Journal of Chromatography A, 1261 (2012) 179–188

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Sequential determination of fat- and water-soluble vitamins in green leafy vegetables during storage

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ARTICLE INFO

Article history:
Available online 3 May 2012

Keywords:
Fresh-cut vegetables
Fat-soluble vitamins
Water-soluble vitamins
LC–MS/MS
Green leafy vegetables

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, B_1, B_2, B_3, B_5, B_6, B_9, 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. Using this optimized methodology, low LOQs were attained for the analyzed vitamins in less than 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.

1. Introduction

Vitamins are biologically active organic compounds that are essential micronutrients involved in metabolic and physiological functions in the human body. There are 13 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

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0021-9673/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.chroma.2012.04.067

by an excess of compounds with similar chemical behavior. 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 B_5, B_6, B_9 and B_{12}); 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, B_1, B_2, B_3, B_5, B_6 and B_9) 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.

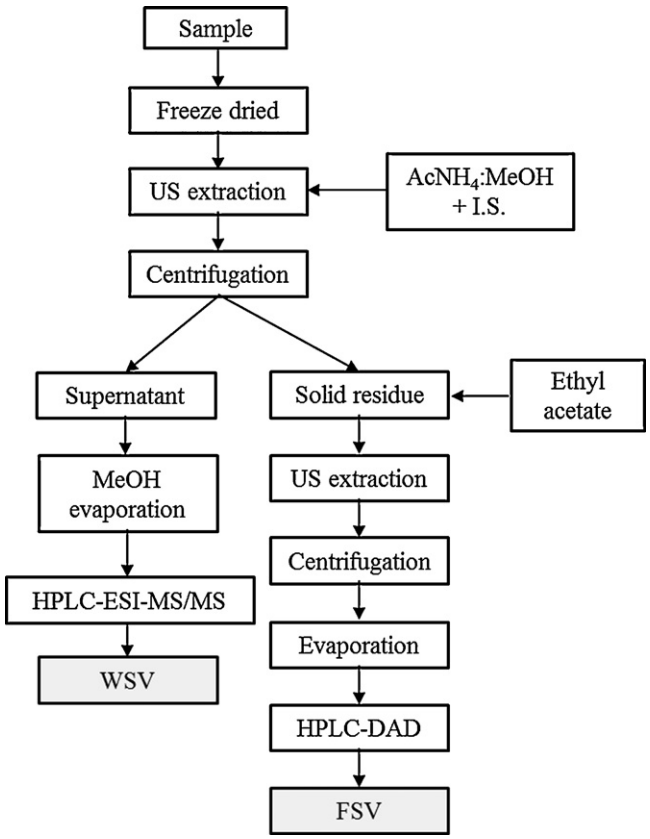

Fig. 1. Extraction scheme for WSV and FSV. US, ultrasounds; $AcNH_4$, ammonium acetate.

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 (B_1), riboflavin (B_2), nicotinamide (B_3), D-calcium pantothenate (B_5), pyridoxine (B_6), folic acid (B_9), 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 (1 mg/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 5 mg/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. During method development, 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; B_1: 0.6 μg/ml; B_2: 0.75 μg/ml; B_3: 1.75 μg/ml; B_5: 0.8 μg/ml; B_6: 0.67 μg/ml; B_9: 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 Fig. 1. During the extraction process, samples were always protected from direct exposition to light and kept on ice to minimize vitamins degradation.