DETERMINATION OF VITAMINS B AND C IN THE BIOMASS GROWN ON THE OLIVE MILL WASTEWATER IN SYRIA BY HPCL METHOD

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Abstract. The study includes the results of the pH effect of the extraction solution on water-soluble vitamins in the biomass that grown on the olive mill wastewater (resulting from Syrian olive presses) diluted by urea solutions (4%) 75:25 (OMW; Urea 4%) and grafted with Geoterichum hypha. The vitamins determined by high performance liquid chromatography (HPLC) after extracted it in different pH solutions; Phosphate solution pH = 7, Acetic acid solution 2% pH =2.6 and Sodium hydroxide solution 0.1N pH = 12. The results showed that B1, B3, B6, B7, B9 and C were found in the biomass in different percent amount, according to the extraction solution, but no vitamin B2 was present. .In the pH = 7 the percentage of vitamins in biomass was B6 = 0.006%, B7 = 0.11% and B9 =0.01%. In the acidic medium at pH = 2.6, the percentages of vitamins were B3 = 0.005%, B6 = 0.03%, B7 = 0.45%, B9 = 0.003% and C = 0.025%. In the alkali medium the percentages were as follows: B1 = 0.28%, B7 = 0.03% and B9 = 0.04%**.**

Keywords: olive mill wastewater; vitamins in biomass

Introduction

World olive oil production is estimated to around 2.9 million tons a year as Table 1. The EU countries alone produce 72 – 75 % of the world production and the Middle East countries produce 15 – 20% and the other countries about 7 – 10% (Malorgio & Felice, 2014). Extraction process yields oil (which accounts for 20 % of the total mass) and two by-products: a solid residue (30 % of the total), and a black wastewater (50 % of the total) called olive mill wastewater (OMW) (Martin et al., 1991). OMW is an important polluter due to dark colour and high concentration of organic and phenolic compounds, which make OMW toxic and resistant to biological degradation (Mekki et al., 2007). The organic load of OMW, expressed as chemical oxygen demand (COD; range 80 g L-1 to 200 g L-1) and biochemical oxygen demand (BOD; 50 g L-1 to 100 g L-1) is 200 to 400 times higher than in a typical municipal sewage (Cossu et al., 1993). The organic fraction includes sugars, tannins, polyphenols, polyalcohols, pec-

tins, and lipids. The phenolic fraction is recalcitrant to biodegradation and is responsible for several biological affects including phytotoxicity and antibiosis (Dragičević et al., 2010).

Table 1. World olive oil production (Malorgio & Felice, 2014)

Countries	Area (10^3ha)	Production (10^3 tons)	Consumption (10^3 tons)	Exports (10^3 tons)
European Union	5,000	2,161.1	1,876.1	419.7
Tunisia	1,850	156.7	36.0	132.0
Turkey	800	144.2	110.7	25.4
Morocco	950	105.8	80.0	12.0
Syria	700	152.0	114.4	23.5
Jordan	135	26.1	24.5	1.6
Argentina	100	22.3	5.3	17.0
Other	465	166.3	679.3	36.8
Total	10000	2,934.4	2,926.3	669.5

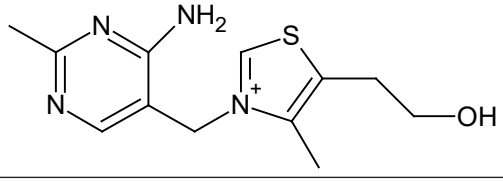
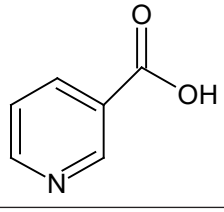
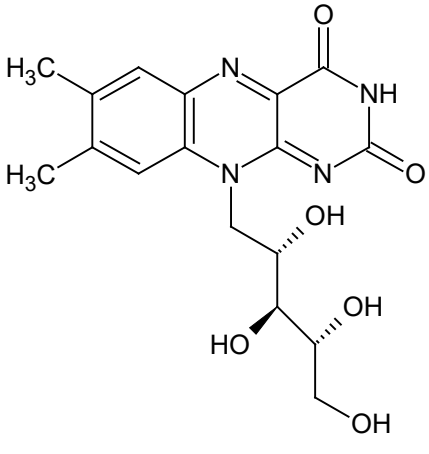
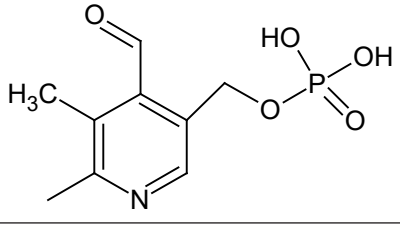
The number of olive presses in Syria reached 841, producing approximately 800 tons of liquid waste per year, which is a real environmental problem because polluted chemicals in soil and groundwater occur (Kholani & Malo, 2012).

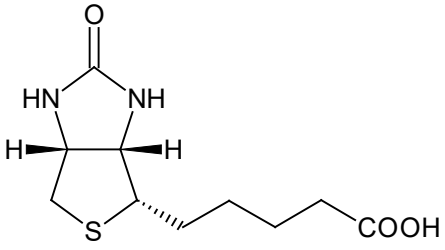
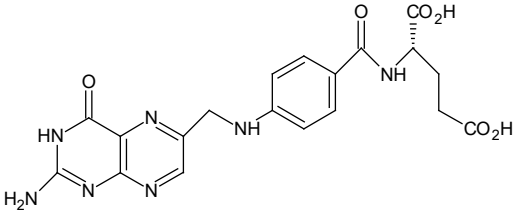
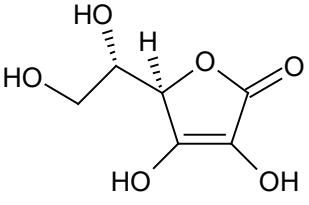
The problem of disposing of olive mill wastewater (OMW) remains unresolved, although, there are several attempts (Di Lecce et al., 2014). Some of these methods used to treat OMW from their chemical components (as compounds harmful to the environment) are both chemical methods and physicochemical methods: filtration of magnetic nanoparticles (Nassar et al., 2014); microfiltration membrane technology (Petrotos et al., 2014); biodegration treatment of olive mill wastewater by non-conventional yeasts (Gonçalves et al., 2009); biodegradation treatment through *Trichosporon cutaneum* and *Geotrichum candidum* (Dragičević et al., 2010); biodegradation treatment by thermophilic bacteria (Al-Qodah et al., 2015).

Some researchers investigated the biomass resulting from biodegradation treatment on OMW medium and found a high quantity of proteins which could be used in animal feeds (Kholani & Malo, 2012). This study includes the determination vitamins B and C in the biomass that grown the OMW diluted with 4% urea solution. Vitamins are complex organic substances found in small quantities in different foods, which should be presented in other nutrients to maintain vital functions. The vitamins are in two parts: ones are dissolved in fat and others are dissolved in water and these groups of vitamins play an important role in all the enzymes necessary to complete the various biological processes.

The body cannot produce vitamins so it is expected the food to supply them in the necessary quantities (Malo et al., 2011).

Table 2. Chemical structure and general characteristics of water-soluble vitamins

Vitamin	Structure	General characteristics
Thiamine, or vitamin B1		Colorless and dissociate with light and heat
Niacin, or nicotinic acid or B3		Colorless and dissolve in hot water and dissociate in alkali pH
Ribovlavine, or B2		yellow color dissolves in the alkaline medium
Pyridoxine or pyridoxol or vitamin B6,		Colorless

Biotin or vitamin B7 or vitamin H or coenzyme R		Colerless
Folate, or folic acid or vitamin B9		yellow color dissolves in the alkaline medium
Ascorbic acid or Vitamin C		Colorless, stable in the acidic medium, dissipated by heat and light and ultraviolet radiation

Materials and methods

Instrumentation and chemicals

High performance liquid chromatograph (Knauer, Germany), centrifuge (Harmonic series, Taiwan). Organic and inorganic solvents of HPLC grade such as methanol and phosphoric acid were obtained from Merck (Germany). All water soluble vitamins: thiamin, riboflavine, nicotinic acid, pyridoxine, biotin, folic acid and ascorbic acid from Laboratory Rasayan; sodium hydroxide and acetic acid - from Panreac (Spain).

Samples collection and preparation: OMW collect

The OMW was collected from a local contemporary (Syria). The press was close to Damascus in countryside called (Adiliya), it collected immediately after the press and were saved by heating them 70C^{o} to inhibit enzymes from the microorganisms (Kholani et al., 2012). The samples were placed in sealed plastic containers and placed in refrigerator 4C^{o} (Takriti et al., 2009).

Preparation of the bio-material

Olive fruits were washed, and the knife was washed, then immersed in water and left in the room temperature for a week (Kholani et al., 2012). And then took the Geotrichum threads of Hypha (Ashkar, 2010) (Fig. 1). The Geotrichum Hypha was growed for seven days at 28C^{o} and in a medium containing 1.6 g/L monohydrogenate phosphate, 0.025 g calcium chloride, 0.5 g ammonium nitrate, 0.4 g mono-potassium phosphate, 0.2 g magnesium sulphate and 0.0025 g ion(III) chloride and 10 g sucrose (Chandra et al., 2011).

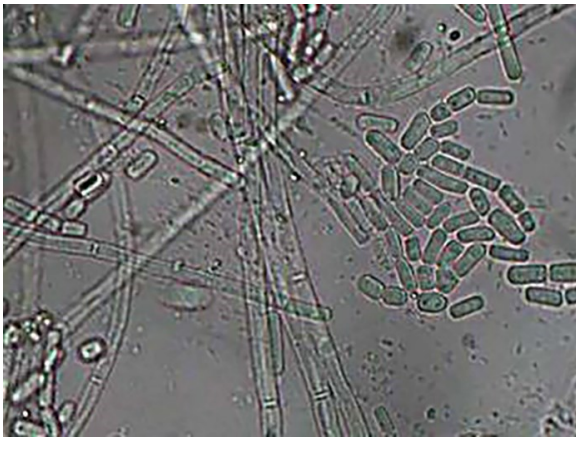


Figure 1. Microscopic checking for the Geotrichum threads of hypha

Figure 2. The biomass that growth on medium containing: monohydrogenate phosphate, calcium chloride, ammonium nitrate, mono-potassium phosphate, magnesium sulphate and ion(III) chloride and sucrose

Preparation of the solutions

Urea 4 %(w/V): dissolve 4g Urea in 10ml ddH_2O; *Buffer phosphate 0.1M pH=7*: dissolve 0.356 g $Na_2HPO_4.2H_2O$ in 10ml ddH_2O(A), dissolve 0.276 g NaH_2PO_4 in 10ml dd H_2O (B), add 3.6ml (A) to 1.4ml (B) then 5ml ddH_2O*; sodium hydroxide 0.1N pH=12*: dissolve 0.04g NaOH in 10 ml ddH_2O; *acetic acid 2% (v/v) pH= 2.6*: add 2ml acetic acid to 8ml ddH_2O; *Vitamins standard solution*: the aqueous stock solution (1mg/ml) of each vitamin was prepared every week, kept in the refrigerator, in an aluminum foil, protected from light; and working standards (in the range of 0.05 – 0.5mg/ml), were used daily by appropriate dilution of the stocks. The standard curves were made by running min. 6 different concentrations of each vitamin on HPLC system (Parlog et al., 2008).

Chromatographic HPLC protocol

For the separation of the six vitamins B and ascorbic acid, a Vertex plus Column 250x4.6mm Eurospher 100-5 C18 was used at ambient temperature. The mobile phase consisted of (A) *phosphoric acid*: water (0.01:100), (B) methanol, at a flow rate 0.8ml/min, UV/Vis, (230, 265,280) nm, the injection volume was 20 microliter, a multi-step gradient was optimized as Table 3.

Table 3. Gradient illusion program that used for vitamin separation

Rentation time (min)	Flow rate (ml/min)	B%	A%
-----	0.8	10	90
10	0.8	10	90
30	0.8	90	10
35	0.8	100	00
40	0.8	10	90
60	0.8	10	90

Biomass sample preparation

The hypha was added to OMW diluted by aqueous urea 4%(75:25) and kept for seven days at 28C°. the Biomess collected and washed by water then kept dry at room temperature (Kholani et al., 2012) (Fig. 3).

Figure 3. The biomass that growth on OMW diluted by aqueous urea 4%(75:25) and kept for seven days at 28C^{o}

Extraction vitamins from the biomass

Two methods that have been relied upon by researchers have been combined (Parlog et al., 2008; Daghestani & Al-Bahra, 2003).

The samples were either analyzed as buffer phosphate extract or after hydrolysis. The samples hydrolysis was done in Sodium Hydroxide (NaOH) 0.1N (alkaline Hydrolysis) or in 2 % acetic acid (CH_3COOH).

The procedure includes: weigh 1 g of the biomass and crush in a porcelain bowl, divide the powder into three equal sections in three plastic test tubes and in each place 0.3 g of them and add to each tube as follows: *first tube*: 5 ml 0.1M phosphate buffer; *second tube*: 5 ml of 0,1 N sodium hydroxide; *third tube*: 5 ml of 2 % acetic acid. The three tubes were placed on a 400 rpm magnetic stirring for four hours at a temperature 30C^{o}, then centrifuged (10000 rpm, 10 min, 20C^{o}), removed the solid mass and collected the supernatant liquid then precipited protein by acetone after adjusted pH =5, kept the tubes in refrigerator to second day, centrifuged (10000 rpm, 5min, 20C^{o}), then adjusted the supernatant to pH=7 by sodium hydroxide and finally transferred the abstract to three glass tubes sealed and covered with aluminum foil to prevent take apart vitamins by light until they were analyzed by the HPLC.

Results and discussion

HPLC Separation of vitamins using pure standards

At first, the HPLC analysis was performed using individual solutions, and then mixed standards as presented in Fig. 4. We selected the most suitable absorption wavelength in order to obtain their simultaneous detection since their spectral prop-

erties differ very seriously, the peak intensity was different at 260nm, the highest being for folic acid then thiamin and lowest B7 (or the Biotin). Then we saved the retention time for each vitamins as shown in Table 4.

Table 4. Specific retention time for the seven vitamins

Vitamin type	Retention time
B1	3.18
C	4.05
B3	4.48
B6	5.45
B9	21.4
B2	23.32
B7	27.7

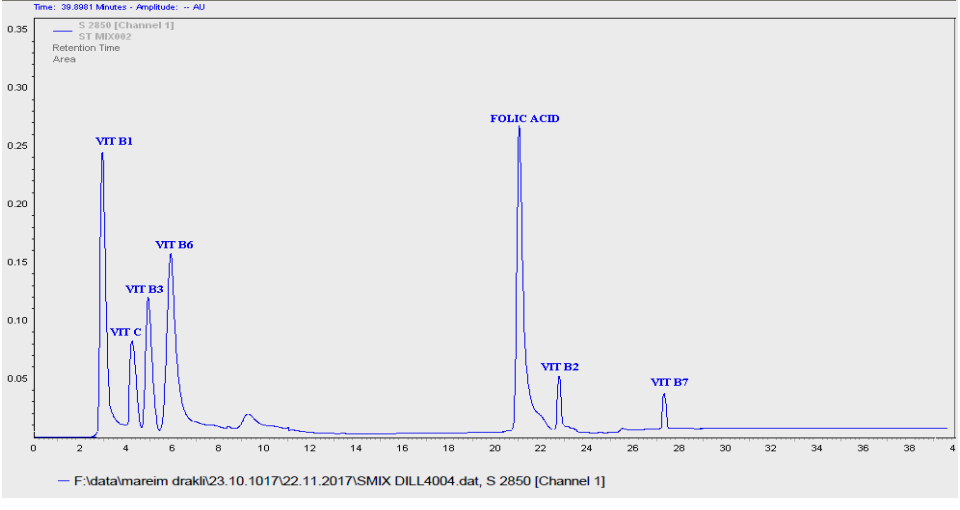


Figure 4. HPLC chromatogram of a mixture of pure standards of vitamins B1, B2, B3, B6, B7, B9, C

The standard chains of each vitamin were studied at the appropriate wavelengths (concentration in terms of area) (Fig.5). Let us take advantage of them later by setting the amount of vitamin according to the area of the appropriate rectal equation.

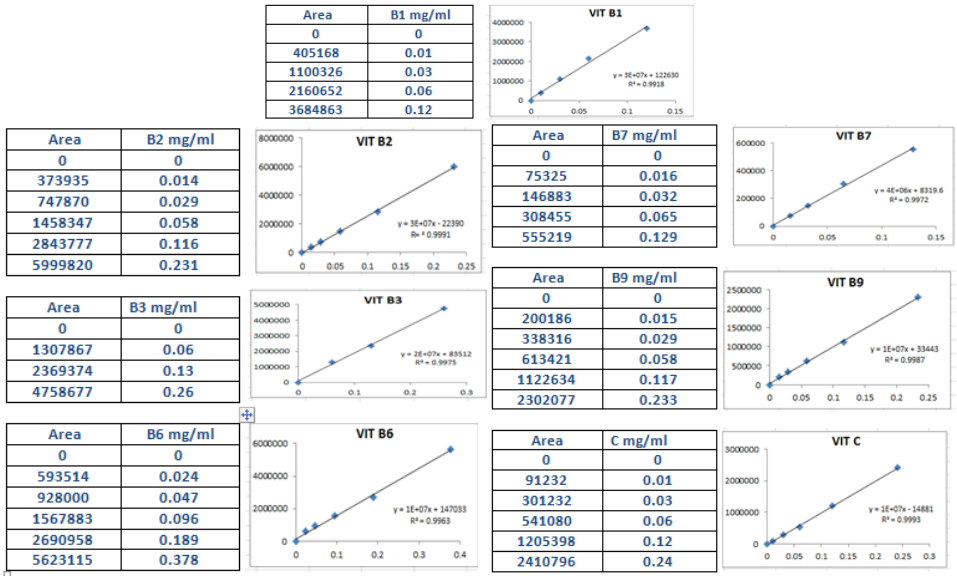


Figure 5. The standard chains of B1, B2, B3, B6, B7, B9,C (concentration mg/ml in terms of area)

HPLC analysis of water- soluble vitamins from biomass that growing on OMW: urea 4% and extraction the vitamins with phosphoric buffer pH=7

Fig. 6 presents the HPLC chromatogram of the biomass that growing on OMW: urea 4%, extracted in phosphoric buffer solution at 30C°. We can identify vitamins B6, B7, B9 and determine the percent amount of them in the biomass depending on standard chain end retention time as shown in Table 5.

Table 5. Percent amount vitamin amounts in the biomass depending on standard chain and retention time

Vitamin type	Retention time	Area	Percent amount %
B6	5.5417	360574	0.006
B9	21.65	55544	0.01
B7	27.317	98731	0.11

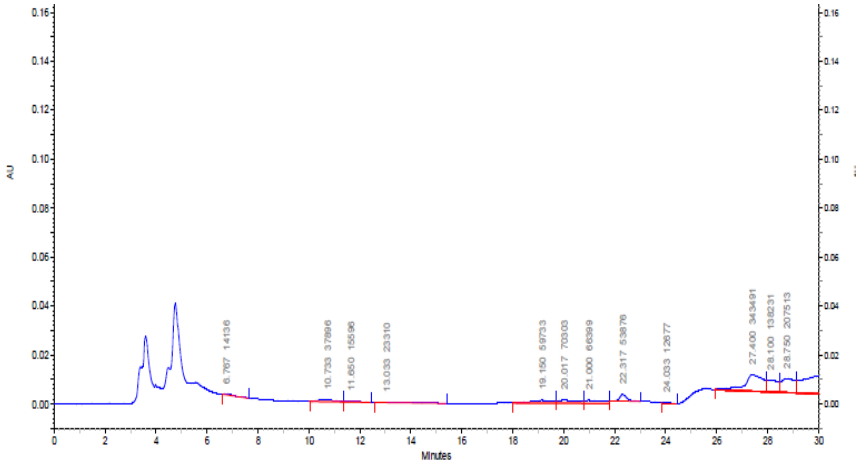


Figure 6. HPLC chromatogram of free vitamins from biomass extraction in phosphoric buffer

HPLC analysis of water- soluble vitamins from biomass that growing on OMW: urea 4% and extraction the vitamins with acetic acid pH=2.6

Fig. 7 presents the HPLC chromatogram of the biomass that growing on OMW: urea 4% extracted in acetic acid solution at 30C° pH=2.6. We can identify vitamins B3, B6, B7, B9, C and determine the percent amount of them in the biomass depending on standard chain end retention time as shown Table 6.

Table 6.1. Percent vitamin amounts in the biomass depending on standard chain and retention time

Vitamin type	Retention time	Area		Percent amount %
C	4.267	66200		0.025
B3	4.783	21957		0.005
B6	5.483	14613		0.03
B9	21.067	7944		0.003
B7	27.483	371016		0.45

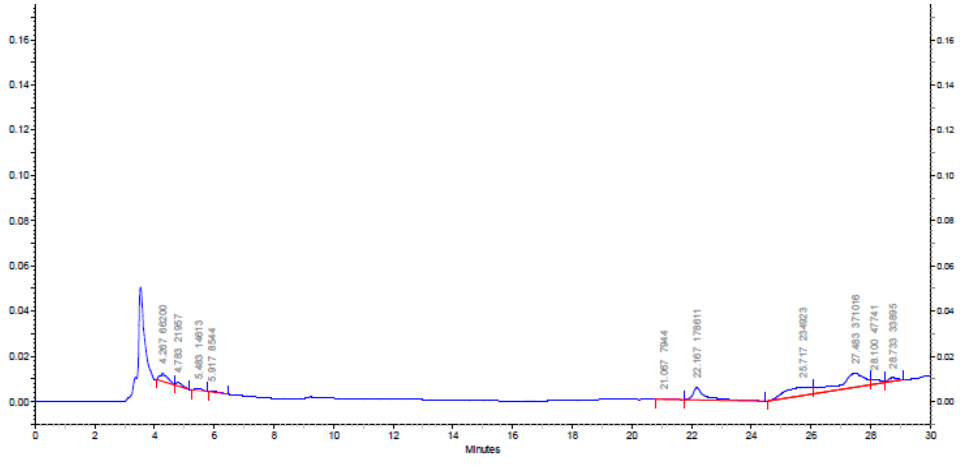


Figure 7. HPLC chromatogram of free vitamins from biomass extraction in acetic acid pH=2.6

HPLC analysis of water- soluble vitamins from biomass that growing on OMW: urea 4% and extraction the vitamins with hydroxide sodium pH=12

Fig. 8. presents the HPLC chromatogram of the biomass that growing on OMW: urea 4%, extracted in hydroxide sodium solution at 30C° pH=12. We can identify vitamins B1, B7, B9 and determine the percent amounts of them in the biomass depending on standard chain end retention time as shown Table 7.

Table 6.2. Percent vitamin amounts in the biomass depending on standard chain end retention time

Vitamin type	Retention time	Area	Percent amount %
B1	3.567	1845466	0.28
B9	21.5	114308	0.04
B7	27.417	32848	0.03

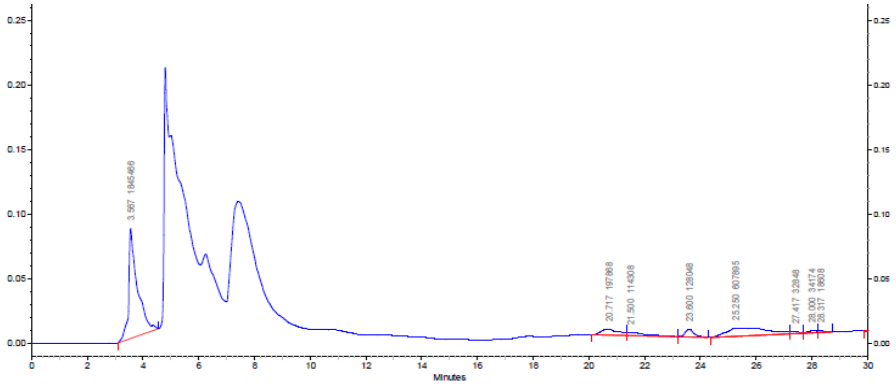


Figure 8. HPLC chromatogram of free vitamins from biomass extraction in hydroxide sodium pH=12

Table 7. Percent vitamin amounts in the biomass depending on pH extraction

pH extraction	B1 %	B2 %	B3 %	B6 %	B7 %	B9 %	C %
pH=2.6	----	----	0.005	0.03	0.45	0.003	0.025
pH=7	-----	----	----	0.006	0.11	0.01	----
pH=12	0.28	----	----	----	0.03	0.04	----

Conclusions

In this work we optimized the protocols for HPLC separation of seven water soluble vitamins: thiamin, riboflavin, nicotinic acid, pyridoxine, biotin, folic acid and ascorbic acid as pure standard solution, using an original and optimized protocol.

Table 7 explains the conclusion of this study: same vitamins were analyzed differently, as phosphoric buffer extract or as acid or alkaline hydrolyzed extracts. The phosphoric buffer extracts contain only small quantities of free vitamins B6, B7, B9 but B6, while the acid hydrolyzed extract contains five vitamins B3, B6, B7, B9 and C and the highest concentration is to B7 comparative with other vitamins and to other pH extract. It should be noted that ascorbic acid or vitamin C does not appear in other extraction medium.

Finally, the alkaline extract contained three vitamins B1, B7 and B9 and the highest concentration was to B1 comparative with other vitamins and to other pH extract. It should be noted that B1 or thiamine does not appear in other extraction medium.

Generally, we cannot depend the specific pH to extract vitamins, but each time we should extract on different pH, because some vitamins appear in the acidic pH and the other with an alkaline pH and other on pH=7. Although some vitamins are extracted in the three circles but their concentration varies from one medium to another.

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