Phenolic profile evolution of different ready-to-eat baby-leaf vegetables during storage

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ABSTRACT

Ready-to-eat baby-leaf vegetables market has been growing and offering to consumers convenient, healthy and appealing products, which may contain interesting bioactive compounds. In this work, the composition and the evolution of the phenolic compounds from different baby-leaf vegetables during refrigerated storage was studied. The phenolic compounds were extracted using pressurized liquid extraction (PLE) and the phenolic profile of each sample was analysed and quantified by using LC-MS and LC-DAD methods, respectively, at the beginning and at the end of a 10-day storage period. The baby-leaf vegetables studied included green lettuce, ruby red lettuce, swiss chard, spinach, pea shoots, watercress, garden cress, mizuna, red mustard, wild rocket and spearmint samples and a total of 203 phenolic compounds were tentatively identified and quantified. The main naturally phenolic compounds identified correspond to glycosylated flavonoids, with exception of green lettuce and spearmint leaves which had a higher content of hydroxycinnamic acids. Quantification of the main compounds showed a 10-fold higher content of total phenolic content of ruby red lettuce (483 mg g^{-1}) in relation to the other samples, being the lowest values found in the garden cress (12.8 mg g^{-1}) and wild rocket leaves (8.1 mg g^{-1}). The total phenolic content only showed a significant change ($p < 0.05$) after storage in the green lettuce (+17.5%), mizuna (+7.8%), red mustard (-23.7%) and spearmint (-13.8%) leaves. Within the different classes of phenolic compounds monitored, the flavonols showed more stable contents than the hydroxycinnamic and hydroxybenzoic acids, although the behaviour of each compound varied strongly among samples.

Keywords: Baby-leaf vegetables; PLE extraction; HPLC-DAD-MS; Phenolic compounds identification and quantification; Storage

45 **Highlights**

46 - A pressurized liquid extraction method was optimized and applied to the extraction of
47 phenolic compounds of 11 baby-leaf samples.

48 - An HPLC-DAD-MS method was developed to identify and quantify different phenolic
49 compounds.

50 - Comparison of the levels of phenolic compounds was made between the beginning
51 and end of storage, showing small difference between sampling days.

52 - Flavonols were the most stable compounds during refrigerated storage.

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1. INTRODUCTION

The consumer demand for more convenient fresh food products led to a rapid growth of the fresh-cut industry, that became a multi-billion dollar sector worldwide in the last years. Fresh-cut vegetables can meet the consumer demands about the relationship between food, healthy lifestyle and convenience [1]. They are elaborated without additives, by minimal processing methods such as washing, cutting and packaging at chilling temperatures with polymeric films. Baby leaf salads have gained popularity over the traditional fresh-cut salads, by adding more variety to the diet and offering a product that attracts consumers and producers. The baby leaves are mixed, washed and packaged as whole, maintaining an appealing 3-D structure, reduced oxidation damage due to a small stem diameter and greater stability during shelf life [2-4]. Lettuces, rocket, watercress, spinach, and mustard greens are among the most used baby leaves, being sold individually or in salad mixtures [3].

The consumption of fresh vegetables is encouraged, not only due to their micronutrient composition (normally rich in vitamins and minerals), but also due to their phytochemicals, that are believed to protect human health [5]. Some antioxidant, anti-inflammatory and antitumor effects have been attributed to certain phytochemicals, that are also related to the vegetable color and flavor [5,6]. Within the European Union there is no specific regulation related to the presence of phytochemicals, but any nutrition and/or health claim made on the labels must be based on scientific studies that take into consideration the composition of phytochemicals and their qualitative and quantitative characteristics [5,7]. The antioxidant properties of the vegetables are one of the most present label claims due to the high levels of carotenoids, tocopherols and ascorbic acid that have epidemiological evidence of benefiting human health [6]. In the other hand, the antioxidant properties of vegetable intake are also closely related to the presence of

phenolic compounds. These are secondary metabolites of the plants, characterized by having at least one aromatic ring with one or more hydroxyl groups [6,8]. Polyphenols can range from simple molecules (phenolic acids) to more complex structures (e.g., phenylpropanoids or flavonoids) or even highly polymerized compounds (such as lignins or tannins), with flavonoids representing the most common and widely distributed sub-group [9]. Moreover, different types and numbers of sugars and functional derivatives such as esters or methyl esters can be conjugated to aglycones, forming numerous structures of phytochemicals, being described more than 8000 natural phenolic compounds [6,8,10]. The phenolic content of a plant is affected by several factors like plant species, cultivar, environmental conditions, water availability, light exposure, germination, maturity, processing and storage [5,11]. In minimally processed fresh-cut products, the shredding step can increase the antioxidant capacity associated with wound-induced phenolic compounds [12]. Reyes, et al. [13] described major changes in the total soluble phenolic content during the storage of fresh-cut vegetables, influenced by the initial levels of reduced ascorbic acid and phenolic compounds. Also light exposure and temperature of storage can induced the synthesis of certain phenolic compounds.

The importance of phenolic compounds as potential antioxidants and their complex chemical structure, variability and distribution creates a challenge to properly assess their content in food products. Traditionally, techniques to extract phenolic compounds from fresh or freeze-dried vegetables use large amounts of hydro-organic solvent mixtures [14] and are normally very laborious, time-consuming and not very selective. Pressurized Liquid Extraction (PLE) has been shown to be a more environment friendly alternative to extract bioactive compounds from a vegetal matrix [15]. PLE combines elevated temperature and pressure with the use of minimum amounts of food-grade

solvents to achieve a fast and efficient extraction of several compounds, while preserving their bioactivity and chemical structure. A better diffusion of the solvent into the matrix is obtained by maintaining the pressures and temperatures below the critical point of the solvents, due to a higher solubility of the analytes in the solvent and to the decrease of solvent viscosity and surface tension [15]. PLE has been successfully applied to the extraction of phenolic compounds from vegetable matrixes, showing high yields, better recoveries, being more time efficient and economic when compared to the traditional methods [14,16,17].

As the popularity of ready-to-eat baby-leaf vegetables increases, there is an urgent need to understand how the profile of important components of these more immature vegetables evolves during storage. In this sense, it has been already demonstrated how some fat- and water-soluble free vitamin losses may be produced during refrigerated storage of these products [18]. The purpose of this work was to study the evolution of the phenolic compounds of a wide group of ready-to-eat baby-leaf vegetables during storage, including green lettuce, ruby red lettuce, swiss chard, spinach, watercress, garden cress, mizuna, red mustard, wild rocket, peashoots and spearmint. To do that, a PLE method was optimized to extract the phenolic compounds from these vegetables and the obtained extracts were analyzed by HPLC–DAD–MS. The information available so far focuses more on the identification of the phenolic compounds present in these baby leaves [19-22]. Moreover, there are only a few publications on the changes of the phenolic compound during the storage of baby-leaf samples, mainly for spinach leaves [23-25], wild rocket [26] and lettuces [27].

2. MATERIALS AND METHODS

2.1. Chemicals and standard solutions

Methanol was of HPLC-grade and acquired from LabScan (Dublin, Ireland) whereas ethanol was purchased from Scharlab (Barcelona, Spain). Folin–Ciocalteu phenol reagent and sodium carbonate (Na_2CO_3) were acquired from Merck (Darmstadt, Germany) and the water used was Milli-Q Water (Millipore, Billerica, MA, USA). Formic acid, gallic acid, 4- hydroxybenzoic acid, sinapic acid, syringic acid, caffeic acid, chlorogenic acid, p-coumaric acid, ferulic acid, quercetin, kaempferol and catechin were supplied by Sigma–Aldrich (Madrid, Spain). The others phenolic standards, i.e. vanillic acid, rosmarinic acid, quercetin-3-rutinoside, quercetin-3-rhamnoside, quercetin-3-glucoside, quercetin-3-galactoside, luteolin-7-glucoside, apigenin-7-glucoside, kaempferol-3-glucoside, apigenin and diosmetin were acquired from Extrasynthese (Genay, France).

Individual phenolic standard solutions were prepared in 70% MeOH solution with the following concentrations: 0.2 mg mL^{-1} for caffeic acid, p- coumaric acid, quercetin, apigenin, apigenin-7- glucoside and kaempferol-3-glucoside; 0.5 mg mL^{-1} for syringic acid; 0.7 mg mL^{-1} for luteolin-7-glucoside, kaempferol and diosmetin; 1.0 mg mL^{-1} for sinapinic acid, rosmarinic acid, salicylic acid, p-hydroxybenzoic acid, vanillic acid, gallic acid, quercetin-3- rhamnoside, quercetin-3-rutinoside and catechin; and 1.4 mg mL^{-1} for chlorogenic acid, ferulic acid, quercetin-3-galactoside and quercetin-3-0-glucoside. All standard solutions were kept under refrigeration at 4°C until analysis.. During development of HPLC-DAD-MS method, a mixture of phenolic standards was prepared by dilution of the individual phenolic stock solutions with 70% MeOH solution.

2.2. Samples

Minimally processed baby leaf vegetables, washed and packaged, were supplied by a producer (Odemira, Portugal) of minimally processed vegetables. The samples were received in the laboratory one day after being processed, individually packaged and in the same conditions normally used for distribution and commercialization of fresh-cut products. The samples comprised 4 of the most common baby leaves used in ready-to-eat salads, namely, green lettuce, ruby red lettuce, swiss chard and spinach, 5 baby-leaf vegetables from the Brassicaceae family characterized by their peppery flavor, namely, watercress, garden cress, mizuna, red mustard and wild rocket, a baby-leaf recently introduced in salad mixtures, pea shoots, and a fresh-cut aromatic herb that can be added to ready-to-eat salads, such as spearmint. Each sample was divided into two batches, corresponding to the two sampling times studied (day 1 and day 10). After sampling, the baby-leaf samples were freeze-dried (Telstar Cryodos-80, Terrassa, Spain) until analysis. The ten-day refrigerated storage (3 ± 1 °C) period was monitored with an EL-USB 2 (Lascar Electronics, Salisbury, UK). The freeze-dried leaves were reduced to a fine powder in a knife mill (GM 200, Retsch, Haan, Germany) and stored protected from light, oxygen and high temperatures. This procedure intended to exclude individual differences and ensure the representativeness of the test sample.

2.3. Pressurized Liquid Extraction (PLE)

The extractions were performed using an accelerated solvent extraction system (ASE 200, Dionex, Sunnyvale, CA) equipped with a solvent controller. The PLE method was first optimized using pea shoots and spearmint as test samples. Methanol/water and ethanol/water mixtures in three different proportions (50%, 70% and 90%) were the solvents tested to extract phenolic compounds from freeze dried vegetable leaves. The extraction temperature (70°C), pressure (10 MPa), flush volume (60% of cell volume

using extraction solvent), sample quantity (0.5 g) and dispersion (2 g of sea sand) were maintained constant during this study. The static extraction time was of 20 min, including an additional 5 min heat-up step prior to any extraction. After choosing the solvents, different durations for the static extraction and number of cycles of extraction were tested in 5 different combinations: i) 10 min $\times$ 1 extraction cycle; ii) 20 min $\times$ 1 extraction cycle; iii) 30 min $\times$ 1 extraction cycle; iv) 5 min $\times$ 2 extraction cycles; v) 10 min $\times$ 2 extraction cycles. The extraction procedure was performed as previously described [16]. All the extractions were done using 11 mL extraction cells and, between extractions, a rinse of the complete system was performed to avoid any carry-over. The extracts were protected from light and stored under refrigeration until dried. A Rotavapor R-210 (Büchi, Labortechnik AG, Flawil, Switzerland) was used to evaporate the organic solvents from the extracts. The remaining aqueous phase was freeze-dried (Labconco Corporation, Missouri, USA) to obtain a completely dried extract. Dried extracts were re-dissolved in 70% MeOH (5 mg mL⁻¹) and filtered through a 0.45 μ m disposable syringe filter. All extractions were made by duplicate.

2.4. Determination of total phenols content (Folin–Ciocalteu method)

The total phenols content of the different PLE extracts was determined by using the Folin–Ciocalteu assay [28] with some small changes [29], and was expressed as mg gallic acid equivalents/g dry extract. Briefly, 10 μ L of sample (5 mg mL⁻¹ in 70% MeOH) and 50 μ L of undiluted Folin–Ciocalteu reagent were added to 600 μ L of water. After 1 minute, 150 μ L of 20% (w/v) Na₂CO₃ was added to the mixture and, finally, the volume was made up to 1 mL with deionized water. This reaction mixture was incubated for 2 h at 25 °C and then, 300 μ L were transferred to a well of a 96-well microplate. Absorbance was measured at 760 nm in a microplate spectrophotometer

reader Powerwave XS (BioTek Instruments, Winooski, VT) and compared to a gallic acid calibration curve (linear range: 0.015–1 mg/mL, $R^2 > 0.997$).

2.5. Analysis of phenolic compounds by HPLC-DAD-MS

The phenolic compounds present in the PLE extracts of the baby-leaf samples were analyzed by HPLC-DAD-ESI-MSⁿ on an Agilent 1200 liquid chromatograph (Agilent, Santa Clara, CA, USA) equipped with an autosampler, a DAD, and directly coupled to an ion trap mass spectrometer (Agilent ion trap 6320) via an electrospray interface. The column used was a Zorbax Eclipse XBD C₁₈ (5 μ m, 150×4.6 mm) (Agilent, Santa Clara, CA) and the mobile phases were water (0.1% formic acid, A) and MeOH (0.1% formic acid, B). The gradient employed was the following: 0 min, 95% A; 4 min, 95% A; 20 min, 73% A; 50 min, 5% A; 57 min, 99% A; 58 min, 99% A; 60 min, 95% A. A flow rate of 0.7 mL min⁻¹ was used together with an injection volume of 10 μ L. The diode array detector recorded the spectra from 200 to 550 nm, being each run monitored at 280, 330 and 370 nm. The MS detector operated under ESI negative ionization mode using the following parameters: dry temperature, 350 °C; dry gas flow, 12 L min⁻¹; nebulizer gas pressure, 40 psi; capillary voltage, 3500 V. The instrument mass scan range was m/z 100 to 1000 and MS/MS automatic mode was used on the more abundant ions in the MS spectra to study their fragmentation patterns.

The phenolic compounds were identified by comparing their retention time, UV-Vis and mass spectra with those obtained from standard solutions, when available. Otherwise, peaks were tentatively identified comparing the obtained information with available data reported in the literature. To identify the presence of acyl flavonoid derivatives on the samples, an alkaline hydrolysis was carried out to eliminate acid

moieties (e.g., p-coumaroyl, caffeoyl, feruloyl and sinapoyl), following the procedure described by Francisco, et al. [30].

For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of other compound from the same phenolic group. The results were expressed in $\mu\text{g g}^{-1}$ of dry weight (d.w.), as mean $\pm$ standard deviation of two extracts. At least, two replicates of each extract were made for quantification purposes.

2.6. Statistical analysis

The results were expressed as mean $\pm$ standard deviation and the differences of phenolic contents between the two sampling days (day 1 and day 10) were tested using one-way ANOVA test. Differences were considered as statistically significant at a value of $p < 0.05$. The statistical analyses were carried out using Statistica 8.0 software (Statsoft Inc., Tulsa, USA).

3. RESULTS AND DISCUSSION

As mentioned, the phenolic profile of a vegetable can be influenced by intrinsic factors related to the plant development stage and genetic variability. Also, some external factors will affect the plants' phenolic metabolism in response to agronomic and environmental conditions, post-harvesting processing and also storage conditions. Therefore, the phenolic profile of baby-leaf vegetables will be the result of the interaction of both intrinsic and external factors on their development. In a previous work, it was already demonstrated how the amount of free vitamins in these products decreased during storage [18]. In the present work the same analysis procedure was

applied to all samples in order to study the stability of another important group of bioactive-relevant compounds, polyphenols. It included an optimization of a PLE method, followed by the development of the HPLC method to separate, identify and quantify the phenolic compounds present. The concentration of the individual phenolic compounds was compared between the samples collected in the beginning and at the end of the storage period.

3.1. Samples extraction

In this work, one of the objectives was to use a “green” extraction method of phenolic compounds that could be applied to different samples of baby-leaf vegetables. In order to optimize the PLE conditions to extract phenolic compounds from the studied vegetables, the total phenols content of the extracts was monitored (Figure 1). First of all, the influence of using different combinations of hydro-organic solutions with MeOH or EtOH was tested. As can be observed in Figure 1A, there were no statistically significant differences ($p < 0.05$) between the use of MeOH or EtOH in the extractions for a particular proportion in the two different vegetables considered as models (pea shoots and spearmint), showing similar values for each concentration of organic solvent. The use of 90% of organic solvent revealed a lower yield of total phenols, compared to the use of 70% and 50%. These latter proportions produced similar results, although the use of 70 % of organic solvent slightly improved the obtained results, and between them, MeOH allowed the attainment of a higher total phenols value (5% and 7% more in pea shoots and spearmint, respectively). These results were similar to those obtained in the PLE of parsley flakes [31]. Consequently, 70% MeOH in water was the solvent chosen to perform the study about the influence of the extraction time and number of extraction cycles.

Regarding the extraction time, no significant differences ($p < 0.05$) were revealed at the different studied extraction times for both vegetables (Figure 1B). These results are in agreement with previous PLE studies, where the time of extraction also showed a minor influence on the total extraction yield and total antioxidant activity of the extracts [32,33]. Spearmint PLE extracts obtained with 2 extraction cycles of 10 min had a 12% higher phenol content than the extract produced with a single static extraction of 20 min. The use of 2 static cycles of 10 min also produced, in both samples, the smaller variation between replicates ($< 1\%$). Even though, a static extraction cycle of 10 min produced similar yield than the longer extractions, the use of 2-ten min cycles was selected due to better results obtained for spearmint and considering that this process could be more appropriate to cover the potential variability that might exist among samples [15,31].

Concerning the rest of the extraction conditions, the influence of the extraction pressure has been described to have a negligible effect on the overall extraction yield once it is enough to maintain the solvent in the liquid state [15,17]. Thus, 10 MPa was set as extraction pressure. On the other hand, temperature is considered to have a major influence on the extraction yield. Normally, high temperatures improved the diffusion rate of the analytes of interest to the solvent, but it can also promote the co-extraction of other compounds, decreasing the selectivity of the extraction [17]. The use of very high temperatures can also affect the stability of more thermo-labile compounds and also may drive to the formation of new compounds [34]. A temperature of 70 °C was chosen based on other published works [30,35], in order to improve the transfer rate of the analytes to the solvent while maintaining the natural phenolic profile present in the baby leaves, avoiding the formation of other compounds.

3.2 Phenolic profile of ready-to-eat baby-leaf vegetables

3.2.1 Characterization of the PLE baby-leaf extracts

In first place, a new HPLC method was optimized to achieve the best separation of the 23 phenolic compounds for which commercial standards were available (see Figure 2 and Table 1 for peak identification). These compounds were preliminarily chosen according to the phenolic composition described in the literature for some of the studied species [6,14,19,21]. Most compounds were correctly separated with very good resolution; however, the separation of quercetin-3-*O*-rutinoside and quercetin-3-*O*-glucoside (peak 14+15) was not achieved, co-eluting in the conditions selected (Figure 2). The chromatograms corresponding to the studied samples are also presented in Figure 2. Each baby-leaf PLE extract showed a particular and distinctive phenolic profile, with a good separation of the compounds. For each phenolic profile the identification of the compounds was attempted by combining the information of the DAD and the MS detector, together with retention times and information available on the literature. A preliminary analysis of the compounds' UV-Vis spectra allowed the classification of the separated peaks into two classes of phenolic acids (namely, hydroxycinnamic and hydroxybenzoic acid derivatives) and into three classes of flavonoids (flava-3-ol, flavonol and flavone derivatives). Hydroxybenzoic acids and flava-3-ols were detected at 280 nm, whereas hydroxycinnamic acids exhibited an absorbance maximum around 320-330 nm, flavonols between 350 and 385 nm, and flavones in the 277–295 nm range with a shoulder at 300–330 nm [36]. Due to the fact that in the nature, polyphenols occur conjugated to sugars and organic acids, the comparison between the UV-Vis spectra and retention time with the available standards did not permit the complete identification of most of compounds present in the samples. The study of the hydrolyzed extracts, comparing the native and the hydrolyzed profile,

permitted the identification of the acyl glycosylated compounds that include in their structure an acid moiety; those compounds were not present in the hydrolyzed extracts, appearing with more intensity the hydroxycinnamic acids (caffeic, p-coumaric, ferulic and sinapic acids) that would be present in a conjugated form in the native extracts, and also other flavonoids mainly conjugated with sugar moieties (see Figure 3). Combining the information obtained from the UV-Vis spectra obtained from the native and hydrolyzed extracts together with the analysis of the MS spectra and fragmentation patterns of the main ions detected, the separated compounds could be tentatively assigned on the different samples. In Tables 2 and 3 the identification of the major phenolic compounds of each sample is presented. In some cases, the low concentration of the compound in the extract, or the high background noise in the MS signal did not permit to clearly identify the main ions of the compound or the fragmentation pattern. In those cases the compounds identification was based only on their UV-Vis spectra and was referred as a derivative of the more similar aglycone. Thorough MS analysis of pure compounds could theoretically make possible to assign the positional isomers of the glycosylated flavonoids. However, in the present work, when it was not possible to unambiguously characterize those flavonoids, the most frequently found form, containing a 7-*O*-linkage, was assumed.

In total, 203 compounds from the 11 baby-leaf samples were tentatively identified. All the samples showed particular chemical compositions with the exception of two baby-leaf lettuces (green and ruby red) which presented a similar phenolic profile (Table 2). Chlorogenic acid (4^L: 5-caffeoylquinic acid) and chicoric acid (10^L) were the two major hydroxycinnamic acids present in these samples, that were also composed by several quercetin derivatives and a flavone (luteolin-7-*O*-glucuronide, peak 13^L). These baby-leaf samples reveal the same composition that other works described for different

lettuce samples [36,37]. Flavonoids (9^{sc} - 19^{sc}) were the main compounds present in swiss chard. Peaks 9^{sc} , 10^{sc} , 12^{sc} and 13^{sc} corresponded to the flavone apigenin, conjugated with sugar moieties, identified by the presence of a ion at m/z 269 $[M-H]^-$ in the MS^2 experiments (Table 2). These compounds were described as characteristic in the phenolic composition of swiss chard leaves [39]. Quercetin (peaks 11^{sc} , 17^{sc} and 18^{sc}) (MS^2 ion at m/z 301 $[M-H]^-$) and isorhamnetin (peaks 14^{sc} , 15^{sc} and 19^{sc}) (MS^2 ion at m/z 315 $[M-H]^-$) derivatives were also identified (Table 2).

Spinach leaves showed a great number of flavonols, especially patuletin (MS^2 ion at m/z 331 $[M-H]^-$) and spinacetin (MS^2 ion at m/z 345 $[M-H]^-$) derivatives (peaks 3^{sp} , 5^{sp} - 18^{sp}), described in the literature as the main phenolic compounds of these leaves [14,23,24,41]. Flavonols were also the main compounds found in pea shoots (peaks 3^{ps} to 15^{ps}). To our knowledge, the phenolic compounds of pea leaves had only been studied in a previous work [42] in which 7 flavonols were identified. In the present work, 5 of the previously referred compounds were detected (peaks 3^{ps} , 4^{ps} , 6^{ps} , 8^{ps} and 9^{ps}), as well as other compounds that were identified for the first time in this baby-leaf product, corresponding to peaks 10^{ps} to 15^{ps} (Table 2). In pea shoots, the presence of hydroxycinnamic acids was not verified as individual compounds, although their presence was confirmed as conjugates with flavonols (6^{ps} , 8^{ps} , 9^{ps} , 12^{ps} , 13^{ps} and 15^{ps}), thanks to the detection of p-coumaric, sinapic and ferulic acids in its corresponding hydrolyzed extract.

Watercress, garden cress, mizuna, red mustard and wild rocket are all plants belonging to the Brassicaceae family, which includes numerous economically important species that had been widely investigated in terms of glucosinolates and phenolic composition [6,21,30]. In these samples, flavonols were the main group of phenolic compounds, showing different quercetin, kaempferol and isorhamnetin derivatives (Table 3). As

could be expected, these samples showed some common phenolic compounds between them. For instance, the presence of ferulic (peaks 10^w, 14^g, 17^m, 23^r) and sinapic (11^w, 15^g, 18^m, 24^r) acids derivatives appeared in watercress, garden cress, mizuna and red mustard. Concerning the flavonoid content, 9 matching compounds were found in the phenolic profile of mizuna and red mustard leaves, corresponding to isorhamnetin 3-O-glucoside-7-O-glucoside (14^m, 21^r), kaempferol-3-(methoxycaffeoyl-diglucoside)-7-glucoside (6^m, 10^r), kaempferol 3-(caffeoyl-diglucoside)-7-glucoside (7^m, 11^r), kaempferol- 3-(sinapoyl-diglucoside)-7-glucoside (9^m, 15^r), kaempferol- 3-(feruloyl-diglucoside)-7-glucoside (10^m, 16^r), kaempferol-3-(p-coumaroyl-diglucoside)-7-glucoside (11^m, 17^r), quercetin 3-hydroxyferuloylsophoroside-7-glucoside (4^m, 7^r), quercetin-3-caffeoyldiglucoside-7-glucoside (5^m, 8^r) and quercetin-3-(sinapoyl-diglucoside)-7-glucoside (8^m, 13^r).

In the PLE extract of watercress, different quercetin-related compounds were identified (peaks 6^w, 7^w, 13^w and 14^w) [21] as well as different isorhamnetin derivatives (peaks 16^w, 18^w). Watercress phenolic profile also showed a significant presence of hydroxycinnamic derivatives (peaks 4^w, 5^w and 9^w-12^w), being mainly caffeic, p-coumaric, sinapic and ferulic acids derivatives (Table 3).

In garden cress leaves, the flavonols found correspond to quercetin (peaks 5^g and 6^g) and kaempferol (peaks 7^g -12^g) conjugated with glucoside, rhamnosyl, caffeoyl, p-coumaroyl and synapoyl. Also the presence of sinapic (peaks 4^g, 13^g and 15^g) and ferulic acid (14^g and 16^g) derivatives was detected. These were the same type of compounds that were also described in the study of the phenolic composition of garden cress sprouts [45]. Mizuna and red mustard leaves showed a flavonoid composition similar to the one described in the literature for those species [21,40]. Red mustard PLE extracts revealed the most complex profile among the studied samples in terms of

number of compounds present (Figure 2). A total of 25 phenolic compounds were tentatively identified; as can be seen in the complete profile obtained (Figure 2), kaempferol, quercetin and isorhamnetin glycosides were the main compounds of this vegetable (6^f to 18^f and 20^f to 21^f), mainly conjugated with ferulic, sinapic and p-coumaric acid. In the case of wild rocket leaves, the MS analysis only permitted the complete identification of 6 compounds (2^{wr} , 5^{wr} , 6^{wr} , 8^{wr} , 10^{wr} and 11^{wr}), being the other classified accordingly to the UV-Vis spectra and complemented with the data obtained from the analysis of the hydrolyzed extract.

Lastly, spearmint is an aromatic herb from the Lamiaceae family that includes plants recognized as being a good source of phenolic compounds, like oregano and rosemary [16,46]. The compounds found in spearmint were mainly caffeic acid derivatives, like chlorogenic (4^s), rosmarinic (12^s), lithospermic (13^s) and salvionic (17^s) acids. Besides the rosmarinic acid (molecular ion at m/z 359[M-H]⁻), that was the main compound of the spearmint phenolic profile, 5 compounds were identified as rosmarinic acid derivatives (15^s and 19^s - 22^s), due to the presence of a fragmentation ion at 359[M-H]⁻.

3.2.2 Quantification and stability of phenolic compounds during storage

Once the characterization of phenolic profiles of the PLE extracts of the baby-leaf vegetables was accomplished, these compounds were quantified. Calibration curves (7 concentration points from 0.2 to 440 mg mL⁻¹) were constructed for the available commercial standards. The linearity, limits of detection (LOD), limits of quantification (LOQ) and repeatability of the HPLC-DAD method were determined and are shown in Table 1, together with their retention time (RT), UV-Vis maxima, [M-H]⁻ and main fragments ions obtained. R² values higher than 0.997 were obtained for all the quantified compounds within the linear range studied. The standards of quercetin and

salicylic acid revealed the lowest and the highest LODs and LOQs, respectively. The method showed good repeatability values in the intra-day precision test (0.2-1.1% for retention times and 0.9-2.8% for peak area, $n = 5$), and between 3 consecutive days (inter-day), showing RSD values always below 3.5% (0.2-1.2% for retention times and 0.9-3.5% for peak area, $n = 15$). Tables 2 and 3 show the results obtained from the quantification of the identified phenolic compounds as well as the total amount of hydroxycinnamic acids, hydroxybenzoic acids, flavones, flavan-3-ols, flavonols and total phenolic compounds determined on each sample. The quantification of the compounds for which there was not a standard available was made using the calibration curve of the compound that was included in their structure. Compounds identified as caffeic acid derivatives (e.g., chicoric acid) were quantified using the caffeic acid calibration curve following the same strategy for the other hydroxycinnamic and hydroxybenzoic derivatives. Flavonols were quantified with the quercetin-3-*O*-glucoside calibration curve in the case of quercetin and isorhamnetin derivatives and with the kaempferol-3-*O*-glucoside calibration curve in the case of kaempferol derivatives. Spinacetin and patuletin were also quantified as a glycosylated flavonol using the quercetin-3-*O*-glucoside calibration curve. On the other hand, flavone compounds were determined using either apigenin or luteolin calibration.

From all the baby-leaf vegetables analyzed, the ruby red lettuce had the highest concentration of phenolic compounds (approximately 483 mg g^{-1} (d.w.)), at least 10-times higher than the rest of samples. On the other hand, the lowest phenolic content was found in the wild rocket leaves, where a concentration of 8 mg g^{-1} (d.w.) was found. Regarding the evolution of the identified compounds during the refrigerated storage, significant changes ($p < 0.05$) were found in almost all cases. In green lettuce the total phenolic content increased a 17.5% from $34.1 \pm 0.5 \text{ mg g}^{-1}$ (d.w.) in the first day of

storage to $41.4 \pm 9.8 \text{ mg.g}^{-1}$ (d.w.) after 10 days, whereas ruby red lettuce phenolic compounds were shown to be more stable. Nevertheless, there were some significant changes ($p < 0.05$) registered in the hydroxycinnamic acids concentration.

Swiss chard, spinach and pea shoot revealed a great stability of their phenolic profile during the 10-day period of refrigerated storage. In fact, their total polyphenols concentration was not significantly modified ($p > 0.05$), although some punctual changes were found (marked with an asterisk in Table 2). The main components of these samples corresponded to flavonols, that represented a 92.9% and 99% of the total phenolics quantified in spinach and pea shoots, respectively. In spinach PLE extracts, 5,3,4-trihydroxy-3-methoxy-6:7-methylenedioxyflavone-4-glucuronide (17^{sp}) was the major phenolic detected, representing approximately 26% of the total phenolics, which is in agreement with previously published reports [23,24,41].

From all the studied samples, the brassicas had the lowest phenolic content. Within these samples, watercress, mizuna and red mustard had similar contents (21.0 ± 1.9 , 18.5 ± 0.3 and $17.1 \pm 0.9 \text{ mg.g}^{-1}$ (d.w.), respectively). The stability of the phenolic content was different among these samples; while watercress, garden cress and wild rocket total phenolic content was not modified, mizuna and red mustard suffered significant ($p < 0.05$) changes during refrigerated storage. In fact, mizuna samples revealed a 7.8% increase, whereas red mustard showed a marked loss (23.7%) in the total phenolic content, as a result of the decrease verified in 4 hydroxycinnamic acids (3^{r} , 4^{r} , 19^{r} and 24^{r}) and 8 flavonols (5^{r} , 7^{r} , 8^{r} , 10^{r} , 11^{r} , 13^{r} , 17^{r} and 18^{r}). In general, flavonols were the main phenolic group in the brassicas leaves studied. More specifically, these compounds represented 65-66% of the phenolics of watercress and mizuna, and 80-90% in red mustard, garden cress and wild rocket. On the other hand, hydroxycinnamic acids, showed a significant evolution during the 10-day period

($p < 0.05$), decreasing a 63.8% in wild rocket, a 7.6% in garden cress and 27.9% in red mustard. The opposite behavior was observed for hydroxybenzoic derivatives increasing 54.9%, 72.6%, 64.7% and 62.1% in garden cress, mizuna, red mustard and wild rocket leaves, respectively.

Regarding spearmint, 81% of total phenolic compounds was formed by hydroxycinnamic acids showing a very different composition from all other studied samples, where flavonoids were the main compounds. However, rosmarinic acid (12^{S}) and the luteolin derivatives (10^{S}) that represented 44% and 8% of the total phenolic compounds, respectively, decreased during storage. These losses combined with the changes suffered by other minor compounds resulted in a total loss of 13.8% of the phenolic content between day 1 and day 10 of refrigerated storage.

The results obtained for the different samples confirm that the phenolic composition of each vegetable is differently affected during the same storage conditions. The phenolic content of green lettuce, mizuna, red mustard and spearmint changed significantly during storage, while in the other samples only punctual changes in different compounds were noticed. The evolution of the phenolic profile was expected, as they are part of the natural defense metabolism of the plant. Accordingly to previous studies about the stability of phenolic content during storage of green leafy vegetables [13,23,27], several intrinsic factors were pointed out to be determinant to the phenolic compounds metabolism, related to the presence of other compounds with strong antioxidant activity (eg. vitamin C and carotenoids) that could act as a first defense against oxidative stress. In a previous study about the vitamin content of these baby-leaf [18], pea shoots, watercress and wild rocket leaves showed the highest content of vitamin C in relation to the other samples, which may have contributed to the stability of their phenolic profile. The fact that hydroxycinnamic and hydroxybenzoic acids

undergo more changes, points out to a greater stability of the flavonoids during the refrigerated storage of baby-leafs.

CONCLUSIONS

In this work, the use of an optimized PLE method revealed to be an efficient and “green” option to extract polyphenols from green leafy vegetables, being the first time that this method was applied to the phenolic extraction of ready-to-eat baby-leaf vegetables. More than 200 polyphenols were tentatively identified in 11 baby-leaf vegetables thanks to the application of a HPLC-DAD-MS method. The quantification of these compounds through a 10-day storage period revealed that the evolution of the phenolic profile during refrigerated storage was different in every sample, being flavonols the more stable compounds of the baby-leaf samples. In general, the differences between the overall phenolic compositions between the two sampling days were small, although statistically significant for green lettuce, mizuna, red mustard and spearmint, showing that the baby-leaf maintained their richness in phenolic compounds that may be beneficial to the human health. Nevertheless, some interesting relationships and variations among the different quantified components have been observed during the studied storage shelf-life period.

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613 **Figure captions:**

614 **Figure 1.** Total phenols content obtained with PLE of the two samples at the indicated
615 conditions (A. Solvents mixtures comparison; B. static extraction time and number of
616 extraction cycles).

617 **Figure 2.** HPLD-DAD chromatograms (280 nm) of the phenolic standards and PLE
618 baby-leaf extracts of all samples. For peak identification and information see Tables 1, 2
619 and 3.

620 **Figure 3.** HPLC-DAD (280 nm) chromatogram of ruby red lettuce phenolic profile. A
621 (black line), native extract and B (grey line), hydrolyzed extract. (For peak
622 identification see Table 2).