Development of a HPLC-DAD- MS/MS method for simultaneous determination of fat- and water- soluble vitamins in green leafy vegetables

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ABSTRACT

The simultaneous analysis of fat- and water-soluble vitamins from foods is a difficult task considering the wide range of chemical structures involved. In this work, a new procedure based on a sequential extraction and analysis of both types of vitamins is presented. The procedure couples several simple extraction steps to LC-MS/MS and LC-DAD in order to quantify the free vitamins contents in fresh-cut vegetables before and after a 10-days storage period. The developed method allows the correct quantification of vitamins C, B₁, B₂, B₃, B₅, B₆, B₉, E and provitamin A in ready-to-eat green leafy vegetable products including green lettuce, ruby red lettuce, watercress, swiss chard, lamb's lettuce, spearmint, spinach, wild rocket, pea leaves, mizuna, garden cress and red mustard, attaining low LOQs. Total analysis time was around 100 min including extraction and vitamin analysis using 2 optimized procedures; good repeatability and linearity was achieved for all vitamins studied, while recoveries ranged from 83% to 105%. The most abundant free vitamins found in leafy vegetable products were vitamin C, provitamin A and vitamin E. The richest sample on vitamin C and provitamin A was pea leaves (154 mg/g fresh weight and 14.4 mg/100 g fresh weight, respectively), whereas lamb's lettuce was the vegetable with the highest content on vitamin E (3.1 mg/100 g fresh weight). Generally, some losses of vitamins were detected after storage, although the behavior of each vitamin varied strongly among samples.

Keywords: fresh-cut vegetables, fat-soluble vitamins, water-soluble vitamins, LC-MS/MS, green leafy vegetables.

1. INTRODUCTION

Vitamins are biologically active organic compounds that are essential micronutrients involved in metabolic and physiological functions in the human body. There are thirteen vitamins identified that are classified according to their solubility into fat-soluble vitamins (FSV) (A, E, D, and K) and water-soluble vitamins (WSV) (B-group vitamins and vitamin C) [1]. These compounds greatly differ in their chemical composition, physiological action and nutritional importance in the human diet, even within the same group [2]. The FSV are involved in complex metabolic reactions related to important biological functions, such as vision (vitamin A), calcium absorption (vitamin D), antioxidant protection of cell membranes (vitamin E) and blood coagulation (vitamin K), among other functions [3]. Several vitamins of the B-group act mainly as coenzymes in the catabolism of foodstuffs to produce energy[1] .

WSV and FSV are one of the micronutrients that are usually labeled in foods. In this sense, minimally processed vegetables (e.g. lettuce, wild rocket, watercress, spinach) are not an exception. These products are basically ready-to-eat foods composed by raw vegetables that retain as much of the naturally occurring vitamin content. However, there are several factors that can lead to vitamin losses in these products such as temperature, the presence of oxygen, light, moisture content, water activity, pH, enzymatic modifications and metal trace elements, particularly iron and copper [1].

The degree of degradation will vary according to the vitamin and could also be affected by the processing and storage time to which the vegetable is submitted. It is known that WSV are more susceptible to leaching losses during washing, while vitamin C is very prone to chemical oxidation during processing and storage stages [1]. Vitamins A and E could be destroyed under the presence of oxygen, light, heat, trace metal ions and storage time [1]. Therefore, monitoring the vitamin content during processing and

storage is of great importance to food technologists and consumers to assure the nutritive value of foods, and also for quality assurance purposes and regulatory compliance. This requirement creates the need for more rapid and specific methods for vitamin determination [4-6].

The development of a single method for the multiple and simultaneous monitoring of WSV and FSV is very challenging due to different reasons. The level of vitamins in food may be as low as few micrograms per 100 g, usually very unstable and accompanied by an excess of compounds with similar chemical behaviour. Traditionally, methods for vitamin determination require the analysis of each vitamin individually by using different physical, chemical and biological methods. Microbiological assays are still the methods of reference as Official Methods of Analysis of AOAC International for some vitamins (vitamin B₅, B₆, B₉ and B₁₂); these are highly sensitive but also laborious to achieve an estimation of mean value with a certain precision [1,6,7]. High performance liquid chromatographic (HPLC) methods are often used for the determination of WSV and FSV. The choice of the method depends on the accuracy and sensitivity required, as well on the interferences encountered in the sample matrix. HPLC, with UV absorbance and/or fluorescence detection is well established for both FSV and WSV measurements, but showed some limitations for certain analytes and also lacks specificity in complex matrices [8]. Liquid chromatography-mass spectrometry (LC-MS) shows more sensitivity and specificity for the determination of vitamins in these matrices, and permits the simultaneous analysis of multiple vitamins in a single analysis [8,9]. The majority of the HPLC multivitamin methods found in the literature focused only either FSV or WSV and were mainly applied to analysis of pharmaceutical preparations or supplemented foods [5,10-13]. Only some of them attempted the determination of naturally occurring vitamins on food

[8,9,14-18]. Moreover, to the best of our knowledge, the determination of a wide group of free vitamins in green-leafy fresh-cut vegetables has not been carried out. Consequently, the objective of the present work is to develop and validate a HPLC-DAD-MS/MS-based method that allows a simple and sequential extraction and monitoring of several free forms of WSV (vitamins C, B₁, B₂, B₃, B₅, B₆ and B₉) and FSV (pro-vitamin A and vitamin E) in raw green leafy vegetables to study their contents as well as their evolution along a typical storage period emulating the market conditions.

2. MATERIALS AND METHODS

2.1. Chemicals and standard solutions

All chemicals used were of analytical reagent grade. Vitamins standards (purity >99.0%), namely, ascorbic acid (C), thiamine hydrochloride (B₁), riboflavin (B₂), nicotinamide (B₃), D-calcium pantothenate (B₅), pyridoxine (B₆), folic acid (B₉), α -tocopherol (E) and β -carotene (provitamin A), were purchased from Sigma Aldrich (Madrid, Spain). The internal standards, hippuric acid and trans- β -Apo-8'-carotenal as well as triethylamine (TEA) and butylated hydroxytoluene (BHT) were also from Sigma Aldrich (Madrid, Spain). Ammonium acetate and acetic acid were from Panreac (Barcelona, Spain) and Scharlau (Sentmenat, Spain), respectively. Methanol (MeOH), methyl *tert*-butyl ether (MTBE) and ethyl acetate were HPLC-grade from Lab-Scan (Gliwice, Sowinskiego, Poland). Distilled water was deionized by using a Milli-Q system (Millipore, Bedford, MA, USA).

Individual WSVs standard solutions and hippuric acid (1mg/ml) solution were prepared in 10 mM ammonium acetate (pH 4.5), and kept in the dark under refrigeration at 4 °C

until analysis. Ascorbic acid was prepared at 5mg/ml, thiamine hydrochloride, nicotinamide, D-calcium pantothenate and pyridoxine at 1 mg/ml, riboflavin at 0.05 mg/ml and folic acid at 0.01 mg/ml. A mixture of WSVs were prepared daily by dilution of the individual vitamins stock solutions with 10 mM ammonium acetate solution with concentrations within the range of the values reported by nutritional tables for the samples under study (C: 3.3 µg/ml; B₁: 0.6 µg /ml; B₂: 0.75 µg/ml; B₃: 1.75 µg/ml; B₅: 0.8 µg/ml; B₆: 0.67 µg/ml; B₉: 0.33 µg/ml). α-tocopherol, β-carotene and trans-β-Apo-8'-carotenal were dissolved in MeOH (1 mg/ml) and stored at -20 °C, protected from light. A mixture of these fat soluble vitamins was also prepared before injection at 0.1 mg/ml with ethyl acetate.

2.2. Samples

Twelve samples of green leafy vegetables from seven different families (Asteraceae, Brassicaceae, Chenopodiaceae, Valerianaceae, Alliaceae, Amaranthaceae, and Fabaceae) were obtained from a producer of minimally processed vegetables (Odemira, Portugal). The samples used were fresh-cut leafs of red ruby lettuce and green lettuce (*Lactuca sativa* var. *crispa*), watercress (*Nasturtium officinale*), swiss chard (*Beta vulgaris*), lamb's lettuce (*Valerianella locusta*), spearmint (*Mentha spicata*), spinach (*Spinacia oleracea*), wild rocket (*Diplotaxis muralis*), pea (*Pisum sativum*), mizuna (*Brassica rapa* var. *japonica*), garden cress (*Lepidium sativum*) and red mustard (*Brassica juncea*). The samples were freeze-dried (Telstar Cryodos-80, Terrassa, Barcelona) upon arrival and after 10 days of refrigerated storage (3 ± 1 °C). The freeze-dried leafs were reduced to a fine powder in a knife mill (GM 200, RETSCH, Haan, Germany) and stored protected from light, oxygen and heat until analysis. The freeze

dried samples were spiked with vitamins standards in order to identify and quantify these vitamins forms in the real samples.

2.3. Samples extraction

A scheme of the extraction procedure developed in the present work to simultaneously extract WSV and FSV is shown in Figure 1. During the extraction process, samples were always protected from direct exposition to light and kept on ice to minimize vitamins degradation.

Briefly, 0.250 g of each sample was first extracted with 16 ml of 10 mM ammonium acetate/methanol 50:50 (v/v) containing 0.1% BHT. Standard solutions (hippuric acid and trans- β -Apo-8'-carotenal) were added at this stage (5 μ g/ml and 3.3 μ g/ml, respectively). After 15 minutes of shaking to achieve good sample dispersion in the extraction liquid, samples were placed in an ultrasound bath for 15 minutes. Bath temperature was always controlled with ice to guarantee that water temperature did not rise above 25 °C. The samples were centrifuged at 14000 g for 15 min and the supernatant was withdrawn and filtered through a 0.45 μ m nylon filter. One ml of the supernatant was concentrated into a nitrogen stream to evaporate the methanol and it was injected into a HPLC-MS/MS system to determine the WSV content. The solid residue from the first extraction was re-extracted twice with ethyl acetate containing 0.1% BHT (6 + 6 ml) for 15 minutes, also in the ultrasonic bath. Finally, the samples were centrifuged (14000 g, 15 min) and the supernatants combined and filtered through a 0.45 μ m nylon filter. The extract was taken to dryness under a N₂ stream. The residue was dissolved in 3 ml of ethyl acetate and injected in a HPLC-DAD system to monitor the pro-vitamin A (β -carotene) and vitamin E (α -tocopherol) contents.

2.4. Analysis of water soluble vitamins (HPLC-DAD-MS/MS)

HPLC–MS/MS analyses of WSV were performed using an Accela liquid chromatograph (Thermo Scientific, San Jose, CA) equipped with a diode array detector (DAD), an autosampler and a TSQ Quantum triple quadrupole analyzer (Thermo Scientific). The chromatograph was coupled to a MS analyzer via an electrospray (ESI) interface. Xcalibur software (Thermo Scientific) was used to analyze and store the data. The column used was an ACE-100 C₁₈ (100 x 2.1 mm i.d., 3µm particle size) (Advanced Chromatographic Technologies, Aberdeen, UK).

The method developed to simultaneously separate the seven forms of WSV in a single run was based on the work of Vazquez et al. [19] with some modifications, using 10 mM ammonium acetate solution (pH 4.5) as mobile phase A, MeOH with 0.1% acetic acid as mobile phase B and MeOH with 0.3% acetic acid as mobile phase C. The gradient used is described on Table 1. The flow rate was 0.2 mL/min whereas the injection volume was 10 µL. The DAD recorded the spectra from 200 to 680 nm. Column and autosampler compartments were thermostated at 20 and 5 °C, respectively. To identify and quantify the WSV, the mass spectrometer was operated first in the negative ESI mode, for 1.7 min. Spray voltage and capillary temperature were set at 3000 V and 250 °C, respectively. These conditions were the most suitable for ascorbic acid detection. A second segment of 10.3 min followed, using positive ESI mode to monitor the presence of the other WSV. In this segment the spray voltage and capillary temperature were set at 5000 V and 250 °C, respectively. Nitrogen was used as sheath and auxiliary gas at pressures of 40 and 19 a.u., respectively. Ion sweep gas pressure was 2 units and collision gas (Ar) pressure, 1.3 mTorr. Scan width and scan time were fixed at 0.020 (m/z) and 0.1 s, respectively, and the system was operated in selected reaction monitoring (SRM). SRM parameters were optimized by direct injection of

standards. Two ion transitions were monitored for identification but only the most intense product ion for each precursor ion was used for quantification. The values corresponding to the tube lens offset voltage and collision energy for each selected ion transitions are indicated in Table 2.

2.5. Analysis of fat soluble vitamins (HPLC-DAD)

The FSV determination was performed in an Agilent 1100 HPLC chromatograph (Agilent, Palo Alto, CA) equipped with an autosampler, a diode array detector (DAD) and an YMC C₃₀ analytical column (5 µm particle size, 250 x 4.6 mm i.d.) (YMC, Schermbeck, Germany). The method used was based on the method previously published by Jaime et al. [20]. Methanol/water/TEA (90:10:0.1, v/v/v) and MTBE/methanol/water/TEA (90:6:4:0.1, v/v/v/v) were employed as mobile phases A and B, respectively. Elution was carried out using the following gradient: 0 min, 6.5 %B; 8 min, 6.5 %B; 43 min, 100 %B; 46 min, 6.5 %B; 55 min, 6.5 %B. The flow rate was 1 mL/min and the injection volume 10 µL. Chromatograms were monitored at 295 nm for α-tocopherol content and at 450 nm for carotenoid compounds.

2.6. Method validation

A recovery study for each vitamin was performed by comparing the peak areas of vitamins from the same sample spiked before and after the extraction process to assess possible matrix effects. The recovery study was performed in three different days, always with the same sample (Spearmint).

Intra-day (n=5) and inter-day (three days, n=15) repeatability assays were performed.

Limits of detection (LOD) and quantification (LOQ) were calculated as a signal-to-

noise ratio of 3 and 10, respectively and expressed in ng/ml. Accuracy of the method was assessed by using the recovery percentages of spiked samples.

2.7. Statistical analysis

IBM SPSS Statistics software v.19 was employed for data elaboration and statistical analysis using a level of significance set at 95 %. One-way analysis of variance (ANOVA) was employed to assess differences among vitamin contents at the different storage times. Differences were considered statistically significant if $p < 0.05$.

3. RESULTS AND DISCUSSION

Two different approaches can be followed to determine several free forms of water- and fat-soluble vitamins in real samples. First one deals with the separation of all the vitamin forms in one single analysis and the second with the optimization of two individual methods for a more accurate measurement of the different vitamin forms. Although several works have been published dealing with the simultaneous determination of several WSV and FSV in a single chromatographic run with DAD and MS detectors [15,19,21,22], none of them refer to the analysis in real complex food samples but instead analyze pharmaceutical preparations, functional drinks and parenteral nutrition admixtures. As mentioned previously, the main goal of the present work was to develop a useful and rapid method for the determination of both types of vitamins in real green leafy vegetables and to use it to follow vitamin evolution during cold storage; therefore, the method should be able to provide an accurate quantification of all the free vitamin forms and its changes. Main problems arisen in the determination of WSV- and FSV- in a single run using real complex samples deal with coelutions among the different vitamins and with other major components such as organic acids

and polyphenols (that obviously are found in higher amounts), with the difficulty of selecting the best MS detection conditions, including the ionization technique, which are different for both groups of vitamins; etc. Considering all these aspects, our efforts were focused in the development of two different sequential methods that could provide good quantification capabilities and that could be employed in parallel. Therefore, optimization was carried out for the separation of WSV by HPLC-MS/MS and FSV by HPLC-DAD; and for the extraction protocol to recover both.

3.1. HPLC-MS/MS determination of water-soluble vitamins

During the optimization procedure for the separation of the seven WSV studied, the performance of two different reversed phase columns was tested. A short UPLC C₁₈ column (50 x 2.1 mm, 1.9 µm particle diameter) and a longer C₁₈ column (100 x 2.1 mm, 3 µm particle size) were compared. The longer column provided a better separation among the tested compounds, including those that were weakly retained in the C₁₈ stationary phase like ascorbic acid (vitamin C) and thiamine (vitamin B₁), while maintaining short analysis times (7 min). In Figure 2, the SRM chromatograms of the 7 WSV studied and hippuric acid are shown. As it can be observed, the separation of all the studied compounds was appropriately achieved. Although other type of columns adequate to perform HILIC separations had been used to separate WSV [5], according to Goldschmidt and Wolf [5], thiamine (B₁), pyridoxine (B₆) and nicotinamide (B₃) were the only vitamins that could benefit from this type of separations. Thus, considering the wider group of WSV studied here, the applicability of the HILIC approach in the present development would be of limited value.

Although the method was developed based on a previously published method [19] using 10 mM ammonium acetate (pH 4.5) and methanol as mobile phases, other alternative

mobile phases were also tested. The use of acidified water (using 1% of either acetic or formic acid) did not improve the obtained results using the buffered aqueous mobile phase. Regarding the organic modifier, acetonitrile was also tested. This solvent has been used in several multivitamin determination methods present in the literature [5,11,23], although in this application the separation was not improved. Thus, the use of methanol was maintained. The acidification of the solvent could be useful to suppress the dissociation of vitamins with an acidic group, like ascorbic acid, pyridoxine, pantothenic acid and folic acid (vitamins C, B₆, B₅ and B₉, respectively), improving peak shapes, and also to promote a better ionization of the basic sites of all vitamins [8]. Acetic acid provided better peak shapes than formic acid. Consequently, different levels of acetic acid were tested, namely 0.1% and 0.3%. It was observed that the higher proportion of acid improved the last eluting peaks, whereas the less retained compounds lost some efficiency. Thus, it was decided to employ a ternary system, allowing the introduction of more acidified methanol as the proportion of the organic modifier increased during the separation (see Table 1). Under these conditions, sufficient resolution between peaks and good peak shapes were obtained for all the studied compounds, as it can be observed in Figure 2.

Once the separation was optimized, the different MS/MS detection parameters were studied. By using the direct infusion of standard solutions, both positive and negative ESI ionization modes were studied for the production of characteristic precursor and product ions of each compound. Precursor ions were selected as the most abundant mass-to-charge (m/z) values. Subsequently, two product ions for each precursor were chosen. The most intense product ion was used for the quantification whereas the other was employed to confirm the identity of the studied compounds. Table 2 shows the precursor and product ions selected for each compound as well as the collision energies

and tube lens offset values employed for their detection. As it can be observed, only ascorbic acid (vitamin C) showed more intense ions in the negative ESI ionization mode. The rest of vitamins as well as the internal standard were detected as $[M+H]^+$. Thiamine (vitamin B₁) was monitored as the loss of associated chloride, whereas calcium pantothenate (vitamin B₅) was determined as pantothenic acid. The rest of parameters involved in the ESI detection of the studied vitamins, namely, capillary temperature (250-350 °C), spray voltage (3000-5000 V), sheath gas pressure (20-60 a.u.) and auxiliary gas pressure (0-40 a.u.), were optimized using a univariate method. The values that provided the best response for all vitamins are described in section 2.4. Once the optimum separation conditions and MS/MS detection parameters were selected, the instrumental intra-day and inter-day precision was evaluated. Intra-day precision was assessed through the consecutive injection (n=5) of a mixture of the seven WSV standards consecutively in the same day, that was repeated for three different days for inter-day precision evaluation (n=15). The obtained results are summarized in Table 3. The RSD values, for the same day, ranged between 0.9% and 7.2 % for peak areas and between 0.1 and 2.0% for retention times. The RSD values between days were slightly higher (3.6-9.1% for peak areas and 0.3-1.5% for retention times), although they were always below 10% for peak areas and 2% for retention times (see Table 3). The linear range was also tested using a least square fit, which showed a good linearity ($R^2 > 0.99$) for all WSV, within the range selected. Instrumental LODs (0.18-42.28 ng/ml) calculated as three times the signal-to-noise (S/N) ratio and LOQs (0.56-128.13 ng/ml) obtained for each WSV as ten times the S/N ratio, are also presented in Table 3. These data verified the suitability of the optimized HPLC-MS/MS method for a rapid and sensitive detection of the 7 WSV in a single run.

3.2. HPLC-DAD determination of fat-soluble vitamins

The method chosen to determine the free fat-soluble vitamins, i.e., provitamin A (β -carotene) and vitamin E (α -tocopherol), was based on a previously published method [20,24] with some modifications. A reversed phase column with C_{30} stationary phase was employed to separate the compounds present on the lipophilic extract. This type of column has demonstrated to be capable to separate different geometric isomers of both carotenoids and tocopherols in different matrices [14,25-27]. In fact, compared to C_{18} stationary phases, the less polar C_{30} phases exhibit superior selectivity for isomer separation of carotenoids, and vitamins A and E [28]. Starting from the conditions already published [20,24], the gradient elution program was modified in order to avoid coelutions between α -tocopherol and some carotenoids present on the real samples. An isocratic period was included at the beginning of the elution, which significantly improved the separation. The particular conditions employed are described in section 2.4.

Besides, the DAD detector was sensitive enough for both FSV analyzed, within the concentration range expected in the studied samples (see Table 3). Although identification and quantification of the selected compounds was achieved by comparison with their corresponding commercial standards, the DAD detector also permitted the spectral analysis of other compounds present in the extract to identify other tocopherols and carotenoids based on spectra similarity. The intra-day ($n=5$) and inter-day ($n=15$, 3 days) values were also assessed. Low RSD (%) values were obtained for both parameters being always below 2.1 and 0.6 % for peak areas and retention times, respectively (see Table 3). The linear calibration curves showed a high coefficient of determination ($R^2 > 0.999$).

3.3. Optimization of the extraction procedure

A sequential and simple method for the extraction of free forms of WSV and FSV from different vegetables is proposed in this work. The method is based on the sequential extraction of the plant material using ultrasounds. The method optimization was carried out following a univariate procedure in order to achieve the maximum extraction of the free forms of the studied vitamins in the shortest total time. Basically, the freeze-dried material was mixed with a volume of 10 mM ammonium acetate (pH 4.5)/methanol (50:50, v/v) containing 0.1 % BHT in order to obtain the water-soluble vitamins. The use of methanol in the mixture allowed the better dissolution and extraction of the less polar studied WSV [8,21]. On the other hand, BHT was included to prevent the oxidation of the some vitamins like vitamin C or β -carotene and α -tocopherol. Despite the fact that BHT is not soluble in water and is normally used to protect fat soluble compounds, the inclusion in this stage protected the sample from oxidation since the beginning of extraction. Although this was not the best stabilizer for vitamin C, its presence has been described to partly inhibit its oxidation [8]. Other chemicals might potentially have a higher protective effect in this process, like metaphosphoric acid or EDTA, but their presence is also referred to cause ion suppression in MS detection [8,29]. Thus, the combined use of BHT and methanol, provided a compromise for all the studied vitamins. A 15 min ultrasonic extraction was performed to promote the dissolution of the vitamins from the vegetal matrix into the solvent. Due to the labile nature of most vitamins, the temperature of the ultrasound bath was always controlled, and all procedures were performed without direct exposure to light. After centrifugation of the extracts, the supernatant was evaporated under a N₂ stream. The solid residue was then re-extracted for 15 min using ethyl acetate as extracting solvent to recover FSVs.

To determine the optimum volume of solvents and the number of extraction cycles suitable for all vitamins, optimization was carried out with spiked samples. Concerning the extraction of WSV, 16 ml was fixed as solvent volume and the possibility of using an extraction step or two extraction steps (8 ml each) was studied (triplicate assays). As it can be observed in Figure 3A, using two consecutive cycles, the amount of extracted vitamins was slightly higher, although the differences were not statistically significant ($p > 0.05$). Thus, a single extraction step was selected in order to make the whole procedure faster. On the other hand, different volumes of ethyl acetate (8, 12 and 15 ml) were tested for the FSV extraction. Results obtained showed an improvement in the final results when 12 ml of solvent were used compared to 8 and 15 ml. Similarly to WSV, the possibility of dividing the extraction volume in different cycles was studied. It was observed that the use of 2 extraction cycles (6 + 6 ml) significantly improved the results obtained compared to just one extraction cycle (see Figure 3B). The use of a third extraction cycle was also explored but discarded considering that did not produce significant gains while increasing the total extraction time. The extracts obtained were concentrated under a N_2 stream. This step was especially necessary for the α -tocopherol detection, due to its low signal in the DAD detector.

Concerning the determination of FSV, a hot saponification procedure is often used to remove chlorophylls and lipids while allowing the release of the free vitamin forms that are found as esters. However, in our approach, this procedure was not suitable due to the degradation of carotenoids by thermal isomerization and the generation of some artifacts that can interfere in the vitamin's determination. Moreover, this step is not recommended for green leafy vegetables, tomatoes and carrots, due to their low contents of FSV esters [14,30]. Instead, the chlorophylls found in the studied vegetables were, in

our optimized method, properly separated from the target compounds during the chromatographic analysis.

3.4. Extraction recovery study

To assess the suitability of the complete method, a recovery study was performed. Namely, 2 sets of extractions were carried out simultaneously following the optimized procedure described in section 2.3. In one of them, a vegetable sample (spearmint) spiked with a known amount of the target compounds was extracted whereas in the other, the same sample was extracted and spiked with the same concentration of vitamins after extraction. Triplicates of the extraction protocols were performed and the extracts were analyzed using the two methods by duplicate. Comparing the values obtained, it was possible to deduce that no significant matrix effect was observed during the extraction (Figure 4). The recoveries ranged between 83 % and 105% (see Table 3) which was considered as appropriate for a method that simultaneously determines compounds with different structures and chemical properties.

3.5. Green leafy vegetables analysis

Once the complete method of extraction and analysis of WSV and FSV was optimized, the procedure was applied to the study of different fresh-cut green leafy vegetables. The aim was to determine how the vitamin contents evolved during storage under conditions similar to those found in the market. To do that, 12 different products (namely, green lettuce, ruby red lettuce, wild rocket, swiss chard, watercress, spinach, lamb's lettuce, spearmint, pea leaves, mizuna, red mustard and garden cress), for which there were not previous published results, were analyzed upon arrival and after a 10-days storage at 3 °C. The results obtained from these determinations are summarized in Table 4,

expressed as μg of vitamin per 100 g fresh weight (fw). As it can be observed, the vitamins found in highest amounts in general were provitamin A, ascorbic acid and vitamin E. The amounts of vitamin C found in the samples varied in great extent, from more than 150 mg/100 g fw in pea leaves to undetectable amounts in swiss chard. Together with pea leaves, garden cress and wild rocket were the richest samples on this component. For most of the samples, the contents of vitamin C significantly decreased after the 10-days storage. Very strong decreases were observed for red mustard and garden cress. Other samples, such as wild rocket or watercress did not experiment these great losses. Interestingly, a slight but no statistically significant increase ($p > 0.05$) was observed for pea leaves, whereas a significant increment was found for lamb's lettuce. It has been shown that over a storage period similar to that employed in this work, ascorbic acid present in fresh-cut vegetables can be oxidized to dehydroascorbic acid as a result of the function of this compound in the plant as protector against oxygen species [31], even regenerating tocopherols [32]. Vitamin C could be also responsible for the decrease in enzymatic browning observed in some fresh-cut vegetables during storage [33].

B-group vitamins were found in less amount in all the studied vegetables, as it could be expected considering that most of B vitamins are mainly contained in higher extent in animal-derived foods and cereals. Among them, vitamin B₅ was generally the richest in all the studied samples. In fact, in all cases, the amount of free vitamin B₅ detected in the samples after the 10-days storage period was always significantly higher than the contents found before. These increments were in the range of a 2-fold increase, although for some vegetables, the increase was even higher (e.g., Mizuna). There are several possible explanations to this observation; on one hand, a catabolic release of pantothenic acid from coenzyme A (CoA) and acyl carrier protein (ACP) mediated by a

chain of enzymatic steps including hydrolases (for ACP) or phosphatases and pyrophosphatases (for CoA) [34] thus freeing the vitamin B₅ from CoA; and on the other hand an increase on the whole vitamin B₅ content due to a higher vitamin B₅-bacterial producing microbiological load of the green leafy ready-to-eat vegetables after storage. A recent study showed an increase in bacterial load of fresh-cut salads commercialized in Portugal [35] that might be susceptible of synthesizing higher amounts of this vitamin. In fact, processes associated to fresh-cut products such as washing, cutting, shredding and slicing have been identified as potential sources of microbial contamination [36]. An increase as a result of the post-harvest metabolism should not be also discarded [37].

Folic acid (vitamin B₉) was only found in spinach and lamb's lettuce, at levels lower than 11.5 µg/100 g; the contents on folic acid in spinach were by far lower than those reported in other published works [38], although it should be kept in mind that only the form as folic acid was determined in the present work. On the other hand, vitamin B₆ was found in all the studied samples, although maintaining very low levels, even as low as 1.5 µg/100 g. In general, the amount of vitamin B₆ after storage was lower than before, with some exceptions. Even in those latter cases, the differences were small.

For the rest of B-group vitamins, the levels varied among samples. Generally, vitamin B₃ was more abundant than vitamins B₂ and B₁ in almost all samples. For some samples, the contents on these vitamins increased after the 10-days storage time, mainly for vitamin B₁ (see wild rocket, spinach, spearmint, mizuna and garden cress in Table 4). Nevertheless, these increments were not too high considering the amounts of these free vitamins found in the studied samples. Similar behaviors have been observed for other vegetables at the end of prolonged storage periods [39]. The increments in some of them, such as vitamin B₂, might be related to microbial growth in the samples[39].

Besides, other authors have observed post-harvest synthesis of these vitamins, which could also explain the increments found [37]. Figure 5 shows the corresponding chromatograms obtained during the quantification of the studied vitamins in garden cress. As it can be observed in Figure 5B, some other compounds could be tentatively assigned to carotenoid or tocopherol family, thanks to the use of a DAD together with the high resolving power of the C₃₀ stationary phase employed in this determination.

The levels of fat-soluble vitamins found in the samples were by far higher than those of the B-group vitamins. Provitamin A was the most abundant FSV in all samples reaching maximum values for pea leaves, garden cress and spinach. The poorest source of provitamin A was ruby red lettuce, with values lower than 3.5 mg/100 g fw. For 4 out of the 12 samples studied, the levels of provitamin A did not varied significantly after the 10-days storage period, namely, mizuna, pea leaves, lamb's lettuce and spinach. For other samples, small to moderate decreases were observed, being green lettuce the fresh-cut vegetable that presented a higher percentage of loss of its initial content on this provitamin (ca. 50 %).

The amount of vitamin E in the studied vegetables after storage ranged from 321 µg/100 g fw for watercress to 3663 µg/100 g fw for pea leaves. A statistically significant increase ($p < 0.05$) on the concentration of vitamin E after storage was detected in spinach, pea leaves, mizuna and garden cress. These samples were also among the richest on vitamin C, which, as it has been already mentioned, is able to regenerate vitamin E [32].

4. CONCLUSIONS

In this study, a methodology to extract and quantify several free water soluble vitamins (vitamins C, B₁, B₂, B₃, B₅, B₆ and B₉) and fat soluble vitamins (vitamin E and

provitamin A) by LC-MS/MS and LC-DAD, respectively, has been optimized. The method has been used to quantify the vitamin's level in 12 different fresh-cut vegetables before and after a 10-days storage period under refrigeration (3°C). The optimized sequential extraction-analysis procedure has revealed as an appropriate methodology for the simultaneous determination of a whole range of free water and fat-soluble vitamins, with different chemical structures, in real complex samples. The procedure allows a complete analysis of all the vitamins in less than 100 min of total analysis time, including extraction and determination, with recoveries ranging from 83 to 105%, suitable LOD and LOQ and appropriate intra-day and inter-day precisions.

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569 **Table 1.** Gradient Elution used on water soluble vitamins analysis.

Time (min)	A (%)	B (%)	C (%)
0	90	10	0
3	90	10	0
4	50	0	50
7	50	0	50
10	0	100	0
17	0	100	0
20	90	10	0
30	90	10	0

570

571 **Table 2.** Retention times (min) and MS/MS detection parameters for the water soluble vitamins analyzed.

Peak	Vitamin	t_r (min)	Precursor ion (m/z)	SRM transitions		Tube lens offset (V)
				Quantifier ion (m/z) (Collision energy, V)	Qualifier ion (m/z) (Collision energy, V)	
1	Ascorbic Acid (C)	1.41	174.9 [M-H] ⁻	115.2 (14)	87.3 (18)	76
2	Thiamine (B ₁)	1.81	265.1 [M+H] ⁺	122.1 (10)	144.1 (16)	48
3	Pyridoxine (B ₆)	2.30	169.9 [M+H] ⁺	152.1 (11)	134.1 (19)	55
4	Nicotinamide (B ₃)	3.26	123.0 [M+H] ⁺	80.3 (16)	78.3 (24)	67
5	Pantothenic Acid (B ₅)	3.72	220.0 [M+H] ⁺	202.1 (12)	184.1 (12)	60
6	Hippuric Acid (IS)	5.90	180.1 [M+H] ⁺	105.2 (10)	77.4 (10)	59
7	Folic Acid (B ₉)	6.24	442.0 [M+H] ⁺	294.9 (13)	176.0 (34)	69
8	Riboflavin (B ₂)	6.82	377.1 [M+H] ⁺	243.0 (23)	147.1 (37)	93

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573 **Table 3.** Method evaluation parameters: LOD and LOQ values, repeatability, linearity and mean recovery percentages

Vitamin	Detection	LOD (ng/ml)	LOQ (ng/ml)	Repeatability (CV %)				Linearity		Mean Recovery		
				Intra-day		Inter-day		Range tested (µg/ml)	R ²	added (µg/ml)	% ^a	RSD
				Area	RT	Area	RT					
Ascorbic Acid (C)	MS/MS	42.28	128.13	5.6	2.0	6.3	2.0	0.07 - 5.28	0.992	3.30	102.8	10.6
Thiamine (B ₁)	MS/MS	0.79	2.41	3.1	0.7	3.6	0.7	0.0015 - 0.96	0.993	0.60	87.3	8.5
Riboflavin (B ₂)	MS/MS	0.07	0.20	2.5	0.2	4.3	0.2	0.0009 - 1.5	0.996	0.75	93.9	7.4
Nicotinamide (B ₃)	MS/MS	4.35	13.17	2.3	0.5	9.8	0.5	0.004 – 2.80	0.995	1.75	85.4	3.6
Pantothenic Acid (B ₅)	MS/MS	7.67	23.25	0.9	0.6	9.1	0.6	0.02 - 1.67	0.998	0.83	88.2	12.6
Pyridoxine (B ₆)	MS/MS	0.18	0.56	1.7	0.6	9.1	0.6	0.0008 – 0.53	0.997	0.67	82.8	8.9
Folic Acid (B ₉)	MS/MS	0.63	1.90	7.2	0.1	8.4	0.0	0.0004 – 0.53	0.995	0.33	104.8	16.6
α-tocopherol (E)	UV	170	520	0.7	0.1	2.1	0.5	6.25 – 100	0.999	50	87.5	4.9
β-carotene (pro-A)	UV	70	200	0.4	0.1	0.3	0.2	6.25 -100	0.999	50	105.3	12.5

574 ^a mean of five extractions

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576

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Table 4. WSV and FSV content (free forms) found in the fresh-cut green leafy vegetables at the indicated storage times. Values shown as mean $\pm$ sd relative to fresh weight (fw). Asterisks indicate values not statistically different ($p > 0.05$) between day 1 and day 10.

SAMPLES	Ascorbic Acid (C)	Thiamine (B ₁)	Riboflavin (B ₂)	Nicotinamide (B ₃)	Pantothenic Acid (B ₅)	Pyridoxine (B ₆)	Folic Acid (B ₉)	α -tocopherol (E)	β -carotene (provit A)
	$\mu\text{g}/100\text{g fw} \pm \text{sd}$	$\mu\text{g}/100\text{g fw} \pm \text{sd}$	$\mu\text{g}/100\text{g fw} \pm \text{sd}$	$\mu\text{g}/100\text{g fw} \pm \text{sd}$	$\mu\text{g}/100\text{g fw} \pm \text{sd}$	$\mu\text{g}/100\text{g fw} \pm \text{sd}$	$\mu\text{g}/100\text{g fw} \pm \text{sd}$	$\mu\text{g}/100\text{g fw} \pm \text{sd}$	$\mu\text{g}/100\text{g fw} \pm \text{sd}$
green lettuce									
day 1	89.42 $\pm$ 12.76*	79.38 $\pm$ 26.52*	28.21 $\pm$ 3.52*	129.67 $\pm$ 20.46	147.38 $\pm$ 24.36	1.56 $\pm$ 0.23	n.d.	n.d.	5466.41 $\pm$ 321.82
day 10	84.97 $\pm$ 4.29*	80.40 $\pm$ 3.28*	30.21 $\pm$ 5.31*	80.33 $\pm$ 5.93	214.97 $\pm$ 22.30	2.74 $\pm$ 0.28	n.d.	n.d.	2636.21 $\pm$ 267.28
ruby red lettuce									
day 1	71.46 $\pm$ 5.34*	67.84 $\pm$ 14.69*	27.65 $\pm$ 3.91	194.92 $\pm$ 13.31	77.29 $\pm$ 19.10	14.59 $\pm$ 1.91	n.d.	1029.04 $\pm$ 60.63	3412.59 $\pm$ 0.47
day 10	71.43 $\pm$ 4.59*	54.74 $\pm$ 5.92*	18.20 $\pm$ 2.63	54.98 $\pm$ 2.55	127.35 $\pm$ 16.15	5.58 $\pm$ 0.63	n.d.	558.33 $\pm$ 25.86	2000.71 $\pm$ 0.47
wild rocket									
day 1	85111.90 $\pm$ 5725.48	11.15 $\pm$ 1.89	42.92 $\pm$ 13.27*	142.90 $\pm$ 19.09	309.28 $\pm$ 75.03	10.11 $\pm$ 1.02	n.d.	1550.71 $\pm$ 128.54*	9306.42 $\pm$ 897.973
day 10	3555.03 $\pm$ 670.74	23.96 $\pm$ 0.79	54.00 $\pm$ 4.08*	85.29 $\pm$ 5.77	790.21 $\pm$ 14.82	28.34 $\pm$ 1.88	n.d.	1435.29 $\pm$ 42.51*	6778.79 $\pm$ 610.20
swiss chard									
day 1	n.d.	13.91 $\pm$ 1.83	120.02 $\pm$ 6.13	201.22 $\pm$ 11.94	246.82 $\pm$ 7.46	21.56 $\pm$ 1.47	n.d.	1185.98 $\pm$ 76.23*	7152.26 $\pm$ 245.79
day 10	n.d.	10.53 $\pm$ 1.81	110.60 $\pm$ 4.05	138.68 $\pm$ 19.31	490.05 $\pm$ 42.11	46.42 $\pm$ 2.62	n.d.	1041.70 $\pm$ 113.86*	6264.68 $\pm$ 246.24
Watercress									
day 1	59556.28 $\pm$ 2269.26	70.38 $\pm$ 21.19*	143.17 $\pm$ 7.05	110.74 $\pm$ 9.90	262.85 $\pm$ 3.40	12.75 $\pm$ 0.13*	n.d.	2501.40 $\pm$ 206.78	8373.47 $\pm$ 42.50
day 10	48202.14 $\pm$ 776.47	98.55 $\pm$ 11.19*	162.91 $\pm$ 9.49	76.53 $\pm$ 5.31	560.15 $\pm$ 63.39	16.26 $\pm$ 1.52*	n.d.	321.47 $\pm$ 138.11	9336.49 $\pm$ 101.07
Spinach									
day 1	14401.95 $\pm$ 2812.95	193.79 $\pm$ 14.45	257.17 $\pm$ 20.59	166.53 $\pm$ 11.77*	349.64 $\pm$ 20.40	8.22 $\pm$ 0.21	1.25 $\pm$ 0.14*	2870.87 $\pm$ 134.62	11228.64 $\pm$ 1217.31*
day 10	1710.22 $\pm$ 18.45	243.93 $\pm$ 23.96	223.24 $\pm$ 17.14	178.60 $\pm$ 14.77*	526.22 $\pm$ 58.54	13.04 $\pm$ 0.56	0.99 $\pm$ 0.12*	3564.92 $\pm$ 297.94	13411.26 $\pm$ 1448.51*
lamb's lettuce									
day 1	59179.16 $\pm$ 10007.28	131.20 $\pm$ 6.46*	169.13 $\pm$ 31.82	241.19 $\pm$ 46.24*	584.54 $\pm$ 37.95	20.23 $\pm$ 4.55	11.49 $\pm$ 0.64	3078.99 $\pm$ 231.95*	9165.61 $\pm$ 69.14*
day 10	136976.9 $\pm$ 8252.66	130.46 $\pm$ 13.49*	111.01 $\pm$ 8.33	193.87 $\pm$ 5.20*	698.49 $\pm$ 45.07	8.51 $\pm$ 1.09	6.41 $\pm$ 1.20	3174.01 $\pm$ 369.88*	9394.65 $\pm$ 129.30*

581 **Table 4. Cont.**

SAMPLES	Ascorbic Acid (C) μg/100g ± sd	Thiamine (B ₁) μg/100g ± sd	Riboflavin (B ₂) μg/100g ± sd	Nicotinamide (B ₃) μg/100g ± sd	Pantothenic Acid (B ₅) μg/100g ± sd	Pyridoxine (B ₆) μg/100g ± sd	Folic Acid (B ₉) μg/100g ± sd	α-tocopherol (E) μg/100g ± sd	β-carotene (provit A) μg/100g ± sd
Spearment									
day 1	475.01 ± 33.10	121.08 ± 10.05	169.08 ± 25.07*	281.62 ± 7.16	556.64 ± 31.05	7.87 ± 0.93	n.d.	1361.54 ± 18.90*	10613.15 ± 1310.79
day 10	318.26 ± 8.33	215.65 ± 12.68	143.66 ± 14.34*	238.88 ± 13.42	1621.72 ± 117.90	4.64 ± 0.06	n.d.	1541.70 ± 205.80*	8772.66 ± 47.84
pea leaves									
day 1	153937.40 ± 2807.18*	177.52 ± 14.86*	112.18 ± 14.11*	130.76 ± 1.67	636.53 ± 29.73	20.26 ± 1.25	n.d.	2649.40 ± 90.72	14410.38 ± 88.44*
day 10	174049.00 ± 19301.63*	189.40 ± 5.95*	127.50 ± 11.19*	103.93 ± 4.83	1185.78 ± 32.52	47.74 ± 1.26	n.d.	3663.98 ± 74.75	13581.52 ± 589.63*
Mizuna									
day 1	33040.30 ± 1578.16	6.47 ± 0.57	53.13 ± 5.36*	58.06 ± 7.80	125.36 ± 11.37	8.24 ± 0.53	n.d.	1132.11 ± 38.85	7756.01 ± 210.40*
day 10	14671.82 ± 1210.97	25.46 ± 4.11	56.05 ± 4.75*	35.29 ± 3.22	692.95 ± 46.82	20.90 ± 3.10	n.d.	2748.15 ± 38.34	9358.89 ± 790.09*
red mustard									
day 1	43926.55 ± 1079.56	11.60 ± 1.71	63.59 ± 3.95	108.07 ± 12.76	113.45 ± 7.30	5.12 ± 1.10*	n.d.	2054.31 ± 256.06*	8217.13 ± 149.46
day 10	98.86 ± 8.00	17.70 ± 1.59	27.95 ± 1.05	66.74 ± 6.40	278.35 ± 22.74	4.02 ± 0.77*	n.d.	1912.56 ± 36.35*	6297.77 ± 75.14
garden cress									
day 1	10187.03 ± 1185.11	36.15 ± 3.17	124.80 ± 10.03*	191.25 ± 20.66	587.00 ± 17.55	3.63 ± 0.24	n.d.	1947.23 ± 186.24	12771.73 ± 232.45
day 10	209.06 ± 28.70	101.93 ± 5.30	121.60 ± 3.40*	159.11 ± 14.64	1222.04 ± 100.57	16.84 ± 0.94	n.d.	2468.14 ± 142.85	10769.94 ± 137.08

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Figure 1. Extraction scheme for WSV and FSV. US, ultrasounds; AcNH₄, ammonium acetate.

Figure 2. HPLC-MS/MS chromatogram of the seven WSV standards and the IS (hippuric acid) under the optimum analysis conditions.

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593

594 **Figure 3.** A) Recoveries of water-soluble vitamins (%) obtained after 1 vs 2 extraction cycles (16 ml vs 8±8 ml) for WSV. B) Recoveries (%) of
 595 fat-soluble vitamins obtained after the one (12 ml) and two (6 + 6 ml) extraction cycles.

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598 **Figure 4.** Mean content of water-soluble vitamins of sample spiked with the same amount of WSV standards before and after extraction.

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601 **Figure 5.** Chromatograms of WSV (A) and FSV (B) from an extracted sample (garden cress). Peak id: t: tocopherols; c- carotenoids.

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