH assay to evaluate antioxidant capacity of leafy vegetables

	References
Sample material	
Freeze-dried	Boo et al. [27], Li et al. [28], Liu et al. [29], Llorach et al. [5]
Frozen lettuce	Khanam et al. [3]
Fresh lettuce	Kang and Saltveit [30], Rajapakse et al. [31], Reyes et al. [32], Rios et al. [33]
Solvent used for extraction	
Ethanol	Kim et al. [4]
Methanol	Kang and Saltveit [30], Reyes et al. [32], Rios et al. [33]
Methanol/water/formic acid	Llorach et al. [5]
Methanol/water/acetic acid	Boo et al. [27], Khanam et al. [3], Li et al. [28]
Acetone/water	Liu et al. [29]
DPPH (μM)	
60	Reyes et al. [32]
80	Llorach et al. [5]
100	Liu et al. [29], Rios et al. [33]
200	Kim et al. [4]
500	Kang and Saltveit [30]
Incubation time of the reaction mixture (min)	
5	Liu et al. [29]
30	Kang and Saltveit [30], Khanam et al. [3], Kim et al. [4]
50	Rios et al. [33]
60	Boo et al. [27], Li et al. [28]
Till steady state	Reyes et al. [32]
Absorbance measurement (nm)	
515	Boo et al. [27], Khanam et al. [3], Li et al. [28], Llorach et al. [5], Reyes et al. [32]
517	Kang and Saltveit [30], Kim et al. [4], Liu et al. [29], Rajapakse et al. [31], Rios et al. [33]
Expression of results	
% Radical scavenging capacity	Kang and Saltveit [30], Liu et al. [29], Rios et al. [33]
Trolox equivalent antioxidant activity (TEAC) (expressed as mg, μg, or μmol of TEAC per g fresh or freeze-dried sample)	Boo et al. [27], Li et al. [28], Llorach et al. [5], Rajapakse et al. [31], Reyes et al. [32]
Ascorbic acid equivalent (AAE) (expressed as mg or μg of AAE per g dry weight)	Khanam et al. [3], Kim et al. [4]

continuous shaking (Orbital Shaker TS-1000, China) for 1 h. After that, samples were centrifuged at 8,000 rpm for 15 min and the supernatant was considered the antioxidant source.

Ethanol (0.25 mL) was mixed with 1 mL of DPPH (100 μM) to determine the initial absorbance of the DPPH solution (A_{DPPH}) at 517 nm. Next, the decrease in the absorbance of a mixture composed by 0.25 mL of lettuce extract and 1 mL of 100 μM DPPH was measured at 0, 1, and every 5 min in the dark and at ambient temperature for 200 min. Blank solutions (without DPPH) were prepared to correct any influence due to lettuce extract color. The wavelength use for measurement and the volumes of DPPH solution and lettuce extract (or ethanol) in the reaction mixture were determined according to the results obtained in the first assay. The absorbance of the sample corrected by the blank (A_S) was used to calculate the remaining DPPH (R_{DPPH}) expressed as a ratio related to the initial DPPH absorbance:

$$R_{DPPH} = A_S/A_{DPPH}. \tag{1}$$

The R_{DPPH} concentration was modeled using a biphasic second order model. Kinetic parameters were adjusted with non-linear least squares routines using SYSTAT 5.0 (SYSTAT Inc. 1992).

Extraction procedure parameters

Once the DPPH assay parameters and the kinetic model were established, a third set of experiments were carried out in order to optimize the experimental conditions of the antioxidant extraction procedure. In this sense, various extraction parameters considered of critical importance were consecutively tested to assess their effect on the lettuce antioxidant capacity measured with DPPH methodology. These parameters were: degree of sample processing, sample state (fresh or frozen), solid:solvent ratio, solvent mixture and number of extractions.

Degree of sample processing

Influence of sample processing on antioxidant extraction was evaluated by grinding lettuce leaves until different degree to obtain different particle sizes. Fresh lettuce leaves were homogenized with a tissue blender for 1 and 3 min. Particle size was measured with a gauge. Three samples of 3 g each from each processing time were added to 9 mL of ethanol for the extraction of bioactive compounds, that was carried out at refrigeration temperature (2 °C) with continuous shaking (Orbital Shaker TS-1000, China) for 1 h. After that, samples were centrifuged at 8,000 rpm for 15 min and the supernatant was considered the antioxidant source. Once lettuce extracts were obtained, antioxidant capacity was evaluated using the DPPH assay parameters set in the previous experiments. The antioxidant capacity was expressed as mg of AAE/100 g fresh weight (FW), using calibration and standard curves.

Sample state

Lettuce leaves were divided into three fractions. Fraction 1 (F1) was immediately homogenized with the tissue blender for 1 min and subjected to the extraction procedure as it was previously described. Fraction 2 (F2) was frozen slowly in a commercial freezer to favor the formation of large ice crystals [13]. The third fraction (F3) was first processed with the tissue blender and then 3 g of the lettuce homogenate were weighed and frozen slowly. F2 and F3 were stored for 24 h at −20 °C. After this period, the two frozen fractions were thawed and F2 was crushed with the tissue blender. F2 and F3 were subjected to the same extraction procedure as F1 and the obtained extracts were used to determine the DPPH scavenging capacity.

Solid:solvent ratio

Fresh lettuce leaves (1 g) were crushed with the tissue blender for 1 min and mixed with different volumes of ethanol (3, 5, 10, 20, and 50 mL) to obtain different solid:solvent ratios ranging between 0.02 and 0.33 g/mL. The extraction and quantification procedure continued as mentioned earlier.

Concentration of ethanol in the solvent and mixture with organic acids

Ten different solvent systems were examined for the extraction of antioxidants from lettuce:

1. Pure ethanol.
2. Ethanol:water (50:50 v/v).
3. Pure ethanol with 0.5 % acetic acid (w/v).
4. Ethanol:water (50:50 v/v) with 0.5 % acetic acid (w/v).
5. Pure ethanol with 1 % acetic acid (w/v).
6. Ethanol:water (50:50 v/v) with 1 % acetic acid (w/v).
7. Pure ethanol with 0.5 % citric acid (w/v).
8. Ethanol:water (50:50 v/v) with 0.5 % citric acid (w/v).
9. Pure ethanol with 1 % citric acid (w/v).
10. Ethanol:water (50:50 v/v) with 1 % citric acid (w/v).

The extraction and quantification procedure continued as described earlier. The extract obtained with the solvent system that allowed obtaining the highest antioxidant capacity was subjected to a second extraction step. Therefore, the supernatant was collected and the precipitate was extracted again under the same time and temperature conditions with 10 mL of two different solvent systems:

1. Pure ethanol with 1 % citric acid (w/v).
2. Acetone:water (70:30 v/v).

Both supernatants were combined and further used for antioxidant capacity determinations.

Stability of the frozen lettuce extract

In a fourth assay, lettuce extracts obtained using the extraction parameters fixed in the previous sections were stored at −20 °C to test the stability of the antioxidants extract. Antioxidant capacity by the DPPH method was performed at 0, 1, 6, and 14 days of storage.

Statistical analysis

Results reported in this paper, are means (obtained by least squares method) of at least three independent experiments together with their standard deviations.

Data were analyzed using R, software version 2.12 (R Development Core Team v2.12 2011). Probability level was fixed to $P < 0.05$.

Results and discussion

DPPH assay parameters

The spectrum of the 100 μM ethanolic DPPH solution yielded a maximum peak at 517 nm. Therefore, this wavelength was selected for use in the subsequent assays. This value is in agreement with other studies on lettuce where 515 and 517 nm were the two working wavelengths most frequently used for measurements (Table 1).

The calibration curve of DPPH solution obtained at 517 nm presented a linear dependency between absorbance and concentration and was fitted by linear regression ($R^2 = 0.9991$):

$$Abs_{517\,nm} = 0.0117 * [DPPH] + 0.0086. \tag{2}$$

Brand-Williams et al. [6] found a similar trend at 515 nm expressing the concentration of DPPH as mol/L instead of μmol/L.

Different lettuce extract to DPPH solution ratios are applied by researchers when applying this antioxidant capacity assay (Table 1). A 100 μM DPPH solution and a volumetric ratio of 1:4 (lettuce extract:DPPH solution) was selected in our work. Is important to note that even though a 100 μM DPPH solution would give absorbances higher than 1 (Eq. 2), a dilution effect occurs when DPPH solution is mixed, in the established ratio, with antioxidant extract in the cuvette. Therefore, the initial concentration of DPPH results lower than 100 μM, resulting in a value of absorbance lower than 1 (Eq. 2), as it is recommended for spectrophotometric determinations to ensure the accuracy and linearity of the measurements.

Therefore, in our study 100 μM DPPH solution and a reaction mixture containing 4 mL of this solution and 1 mL of the lettuce extract (or ethanol) were chosen as appropriate parameters for concentration and proportions in reaction mixture.

One of the most common way used for the expression of results from DPPH assays is through percentage of radical scavenging capacity (%RSC), that represents the percentage of inhibition of the DPPH achieved in the assay, and is obtained by the difference between the change in absorbance caused by a blank and by the sample. Among studies reported in Table 1, several presented their results in terms of %RSC. Although this inhibition percentage is easy to calculate, it depends on the initial concentration of DPPH solution and on the concentration of the extract sample, and then, it is not completely correct to compare studies that use different initial values in these parameters [14].

Brand-Williams et al. [6] expressed antioxidant capacity results using the EC_{50} parameter, defined as the sample concentration needed to scavenge 50 % of the original radical concentration. Sánchez-Moreno et al. [8] also introduced kinetic parameters to express antioxidant capacity results and defined the tEC_{50} as the time taken by EC_{50} concentration to reach equilibrium. These authors combined then both parameters (EC_{50} and tEC_{50}) and defined the "antiradical efficiency" as $AE = 1/(EC_{50} \times tEC_{50})$. Although AE values are useful because it combines both factors affecting the antiradical capacity of the sample (concentration of antioxidant extract and reaction time), its evaluation demands skilled labor and laboratory resources since it requires the testing of different sample concentrations. It is also more time-consuming than simple determinations at a fixed end-point since measurements must be made until a steady state is reached, with the drawback that the time needed for one assay will depend on each sample [14].

Some authors express their results in terms of Trolox equivalent activity (TEAC). This methodology, together with %RSC, is one of the most used forms to express results of DPPH assay (Table 1). However, ascorbic acid is a more suitable standard for food matrices as it is a common antioxidant present in vegetables [3, 4, 6, 8]. Therefore, our propose is to use this compound as standard to express DPPH assay results. Figure 1 presents the standard curve obtained for ascorbic acid which was fitted by linear regression ($R^2 = 0.9696$). The application of this equation allows presenting the DPPH scavenging capacity of lettuce in terms of mg AAE/100 g FW.

Kinetic study of the reaction among DPPH and lettuce antioxidants

The reaction between the DPPH radical and an antioxidant is a kinetics-driven process that depends on the nature of the antioxidant [6]. In the present study, an attempt was made to characterize the kinetic behavior of DPPH disappearance with the antioxidants present in lettuce extracts. Figure 2 shows the evolution of R_{DPPH} during the reaction. As it can be observed, an exponential behavior was found and R_{DPPH} presented a sharp initial decrease, but the reaction did not reach a steady state and persisted over time. Accordingly, a biphasic first-order model was proposed to mathematically characterize these results:

$$R_{DPPH\bullet} = f_S e^{-k_S t} + f_R e^{-k_R t}, \tag{3}$$

where R_{DPPH} is the remaining DPPH, f_S and f_R are the fractions of slow and rapid antioxidants, respectively ($f_S + f_R = 1$); k_S and k_R are the specific rates of DPPH scavenging capacity (min^{-1}) due to compounds that react slowly or rapidly with DPPH, respectively. According to this model, lettuce extract is composed by two fractions of antioxidants with different kinetic parameters. One fraction has a lower capacity to scavenge DPPH radical, and the other reacts faster with this radical. The estimated model
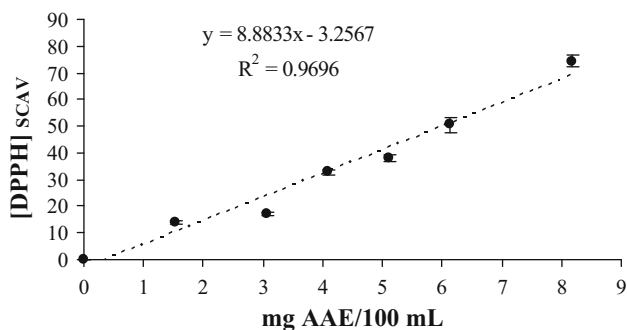
Fig. 1 Standard curve of ascorbic acid (AA) concentration and scavenged DPPH ([DPPH]$_{SCAV}$). *Bars* represent the standard error of the mean. The *dotted line* represents the fitting of the model to the data
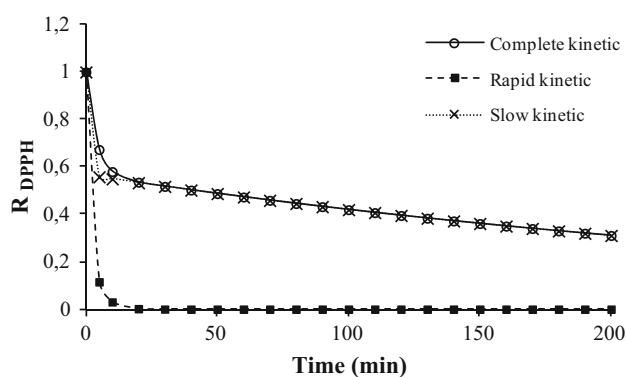

Fig. 2 Biphasic first-order kinetic model described by Eq. (3) and evolution of DPPH concentration during reaction with lettuce antioxidants of slow and rapid action

parameters were $f_S = 0.566$, $k_S = 0.003$, $f_R = 0.434$ and $k_R = 0.265$ ($R^2 = 0.904$). The evolution of R_{DPPH} due to the action of the two antioxidant fractions predicted by this model is also shown in Fig. 2. These results are in consonance with the fact that lettuce heads present a large diversity of antioxidants with different structural conformation. On the one hand, lettuce contains ascorbic acid, which reacts rapidly with the DPPH. On the other hand, phenolic acids are present in lettuce, the dominant ones being chicoric, chlorogenic, and caffeic acids [15], which react quite slowly with the DPPH [6].

In order to verify that the slow kinetic behavior was actually due to the effect of antioxidants of slow action and not to a decomposition of the DPPH radical, the stability of the DPPH alone was studied. For this purpose, 0.25 mL of ethanol was added to 1 mL of 100 μM DPPH solution and the absorbance was measured at 0, 1, and every 5 min in the dark and at ambient temperature for 200 min (data not shown). A slight decrease in the absorbance was found with a rate of 0.0001 min^{-1}. This value is negligible compared to that of slow action antioxidants ($k_S = 0.003$),

confirming that these compounds are the ones responsible for the slow kinetic behavior.

Although the reaction did not reach a steady state, it was considered that most of the DPPH scavenging had occurred at 60 min; therefore, this time was suitable for the determination of the antiradical capacity of lettuce. Some studies performed on lettuce used very short reaction times (as low as 5 min) with a consequent sub-estimation of its antioxidant capacity. Our findings support the idea that kinetic scans and characterization must be performed when a new sample is studied in order to establish the time required to finish the reaction.

Extraction procedure

All experiments carried out in this section used the DPPH assay parameters settled in the previous assays: 517 nm for measuring wavelength, 100 μM DPPH solution, volumetric ratio 1:4 of lettuce extract:DPPH solution, 60 min of contact between lettuce extract and DPPH, and expression of results as mg AAE/100 g FW. It is important to mention that temperature during the extraction process was not considered as a factor and was maintained at 2 °C since it is known that higher temperatures may degrade antioxidant compounds [13].

Degree of sample processing

After 3 min of processing, a lettuce homogenate with a more disaggregated tissue and an average diameter of 1.53 ± 0.64 mm was obtained in comparison with 1 min (2.47 ± 1.06 mm). However, this difference did not affect the DPPH scavenging capacity of the lettuce extracts showing no significant differences in values between samples obtained with each processing time, with mean values of 21.85 ± 4.18 and 23.93 ± 5.24 mg AAE/100 g FW for 1 and 3 min, respectively. It is known that reduction of particle size is required to facilitate the extraction process. This operation breaks up certain structures of the food matrix, releasing bound antioxidants and reducing the distance the analyte has to travel to reach the surface. At the same time, enlargement of the particle surface improves solvent penetration [13]. However, when this treatment is excessive, friction between particles may increase the sample temperature, reducing the stability of the antioxidant compounds and consequently favoring its degradation. Additionally, when the material is more finely milled, it is more unstable, since the breakage of cell walls may expose phenolic compounds (which exhibit antioxidant activity) to the action of the enzyme polyphenol oxidase (PPO) that degrades phenolics [16]. After 1 min of processing the bulk sample temperature rose, on average 1.2 °C, while after 3 min this increase was of 2.6 °C. Such

increase in temperature was not enough to assume that the PPO was more active after 3 min of processing. Therefore, in order to preserve the stability of the lettuce antioxidants and taking into account that both analyzed times provided the same result, it was considered as appropriate to choose 1 min of sample processing.

Sample state

Freezing samples prior to extraction could be advisable since ice crystals produce lesions in the cell structure facilitating the release of cellular components and enhancing the extraction process [13]. Table 2 presents results obtained for extraction of fresh and frozen samples. Significant differences ($P < 0.05$) were found in the antioxidant capacity obtained, but contrary to expectations, both frozen fractions (F2 and F3) exhibited lower radical scavenging capacity than that found for fresh material (F1), being antioxidant activity of F2 and F3, 2.7 and 2.1 lower than that of F1, respectively. The larger cell disruption caused by slow freezing, may put in contact phenolic compounds with the enzyme PPO during sample thawing. Therefore, losses of phenolics and, consequently, a lower antiradical activity can be derived from enzymatic reactions. Moreover, these losses could also be attributed to physical phenomena like changes in solubility of phenolics and ionic charge due to osmotic imbalance during freezing. These changes could affect the structure of the bioactive compounds and diminish their antioxidant action.

Some authors reported that freezing the samples in liquid nitrogen did not reduce significantly the DPPH scavenging capacity of different plant materials [17, 18]. However, Sikora et al. [19] observed that the freezing

process at a temperature of -22 °C decreased the antioxidant activity in kale, green cauliflower, and Brussels sprouts. In this way, the effect of freezing on the antioxidant capacity of vegetables is plant species-dependent and is influenced by the freezing process conditions. According to our results, it is recommended to use fresh material to perform the extraction of antioxidants from lettuce.

Solid:solvent ratio

The concentration gradient of antioxidants between the vegetable matrix and the bulk of the solvent is the driving force of mass transfer for the extraction and is greater when the solvent-to-solid ratio is higher [20]. Although this parameter has been previously discussed by other authors [17, 21], no studies were performed on lettuce and it is important to find an optimal ratio for lettuce samples that increases the extraction efficiency of antioxidants at a relatively low cost. Solid:solvent ratio studied in the present research were selected covering different values found in the literature for lettuce [3, 15, 22, 23].

Figure 3 shows the influence of solid:solvent ratio on the extraction of lettuce antioxidants. As it can be observed in Fig. 3, the higher the solvent-to-solid ratio, the higher the antioxidant capacity. In fact, the DPPH scavenging capacity obtained when 50 mL of solvent was used (73.77 ± 9.53 mg AAE/100 g FW) was the highest among the volumes tested and proved to be significantly different ($P < 0.05$) from the others. No significant differences were detected between 10 and 20 mL of solvent (43.96 ± 6.55 and 50.12 ± 4.64 mg AAE/100 g FW, respectively) and between 3 and 5 mL (25.13 ± 2.44 and 33.26 ± 2.40 mg AAE/100 g FW, respectively).

As a compromise between extraction yield and cost, it was considered as appropriate to use 10 mL of extraction solvent per 1 g of fresh lettuce. Greater solvent volume would increase significantly the cost of the assay, while with a minor solvent volume the mass transfer would be inefficient.

Solvent mixture for antioxidant extraction and number of extraction steps

Methanol, ethanol, and acetone or mixtures of them with water are the solvents most widely used for the recovery of antioxidants of diverse structures from foods [21]. Although methanol is the most widely employed solvent, several studies have reported good extraction yields with ethanol [24, 25]. In this research ethanol was used, avoiding the use of methanol due to its toxicity. Additionally, as one of the major groups of antioxidants in lettuce is constituted of phenolic acids, the solvent acidification may improve the solubility and extractability of

Table 2 DPPH scavenging capacity of lettuce extracts with regards to sample state and extraction steps

Experiment	DPPH (mg AAE/100 g FW)
Sample state	
F1 (fresh sample)	24.90 ± 2.54[a]
F2 (first frozen and then homogenized)	9.07 ± 0.99[c]
F3 (first homogenized and then frozen)	11.75 ± 1.52[b]
Extraction steps	
1 step with pure ethanol with citric acid (1 %) as extractor solvent	72.47 ± 4.61[c]
2 steps with pure ethanol with citric acid (1 %) as second extractor solvent	99.59 ± 3.24[b]
2 steps with acetone:water (70:30 v/v) as second extractor solvent	122.29 ± 7.54[a]

[a,b,c] Comparison within the column. Mean values with different lower case letters within the same column and experiment are significantly different ($P < 0.05$)
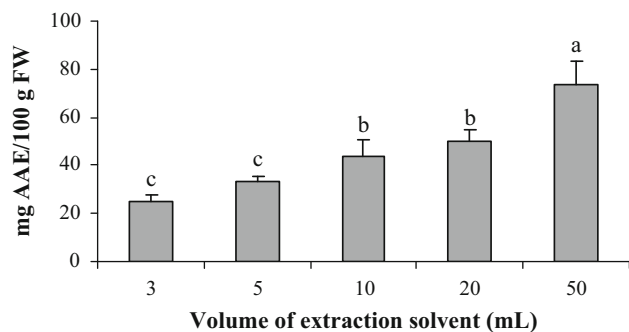
Fig. 3 DPPH scavenging capacity of lettuce using *five* different solvent volumes of pure ethanol per gram of fresh lettuce: 3, 5, 10, 20, and 50 mL. Significant differences as determined by the Tukey–Kramer test are indicated by *different letters*. *Bars* represent the standard error of the mean
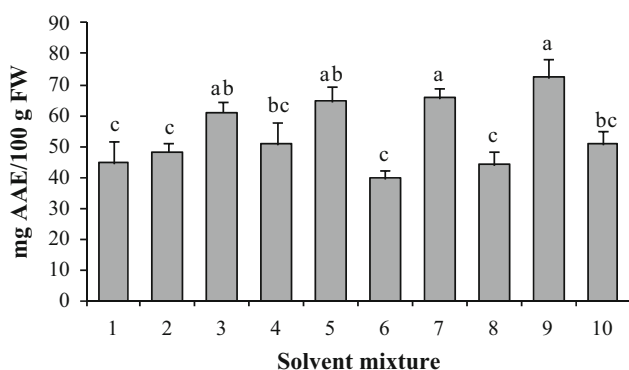

Fig. 4 DPPH scavenging capacity of lettuce using ten different solvent mixtures: *1* ethanol, *2* ethanol:water (50:50 v/v), *3* ethanol with 0.5 % acetic acid (w/v), *4* ethanol:water (50:50 v/v) with 0.5 % acetic acid (w/v), *5* ethanol with 1 % acetic acid (w/v), *6* ethanol:water (50:50 v/v) with 1 % acetic acid (w/v), *7* ethanol with 0.5 % citric acid (w/v), *8* ethanol:water (50:50 v/v) with 0.5 % citric acid (w/v), *9* ethanol with 1 % citric acid (w/v), *10* ethanol:water (50:50 v/v) with 1 % citric acid (w/v). Significant differences as determined by the Tukey–Kramer test are indicated by *different letters*. *Bars* represent the standard error of the mean

these compounds. The decrease in the pH of the medium due to acidification may also preserve phenolic compounds from oxidation by PPO. The acids employed are among those which naturally occur in tissues, particularly citric and acetic acids [13].

Figure 4 shows the results obtained with the different extraction solvent mixtures. The highest antioxidant capacity was attained when ethanol acidified with 0.5 and 1 % citric acid was used (65.62 ± 3.26 and 72.47 ± 5.61 mg AAE/100 g FW, respectively), followed by ethanol acidified with 0.5 and 1 % acetic acid (60.84 ± 3.40 and 64.68 ± 4.67 mg AAE/100 g FW, respectively). Citric acid possesses a double inhibitory effect on PPO, not only by lowering the pH, but also by chelating with the copper moiety of

the enzyme, which is necessary to enable the reaction catalyzed by this enzyme [26]. This effect, in conjunction with the polarity of the ethanol and the solubility of the phenolic acids in this mixture may be responsible for the higher results obtained using ethanol acidified with citric acid.

The presence of water in the acidified solvents had a negative effect since it presented lower DPPH scavenging values when comparing with the same acidified matrix in pure ethanol. Our observations are consistent with those of Tabart et al. [18] who obtained lower results with water alone than with organic solvents for extraction of antioxidants from black currant leaves and buds.

In view of the results, ethanol acidified with citric acid (1 %) was chosen as the best extractor solvent in the first extraction step. A second extraction step increased the values obtained of antioxidant capacity with respect to only one extraction cycle (Table 2). However, the efficiency of this second extraction was better when acetone:water (70:30 v/v) was used, since it increased the antioxidant capacity by 68.8 % in contrast to 37.4 % when using ethanol with citric acid (1 %). As mentioned Pérez-Jiménez et al. [14], a procedure for the extraction of antioxidants from plant foods should combine at least two extraction cycles performed with aqueous-organic solvents with different polarities in order to extract antioxidant compounds with different chemical structures.

Stability of the frozen lettuce extracts

No significant differences were detected in the DPPH scavenging capacity of the lettuce extracts as a function of storage days, with an average value of 115.61 ± 7.32 mg AAE/100 g FW. These results indicate that lettuce extracts were stable until day 14 under the conditions used in this study. Michiels et al. [17] also studied the effect of frozen storage (-20 °C) on the antioxidant activity of liquid extracts of apple, orange, broccoli, and leek, and they found that these extracts were stable up to 7 days of storage.

Summarizing, the method proposed for the determination of the DPPH scavenging capacity in lettuce consists in a solid–liquid extraction of 1 g of fresh sample material (processed for 1 min in a tissue blender) with 10 mL of ethanol acidified with 0.5 or 1 % citric acid. A second extraction step with acetone:water (70:30 v/v) with the same solid:solvent ratio improves extraction yield and the extract obtained is stable during 14 days of storage at -20 °C. For measurement, specific operating parameters of the DPPH technique are recommended to be settled in: concentration of DPPH solution: 100 μM, lettuce extract:DPPH volume relation: 1:4 v/v, incubation time: 60 min, wavelength use for measurement: 517 nm, and expression of results as mg AAE/100 g FW. Using all these adjusted parameters, values

of DPPH scavenging capacity obtained in this work (122.29 ± 7.54 mg AAE/100 g FW) were considerably higher than those reported by Khanam et al. [3] also for lettuce (11.56 μg AAE/g dry weight which is approximately equivalent to 0.06 mg AAE/100 g FW). These results show the effectiveness of the proposed methodology for the extraction of antioxidants from lettuce plants and the measurement of its DPPH scavenging capacity, which allows getting a closer approach to the estimation of the true antioxidant properties of this worldwide consumed vegetable.

Conclusions

The DPPH method is routinely utilized for the assessment of antiradical properties of fruits and vegetables. However, different protocols for extraction and measurement are used, which influences the results and obstructs the comparison of values from equivalent products. Therefore, the importance of sample preparation and quantification procedures in analytical methods should not be undervalued. All the parameters tested in this paper are crucial for antioxidant extraction and DPPH scavenging capacity determination of lettuce. This work highlights the necessity of standardizing extraction of antioxidants and DPPH method for each vegetable; it also reveals the complexity of an adequate estimation of antioxidant capacity for different plant sources.

Conflict of interest The authors of this work declare that there is no conflict of interest with any people or organization.

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