



Received for publication, November, 2, 2018

Accepted, May, 4, 2019

## Original paper

# Internal Validation of the Methods for Determination of Water-Soluble Vitamins from Frozen Fruits by HPLC-HRMS

ADRIAN CONSTANTIN ASĂNICĂ<sup>1</sup>, LUMINIȚA CATANĂ<sup>\*2</sup>, MONICA CATANĂ<sup>2</sup>,  
ANDA GRAȚIELA BURNETE<sup>2</sup>, MONICA ALEXANDRA LAZĂR<sup>2</sup>, NASTASIA BELC<sup>2</sup>,  
ANGEL MARTÍNEZ SANMARTÍN<sup>3</sup>

<sup>1</sup>University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Horticulture, 59 Marasti Blvd, District 1, 011464, Bucharest, Romania

<sup>2</sup>National Research and Development Institute for Food Bioresources – IBA Bucharest, 6 Dinu Vintila Street, 021102, Bucharest, Romania

<sup>3</sup>Centro Tecnológico Nacional de la Conserva y Alimentación, Calle Concordia s/n. 30500. Molina de Segura Murcia, Spain

### Abstract

The analytical methods were developed and validated for separation, detection and quantification of water-soluble vitamins (C, B2, B5, B6 and B7) from frozen fruits by high performance liquid chromatography coupled with high resolution mass spectrometry. Extraction of vitamin C was achieved in 0.1% formic acid solution at room temperature, and extraction of B-group vitamins in 0.1% formic acid solution at 70°C. Extracts purification was achieved on SPE C18 cartridges, 500 mg/3 mL. Water-soluble vitamins and hippuric acid (IS) were separated on a reverse-phase C18 Hypersil GOLD aQ 150 x 2.1 mm, 3 µm particle size, using mobile phases consisting of 995 mL water LC-MS, 5 mL 2M ammonium formate, 1 mL formic acid (A) and 995 mL metanol LC-MS, 5 mL 2M ammonium formate, 1 mL formic acid (B). Vitamin C was detected and quantified in the Electrospray Ionization negative ion mode (ESI-), and B-group vitamins in the Electrospray Ionization positive ion mode (ESI+). Hippuric acid (internal standard) was detected both the ESI- and ESI+ ion modes. The methods have a good sensitivity (vitamin C: LOD = 340.32 µg/L and LOQ = 1031.27 µg/L; vitamins B: LOD = 0.862-8.451 µg/L and LOQ = 2.585-25.355 µg/L). The methods showed a good precision, RSD < 10% for water-soluble vitamins analysis in frozen fruits. Methods developed and validated were applied for determination of water-soluble vitamins content of frozen fruits. Frozen Aronia melanocarpa fruits have the highest vitamin C content (39.785 mg/100g), and the frozen strawberries the highest vitamin B2 content (1.735 mg/kg), vitamin B5 content (3.703 mg/kg) and vitamin B6 content (3.553 mg/kg), respectively. The vitamin B7 content of the frozen fruits taken into study was low, ranging from 0.375 to 0.615 mg/100g.

### Keywords

Water-soluble vitamins, frozen fruits, liquid chromatography, mass spectrometry.

**To cite this article:** ASĂNICĂ AC, CATANĂ L, CATANĂ M, BURNETE AG, LAZĂR MA, NB, ANGEL SANMARTÍN M. Internal Validation of the Methods for Determination of Water-Soluble Vitamins from Frozen Fruits by HPLC-HRMS. *Rom Biotechnol Lett.* 2019; 24(6): 1000-1007. DOI: 10.25083/rbl/24.6/1000.1007

✉ \*Corresponding author: LUMINIȚA CATANĂ, National R&D Institute for Food Bioresources IBA Bucharest, 6 Dinu Vintilă Street, District 2, 021102, Bucharest, Romania  
Phone: +4021.210.91.28; +4021.211.36.39  
E-mail: [e-mail: lumi\\_catana@yahoo.co.uk](mailto:lumi_catana@yahoo.co.uk)

## Introduction

Consumption of fruits and vegetables plays an important role in preventing diseases and health maintaining (CHO et al, 2004). Fruits and vegetables are important elements in a healthy diet due to their low calorie and fat content, but are important sources of vitamins, minerals and fibers (KYUREGHIAN et al, 2010). Frozen vegetables can be an alternative to the fresh ones, as freezing allows the storage, transport and safe consumption of these foods during entire year.

Vitamins are micronutrients that need to be introduced into the diet in small amounts because these are not synthesized by human body (BELITZ et al, 2004). Vitamins are biologically active organic compounds, involved in metabolic and physiological functions in the human body. Depending on their solubility, vitamins are classified into two groups: water-soluble vitamins (WSV) (B-group vitamins and vitamin C) and fat-soluble vitamins (FSV) (A, E, D, and K) (BALL, 2004). These compounds differ greatly in term of chemical composition, physiological action and nutritional importance in the human diet, even within the same group (FINGLAS et al, 1993).

Traditionally, methods for determination of vitamins involve the individual analysis of each vitamin, using various physical, chemical and biological methods (SANTOS et al, 2012). For some vitamins (vitamins B5, B6, B9 and B12) microbiological assays are reference methods. These methods are very sensitive, but very laborious (BALL, 2006; BLAKE, 2007; CHEN et al, 2009). 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 as 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 (GENTILI et al, 2008). 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 (GENTILI et al, 2008).

SANTOS et al (2012) developed and validated 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. 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 sequential determination of a whole range of free water and fat-soluble vitamins, with different chemical structures, in real complex samples. The mean recoveries were ranged between 82.8% and

104.8% in case of water-soluble vitamins, and between 87.5% and 105.3%, respectively, in case of fat-soluble vitamins (SANTOS et al, 2012).

Tayadea et al (2013) developed and validated a rapid method to analyze sequentially fat (A, E, D2, D3, K1, and K2) and WSV (B1, B2, two B3 vitamins, B5, B6, B7, B9, and B12) in *Rhodiola imbricata* root from trans-Himalaya with rapid resolution liquid chromatography/tandem mass spectrometry. The mean recoveries were ranged between 88.95% and 107.07%. Sensitivity and specificity of this method allowed the limits of detection (LOD) and limits of quantitation (LOQ) of the analytes at ppb levels. The linear range was achieved for fat- and water-soluble vitamins at 100–1000 ppb and 10–100 ppb (Tayadea et al, 2013).

BOUZARI et al (2014) assessed vitamin retention in eight fruits and vegetables (corn, carrots, broccoli, spinach, peas, green beans, strawberries, and blueberries) during refrigerated and frozen storage, using high performance liquid chromatography (HPLC-DAD) and liquid chromatography coupled with mass spectrometry (LC-MS). Samples of each commodity were harvested, processed, and analyzed for nutrient content at three storage times per treatment. Ascorbic acid showed no significant difference for five out of the eight commodities and was higher in frozen samples than fresh for the remaining three commodities. Apart from broccoli and peas, which were higher and lower in frozen vs fresh samples, respectively, none of the commodities showed significant differences with respect to riboflavin content. Three commodities had higher levels of  $\alpha$ -tocopherol in the frozen samples, while the remaining commodities showed no significant difference between fresh and frozen.  $\beta$ -Carotene was not found in significant amounts in blueberries, strawberries, and corn. Peas, carrots, and spinach were lower in  $\beta$ -carotene in the frozen samples, while green beans and spinach showed no significant difference between the two storage methods (BOUZARI et al, 2014).

This paper presents the study for internal validation of the methods for determination of WSV from frozen fruits by high-performance liquid chromatography coupled with high resolution mass spectrometry (HPLC-HRMS). Moreover, assessing the water-soluble content of frozen fruits, was performed.

## Materials and Methods

### 1. Materials

In order to internal validate the methods for determination of water-soluble vitamins from frozen fruits by HPLC-HRMS, there were used frozen fruits which were obtained within the Pilot Experiments Plant for Fruits and Vegetables Processing, of IBA Bucharest. Fresh fruits were purchased from indigenous farmers, except the fruits of *Aronia melanocarpa*, which were provided by University of Agronomic Sciences and Veterinary Medicine Bucharest (Faculty of Horticulture).

## 2. Methods

### Reagents and Materials

Vitamins standards (purity > 99.0%), namely, ascorbic acid (C), riboflavin (B2), D-calcium pantothenate (B5), pyridoxine (B6), D-biotin (B7), were purchased from Sigma Aldrich. Also, the internal standards, hippuric acid and ammonium formate eluent additive for LC-MS were purchased from Sigma Aldrich. Water for LC-MS Optigrade, Formic acid UHPLC-MS, Methanol LC-MS were purchased from LGC Standards.

### Standard solutions

Individual WSVs stock standard solutions and hippuric acid solution were prepared and kept in the dark under refrigeration at 3°C until analysis. Vitamin B stock solutions were stored in these conditions for a month. Vitamin C stock solution was daily prepared and was kept in the dark under refrigeration at 3°C until analysis. Ascorbic acid (5 mg/mL), D-calcium pantothenate (1 mg/mL) and pyridoxine (1mg/mL), and hippuric acid were prepared in 0.1% formic acid solution. D-biotin (0.05 mg/mL) was prepared in water. Also, riboflavin (0.05 mg/mL) was prepared in 0.7% NH<sub>3</sub> solution.

During method validation, a mixture of WSVs was prepared daily by dilution of the individual vitamins stock solutions with 0.1% formic acid solution.

Calibration curves ( $y = ax + b$ ) were constructed by plotting the peak area ratios ( $y$ ) of analyte to internal standard versus the concentrations ( $x$ ) of the calibration standards. As internal standard (IS) was used hippuric acid. In case of calibration curve of vitamin C, concentration of hippuric acid (IS) was 1000 µg/L, and in case of calibration curves of B-group vitamins, concentration of hippuric acid (IS) was 500 µg/L.

Vitamin C and hippuric acid (internal standard) were detected and quantified in Electrospray Ionization negative mode (ESI-), having as characteristics ions: ion  $m/z = 175.02391$  (mass variation of max 5 ppm), identified at retention time  $1.14 \text{ min} \pm 30 \text{ s}$ , for vitamin C, and ion  $m/z = 178.04991$  (mass variation of max 5 ppm), identified for hippuric acid.

B-group vitamins and hippuric acid (internal standard) were detected and quantified in the Electrospray Ionization positive mode (ESI+) based on the following characteristics ions:

✓ Vitamin B2 was identified at retention time  $6.05 \text{ min} \pm 30 \text{ s}$ , through ion  $m/z = 377.14493$  (mass variation of max 5 ppm)

✓ Vitamin B5 was identified at retention time  $4.92 \text{ min} \pm 30 \text{ s}$ , through ion  $m/z = 220.11757$  (mass variation of max 5 ppm)

✓ Vitamin B6 was identified at retention time  $2.06 \text{ min} \pm 30 \text{ s}$ , through ion  $m/z = 170.08086$  (mass variation of max 5 ppm)

✓ Vitamin B7 was identified at retention time  $5.92 \text{ min} \pm 30 \text{ s}$ , through ion  $m/z = 245.09503$  (mass variation of max 5 ppm)

✓ Hippuric acid was identified at retention time  $5.48 \text{ min} \pm 30 \text{ s}$ , through ion  $m/z = 180.06511$  (mass variation of max 5 ppm).

### Sample preparation

Extraction of vitamin C was achieved in 0.1% formic acid solution at room temperature, and extraction of B-group vitamins in 0.1% formic acid solution at 70°C. Then, samples were centrifugated (centrifuge Eppendorf 5408 R) at 10000 rpm, at 4°C for 50 min. Supernatant was collected in a centrifuge vial of 50 mL and purified on SPE cartridges (Finisterre) C18, 500 mg/3 mL. For extract purification on SPE cartridges (Finisterre) C18, 500 mg/3 mL, were performed the following steps:

- Cartridge conditioning with 3 mL LC-MS methanol and 3 mL LC-MS water with pH = 4.2 (flow 1 mL/min);

- Introduction of 1.5 mL extract in cartridge (flow 1 mL/min);

- Elution of water-soluble vitamins with 1.5 mL LC-MS water with pH = 4.2 and 3 mL LC-MS methanol (flow 1 mL/min);

- Homogenisation of the purified extract of sample, through vortexation for 3 min.

Then, 1 mL of the purified extract was evaporated to dryness, under nitrogen atmosphere, at  $40^\circ\text{C} \pm 0.2^\circ\text{C}$ . Residue was resolved in 0.1% formic acid solution. Also, it was added internal standard (hippuric acid). In case of the extract obtained for analysis of vitamin C, concentration of internal standard was 1000 µg/L, and in case of that obtained for determination of B-group vitamins it was 500 µg/L. Then, residue, resolved in 0.1% formic acid solution, is filtrated through PTFE syringe filter (0.45 µm), introduced in an autosampler amber vial of 1.8 mL and analyzed by LC-MS.

### Parameters and conditions of HPLC-HRMS methods for determination of water-soluble vitamins from frozen fruits

The separation of water-soluble vitamins and hippuric acid was carried out using an Accela HPLC system consisting vacuum degasser, quaternary pump, autosampler with PELTIER sample temperature control, column compartment with PELTIER temperature control, Diode Array Detector (Thermo Scientific) equipped with a C18 Hypersil GOLD aQ 150 x 2.1 mm, 3 µm particle size. The sample thermostat was maintained at 4°C and the column at 40°C. The injected sample volume was 25 µL and the flow rate of the mobile phase was 0.4 mL/min. The mobile phases consisting of A (995 mL water LC-MS, 5 mL 2M ammonium formate, 1 mL formic acid) and B (995 mL methanol LC-MS, 5 mL 2M ammonium formate, 1 mL formic acid). The chromatographic separation was performed using gradient elution (Table 1).

**Table 1.** Gradient elution for the chromatographic separation of water-soluble vitamins, frozen fruits extract

Time (min)	Mobile phases	
	A	B
0.0	98	2
2.5	98	2
5.0	40	60
6.0	40	60
6.5	98	2
17.0	98	2

The mass spectral analysis was performed on an LTQ Orbitrap XL™ Hybrid FT Mass Spectrometer equipped with an ESI interface operating in negative and positive ion mode, at Resolution = 60000 (Analyzer: FTMS). In positive ion mode the operation parameters were: Mass range: 100 – 700; Heater Temperature (°C): 275; Sheath Gas Flow Rate (arb): 40; Aux Gas Flow Rate (arb): 20; I Spray Voltage (kV): 4; Capillary Temperature (°C): 350; Capillary Voltage (V): 10; Tube Lens (V): 50. In negative ion mode the operation parameters were: Mass range: 100 – 250; Heater Temperature (°C): 275; Sheath Gas Flow Rate (arb): 50; Aux Gas Flow Rate (arb): 40; Spray Voltage (kV): 4; Capillary Temperature (°C): 275; Capillary Voltage (V): -10; Tube Lens (V): -100.

### Validation studies

In the case of methods for determination of WSVs from frozen fruits it was achieved an “*in house*” validation study, being evaluated the following performance characteristics: linearity, accuracy, precision (repeatability, reproducibility), selectivity and sensitivity (limit of detection, limit of quantification).

### Statistical analysis

All analyses were performed in triplicate and the data are presented as mean ± standard deviation. Regarding validation parameters, average for concentration values (expressed as µg/L and mg/100 g), standard deviation in

repeatability SD(r) and reproducibility conditions SD(R), as well as relative standard deviation in repeatability RSD(r) and reproducibility conditions RSD(R) in % were calculated using Microsoft Excel.

## Results

### Performance of the method

#### Calibration curve and linearity

Linearity was investigated with the aid of a regression line with 6 calibration levels by the method of least squares. There were achieved the calibration curves of the water-soluble vitamins taken into study, in the following concentration ranges: 1500 – 10000 µg/L in case of vitamin C; 21.788 – 261.450 µg/L and 87.150 – 1394.40 µg/L in case of vitamin B2; 29.88 – 358.56 µg/L and 358.56 – 1912.32 in case of vitamin B5; 10.334 – 124.002 µg/L and 124.002 – 661.344 µg/L in case of vitamin B6 and B7. Parameters of calibration curves (regression equations and regression coefficient R<sup>2</sup>) and relative standard deviation RSD(r) are presented in Table 2. The calibration model was considered correct if RSD (r) was within the limits of ± 10% for all the levels investigated. The correlation was assessed to be linear for a value greater than 0.99 for the regression coefficient (R<sup>2</sup>). The results showed that RSD(r) for all the levels of concentration studied ranged between 0.3 and 8.2%, being within the limits of ± 10%.

**Table 2.** Parameters of calibration curves and RSD (r) for all the levels of concentration

Analyte	Concentration range (µg/L)	Linear regression equation	Regression coefficient (R <sup>2</sup> )	RSD (r) (%)
Vitamin C	1500 – 10000	$y = 0.0002x - 0.2528$	0.9990	1.32 – 3.05
Vitamin B2	21.788 – 261.450	$y = 0.001x - 0.0012$	0.9991	0.9 – 8.00
	87.150 – 1394.40	$y = 0.0011x + 0.0494$	0.9959	0.6 – 7.20
Vitamin B5	29.88 – 358.56	$y = 0.0026x - 0.0125$	0.9994	0.3 – 3.60
	358.56 – 1912.32	$y = 0.0022x + 0.1504$	0.9981	0.6 – 4.20
Vitamin B6	10.334 – 124.002	$y = 0.0218x + 0.155$	0.9925	0.6 – 7.40
	124.002 – 661.344	$y = 0.0125x + 1.6471$	0.9969	0.6 – 3.10
Vitamin B7	10.334 – 124.002	$y = 0.0039x - 0.0088$	0.9998	0.6 – 8.20
	124.002 – 661.344	$y = 0.0035x + 0.0828$	0.9978	0.6 – 2.20

The linearity range of the methods for determination of WSV from frozen fruits by HPLC-HRMS was verified according to ISO8466-1:1990. The linearity range of the method for vitamin C analysis from frozen fruits was in the range of: 1071.734 – 8028.023 µg/L (linear regression equation:  $y = 0.0002x - 0.2416$ ;  $R^2 = 1$ ). The linearity range of the method for B-group vitamins analysis from frozen fruits was in the next ranges: 4.307 – 321.113 µg/L, for vitamin B2 (linear regression equation:  $y = 0.0011x - 0.0102$ ;  $R^2 = 0.9976$ ); 25.355 – 536.045 µg/L, for vitamin B5 (linear regression equation:  $y = 0.0026x - 0.0123$ ;  $R^2 = 1$ ); 13.250 – 225.488 µg/L, for vitamin B6 (linear regression equation:  $y = 0.0218x + 0.155$ ;  $R^2 = 1$ ); 2.585 – 156.719 µg/L for vitamin B7 (linear regression equation:  $y = 0.0039x - 0.0088$ ;  $R^2 = 1$ ).

### Accuracy

In the absence of certified reference materials for vitamins taken into study, accuracy was investigated by recovery (Thompson et al, 2014). Samples of frozen fruits with determined concentration of vitamin C and vitamin B were spiked with known concentrations of analytes. These samples were analyzed by HPLC-HRMS method and it was determined the concentration of vitamin C and vitamins B (B2, B5, B6, B7) (there were analyzed 7 parallel samples for each addition level, and each sample was analyzed in triplicate). After performing HPLC-HRMS analysis, the recovery of each vitamin was calculated by

$$R (\%) = [(C_s - C_p)/C_a] \times 100,$$

where R(%) is percent recovery,  $C_s$  is total vitamin content in the spiked sample,  $C_p$  is endogenous vitamin content in the sample, and  $C_a$  is the amount of vitamin standard added to the sample.

In case of the method for determination of vitamin C from frozen fruits, there were obtained average recovery factors in the range 92.50 – 95.23%, as follows: strawberries,  $R = 92.50\%$ ; sour cherries,  $R = 93.25\%$ ; *Aronia melanocarpa*,  $R = 93.85\%$ ; raspberries,  $R = 94.10\%$ ; bilberries,  $R = 95.23\%$ .

In case of the method for determination of B-group vitamins (B2, B5, B6 and B7) from frozen fruits, there were obtained average recovery factors in the range 92.85% – 97.20%, as follows:

- ✓ Vitamin B2:  $R = 92.85\%$  for strawberries;  $R = 93.18\%$  for fruits of *Aronia melanocarpa*;
- $R = 93.45\%$  for bilberries;  $R = 94.80\%$  for raspberries

- ✓ Vitamin B5:  $R = 96.70\%$  for strawberries;  $R = 96.85\%$  for fruits of *Aronia melanocarpa*;
  - $R = 97.05\%$  for bilberries;  $R = 97.20\%$  for raspberries
  - ✓ Vitamin B6:  $R = 93.15\%$  for strawberries;  $R = 93.55\%$  for fruits of *Aronia melanocarpa*
  - $R = 93.78\%$  for bilberries;  $R = 93.95\%$  for raspberries.
  - ✓ Vitamin B7:  $R = 93.42\%$  for strawberries;  $R = 93.65\%$  for fruits of *Aronia melanocarpa*;
  - $R = 93.95\%$  for bilberries;  $R = 94.25\%$  for raspberries
- The obtained results for recovery are in accordance with the provisions of the Commission Decision 2002/657/EC (Recovery = 80 – 120%).

### Precision

Precision was determined by repeatability (injection standards of each vitamin and analysis repeatability) and intra-laboratory reproducibility. Injection repeatability was achieved by carrying out 10 consecutive injections in a short period of time (the same day). Analysis repeatability (intra-day) was determined by 7 repeated analyses of the same sample, in the same day by the same analyst and under the same experimental conditions. For evaluating the intra-laboratory reproducibility, 6 samples have been analyzed by two analysts (analyst A—3 samples, analyst B—3 samples) of frozen fruits, performed in the same laboratory and using the same instrument. Repeatability was expressed as RSD(r). Intra-laboratory reproducibility was expressed as RSD(R). Performance parameters of the methods are presented in Tables 3 and 4. In case of the method for determination of vitamin C, RSD(r) was 1.85 and 1.96% for injection repeatability and 2.31% for repeatability analysis, respectively. Also, the RSD(R) in intra-laboratory reproducibility, was 3.72%. In case of the method for determination of B-group vitamins, RSD(r) was between 2.11 and 2.89% for injection repeatability and between 3.08 and 3.15% for repeatability analysis, respectively. Also, the RSD(R) in intra-laboratory reproducibility, was between 3.82 and 3.93%. The applied methods showed a good precision,  $RSD < 10\%$  for water-soluble vitamins analysis in frozen fruits and the results were within the limits set by the following regulations: Commission Decision 2002/657/EC, VICH GL49, CAC/GL 16.

**Table 3.** Performance parameters of the method for determination of vitamin C in frozen strawberries

Performance parameter		Vitamin C
Injection repeatability (vitamin C analytical standards; n =10)	C (µg/L)	4511.59±83.37
	RDS (r) (%)	1.85
Injection repeatability (n =10)	C (mg/100g)	28.98±0.57
	RDS (r) (%)	1.96
Analysis repeatability (intra-day; n= 7)	C (mg/100g)	29.86±0.69
	RDS (r) (%)	2.31
Intra-laboratory reproducibility (n = 6)	C (mg/100g)	32.12±1.19
	RDS (R) (%)	3.72

**Table 4.** Performance parameters of the method for determination of B-group vitamins from frozen fruits (strawberries, bilberries)

Performance parameter		Analyte			
		Vitamin B2	Vitamin B5	Vitamin B6	Vitamin B7
Injection repeatability (mix of vitamins B analytical standards)	C (µg/L)	559.46±13.85	721.06±16.11	268.01±5.66	258.75±6.35
	RDS (r) (%)	2.47	2.23	2.11	2.45
Injection repeatability (strawberries)	C (mg/kg)	1.559±0.044	2.756±0.077	1.124±0.032	0.521±0.015
	RDS (r) (%)	2.82	2.80	2.89	2.85
Analysis repeatability (intra-day) (bilberries)	C (mg/kg)	0.670±0.021	1.812±0.056	0.963±0.030	0.472±0.015
	RDS (r) (%)	3.12	3.08	3.15	3.10
Intra-laboratory reproducibility(strawberries)	C (mg/kg)	1.804±0.071	3.109±0.121	1.250±0.050	0.612±0.023
	RDS (R) (%)	3.93	3.88	3.84	3.82

### Selectivity

Selectivity was demonstrated by chromatographic identification, separation and quantification of (vitamin C and B-group vitamins) in the presence of other components from frozen fruits. Each vitamin taken in the study is identified at a specific retention time and is also identified and quantified based on the characteristic ion (figures 1-5).

### Sensitivity

Sensitivity was characterized by limit of detection (LOD) and limit of quantification (LOQ). Assessment of

LOD and LOQ for water-soluble vitamins analysis in frozen fruits by HPLC-MS was performed applying ICH approach (ICH, 2005). Also, the LOD and LOQ of the method for determination of B-group vitamins from frozen fruits have been experimentally determined. It is worth noting that the values determined experimentally were lower than those calculated. The criteria imposed for LOQ were: S/N > 10, precision (expressed as RSD (r) ≤ 20%) and accuracy (expressed as recovery in the range 80-120%). LOD and LOQ for water-soluble vitamins from frozen fruits are presented in Table 5.

**Table 5.** Sensitivity of the method for determination of vitamin C and B-group vitamins from frozen fruits

No.	Analyte	LOD (µg/L) (ICH method)	LOQ (µg/L) (ICH method)	LOD (µg/L) (experimental)	LOQ (µg/L) (experimental)
1.	Vitamin B2	9.917	30.052	1.435	4.307
2.	Vitamin B5	11.408	34.570	8.451	25.355
3.	Vitamin B6	13.544	41.044	4.416	13.250
4.	Vitamin B7	2.288	6.933	0.862	2.585
5.	Vitamin C	340.32	1031.27	-	-

## Discussions

Methods developed and validated were applied for determination of WSVs content of frozen fruits, which were obtained within the Pilot Experiments Plant for Fruits and Vegetables Processing, of IBA Bucharest. Results obtained are presented in Table 6. Content of vitamin C of the frozen fruits varied in the range 10.675-39.785 mg/100g (the minimum value was registered in case of sour cherries and the maximum one in case of *Aronia melanocarpa* fruits). The vitamin C content of frozen *Aronia melanocarpa* fruits in this study is about 2.32 times higher than that reported by Karakasova et al (2014) (17.15 mg/100g). Vitamin C is an important antioxidant, because reacts with free radicals, reducing reactive oxygen species to protect against the oxidation of lipids, proteins, and DNA. It also functions

as a cosubstrate for a series of enzymes, including those involved in collagen synthesis (Combs and McClung, 2017). To provide antioxidant protection a recommended dietary allowance of 90 mg day<sup>-1</sup> for adult men and 75 mg day<sup>-1</sup> for women has been established.

Among B-group vitamins taken into study, vitamin B5, recorded the highest values in the range 1.745-3.703 mg/kg (the minimum value was registered in case of bilberries and the maximum one in case of strawberries). The highest vitamin B2 content (1.735 mg/kg) was recorded in the case of strawberries, the obtained value being 1.23 times higher than that reported by BOUZARI et al (2014): 1.41 mg/kg. Sour cherries and *Aronia melanocarpa* fruits had the highest values of the vitamin B6 content: 1.435 mg/kg and 1.132 mg/kg, respectively. The vitamin B7 content of frozen fruits analyzed in this study was low, in the range 0.375 - 0.615 mg/kg.

**Table 6.** Content in water-soluble vitamins of frozen fruits

Fruits	Vitamin C (mg/100g)	Vitamin B2 (mg/kg)	Vitamin B5 (mg/kg)	Vitamin B6 (mg/kg)	Vitamin B7 (mg/kg)
Bilberries	23.750±0.216	0.575±0.005	1.745±0.017	0.908±0.007	0.375±0.003
Strawberries	35.874±0.323	1.735±0.013	3.703±0.023	3.553±0.023	0.563±0.004
Raspberries	28.435±0.253	0.412±0.004	3.405±0.022	0.925±0.008	0.437±0.003
Sour cherries	10.675±0.096	0.685±0.006	2.538±0.017	1.435±0.012	0.385±0.002
<i>Aronia melanocarpa</i>	39.785±0.345	0.873±0.008	2.845±0.019	1.132±0.010	0.615±0.005

## Conclusions

The analytical methods were developed and validated for separation, detection and quantification of water-soluble vitamins (C and B2, B5, B6 and B7) in frozen fruits by HPLC-HRMS. Method proposed for determination of vitamin C has a good sensitivity (LOD = 340.32 µg/L and LOQ = 1031.27 µg/L). Also, method proposed for determination of B-group vitamins has a good sensitivity (LOD = 0.862 - 8.451 µg/L and LOQ = 2.585-25.355 µg/L).

The methods showed a good precision, RSD < 10% for water-soluble vitamins analysis in frozen fruits. Methods developed and validated were applied for determination of water-soluble vitamins content of frozen fruits which were obtained within the Pilot Experiments Plant for Fruits and Vegetables Processing, of IBA Bucharest. Frozen *Aronia melanocarpa* fruits recorded the highest vitamin C content (39.785 mg/100g). Also, frozen strawberries recorded the highest values for the content of vitamin B2 (1.735 mg/kg), vitamin B5 (3.703 mg/kg) and vitamin B6 (3.553 mg/kg), respectively. Vitamin B7 content was low in the case of frozen fruit species taken into study. According to the results obtained, frozen fruits can be considered as sources of water-soluble vitamins in a healthy diet.

## Acronyms and abbreviations

ESI- – Electrospray Ionization negative mode  
 ESI+ – Electrospray Ionization positive mode  
 FSV– fat-soluble vitamins  
 HPLC – High performance liquid chromatography  
 HPLC-DAD – high performance liquid chromatography- Diode array detector  
 HPLC-HRMS – high-performance liquid chromatography coupled with high resolution mass spectrometry  
 LC-DAD – Liquid chromatography-Diode array detector  
 LC-MS, LC-MS/MS – Liquid chromatography–mass spectrometry  
 LOD – limit of detection  
 LOQ – limit of quantification  
 RSD(r) – relative standard deviation in repeatability  
 RSD(R) – relative standard deviation in reproducibility  
 WSV– water-soluble vitamins

## Acknowledgements

This study was achieved through Core Program, with the support of the Ministry of Research and Innovation (MCI), contract 29N/2016, project PN 16 46 01 03 and by a grant of the Romanian Ministry of Research and Innovation, CCCDI – UEFISCDI, project number PN-III-P1-1.2-PCCDI-2017-0662 / 12, within PNCDI III.

## References

1. BALL GFM, Vitamins In Foods: Analysis, Bioavailability, and Stability, CRC Press, Boca Raton, 1<sup>st</sup> edition/2005.
2. BELITZ HD, GROSCH W, SCHIEBERLE P. Vitamins. In Food Chemistry, (3<sup>rd</sup> revised edn). Springer-Verlag: Berlin Heidelberg, Germany, 2004.
3. Blake C. Analytical procedures for water-soluble vitamins in foods and dietary supplements: a review. *Anal Bioanal Chem.* 2007; 389(1):63-76. doi: 10.1007/s00216-007-1309-9
4. BOUZARI A, HOLSTEGE D, BARRETT DM. Vitamin Retention in Eight Fruits and Vegetables: A Comparison of Refrigerated and Frozen Storage. *Journal of Agricultural and Food Chemistry.* 2014; 63(3):957-962. doi: 10.1021/jf5058793.
5. CHEN P, ATKINSON R, WOLF WR. Single-Laboratory Validation of a High-Performance Liquid Chromatographic-Diode Array Detector-Fluorescence Detector/Mass Spectrometric Method for Simultaneous Determination of Water-Soluble Vitamins in Multivitamin Dietary Tablets. *J. AOAC Int.* 2009; 92(2): 680-687.
6. CHO EY, SEDDON JM, ROSNER B, WILLETT WC, HANKINSON SE. Prospective study of intake of fruits, vegetables, vitamins, and carotenoids and risk of age-related maculopathy. *Arch. Ophthalmol.* 2004; 122(6):883-892. doi: 10.1001/archoph.122.6.883
7. COMBS GF, MCCLUNG JP. The Vitamins (Fifth Edition), 2017; Academic Press. Chapter 10 - Vitamin C (p. 267-295).
8. FINGLAS PM, FAURE U, WAGSTAFFE PJ. Improvements in the determination of vitamins in



- food through intercomparisons and preparation of RMs for vitamin analysis within the BCR<sup>1</sup> programme. *Fresenius J. Anal Chem.* 1993; 345(2-4):180-184. doi: 10.1007/BF00322584
9. GENTILI A, CARETTI F, D'ASCENZO G, MARCHESE S, PERRET D, DI CORCIA D, ROCCA L. Simultaneous determination of water-soluble vitamins in selected food matrices by liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 2008, 22(13):2029-43. doi: 10.1002/rcm.3583
  10. KARAKASOVA L, BABANOVSKA-MILENKOVSKA F, STOJANOVA M, KARAKASOV B. Comparison of Qualities Properties of Fresh, Frozen and Solar Dried Chokeberry Fruits, 25<sup>th</sup> International Scientific-Experts Congress on Agriculture and Food Industry, 2014; Izmir, Turkey. DOI: 10.13140/RG.2.1.4506.1209
  11. KYUREGHIAN G, STRATTON J, BIANCHINI A, ALBRECHT J. Nutritional Comparison of Frozen and NonFrozen Fruits and Vegetables: Literature Review, 2010; The Food Processing Center, University of Nebraska-Lincoln.
  12. SANTOS J, MENDIOLA JA, OLIVEIRA MBPP, IBÁÑEZ E, HERRERO M. Sequential determination of fat- and water-soluble vitamins in green leafy vegetables during storage. *Journal of Chromatography A.* 2012; 1261:179-188. doi: 10.1016/j.chroma.2012.04.067
  13. TAYADEA AB, DHARA P, KUMARA J, SHARMA M, CHAURASIA OP, SRIVASTAVA RB. Sequential determination of fat- and water-soluble vitamins in *Rhodiola imbricata* root from trans-Himalaya with rapid resolution liquid chromatography/tandem mass spectrometry. *Analytica Chimica Acta.* 2013; 789:65-73. doi: 10.1016/j.aca.2013.05.062
  14. THOMPSON M, ELLISON SRL, WOOD R. Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report). *Pure Appl. Chem.* 2002; 74(5):835-855.
  15. ISO 8466-1:1990: *Water Quality. Calibration and Evaluation of Analytical Methods and Estimation of Performance Characteristics, Part 1: Statistical Evaluation of the Linear Calibration Function*, ISO: Geneva, 2010.
  16. European Commission; *Commission Decision of 12 August 2002 Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results*, (2002/657/EC), L 221/8-36.
  17. Veterinary International Conference on Harmonization (VICH); *VICH GL49-Guidance for Industry Studies to Evaluate the Metabolism and Residue Kinetics of Veterinary Drugs in Food – Producing Animals: Validation of Analytical Methods Used in Residue Depletion Studies*, VICH: Rockville, 2015.
  18. CAC/GL 16: *Codex Guidelines for the Establishment of a Regulatory Programme for Control of Veterinary Drug Residues in Foods, Part III – Attributes of Analytical Methods for Residue of Veterinary Drugs in Foods*, Rome, 1993, p. 41.
  19. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH); *Validation of Analytical Procedures: Text and Methodology Q2(R1)*; ICH: London, 2005.