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ENZYMATIC METHODS USED FOR DETERMINATION VITAMIN C AND STARCH CONTENT OF POTATO TUBERS

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Abstract: *The accuracy of the enzymatic analysis results depends on the enzymes specificity, respecting the reaction conditions and the mode of sample preparation. The principle of all enzymatic methods is based on measuring the absorbance values of some solutions to a specific wavelength (340 nm for starch and 578 nm for L – ascorbic acid). One of the objectives of the paper is to perform a comparative study of enzymatic methods for determination of starch and vitamin C in food products (newest Romanian potato varieties). The advantages of methods that uses enzymatic kits are the simplicity of the test, the safety, high sensitivity, precision and the equipment that do not require a costly investment. The essential condition for safety testing is to respect the work conditions imposed by the providing company. Because the methods are based on the use of enzymes, the pH and the temperature of the solutions have to be respected for safety outcomes.*
Keywords: *Starch, L-Ascorbic Acid, enzymatic kits, potato*

1. INTRODUCTION

Due to the advantages of using enzymatic kits (simplicity, safety, high sensitivity, equipment and devices that do not require a costly investment), these tests have gained in last years more and more land, being widely used in food industry (Figure 1). [1]

Figure 1: Advantages of - using enzymatic methods (including enzymatic kits).

This paper presents the results obtained using enzymatic kits for the determination of Starch and vitamin C in food products (potato tubers of several Romanian varieties). The methods used were based on the use of specific kits that can identify a specific component of the food matrix. To estimate the concentrations of the tested chemical compounds were found the absorbance values at:

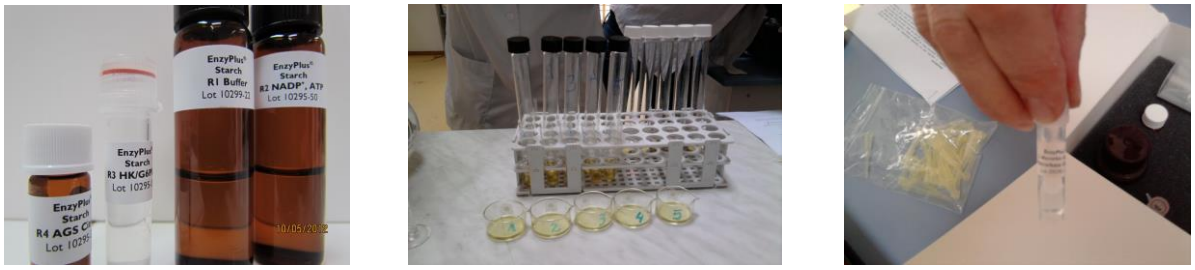
- ✓ 340nm ($NADPH + H^+$), for starch dosing [2]
- ✓ 586nm (Tetrazolium salt MTT [3–(4,5dimethylthiazolyl–2)–2,5–diphenyltetrazolium bromide]), for L-ascorbic acid dosing [3]

2. MATERIAL AND METHODS

2.1. Determination of starch in potato tubers

Biological material used for analysis: romanian varieties of potato tubers from the National Research Institute for Potato and Sugar Beet Brasov (Christian, Roclas, Sevastia, Castrum, Blue Congo).
The kits from the set "*EnzyPlus Starch*" (Biocontrol company, www.biocontrolsys.com) used for the determination of Starch have the following supplies components (Figure 2A):

- ✓ R1 – Buffer Solution: Imidazole buffer, magnesium chloride and preservative;
- ✓ R2 – $NADP^+$, ATP;
- ✓ R3 – Hexokinase (HK) (300 U/ml), Glucose– 6–Phosphate Dehydrogenase (G6PDH) (400 U/ml);
- ✓ R4 – (Lyophilized) Citrate, pH about 4.6, 250U amyloglucosidase (AGS).



A B C

Figure 2: A. The main components of the kit "EnzyPlus Starch" B. Samples and blanks prepared for incubation. C. Ascorbate oxidase - an important component of the "L ascorbic acid EnzyPlus" kit

The principle of the method arises from the reactions listes below, reactions which are actually also the main stages. (Brautechnische Analyzenmethoden, Band III, S. 1982)

I. Starch is hydrolysed to D–Glucose at pH 4.6 in the presence of the enzyme AGS (myloglucosidase):

$$starch + (n-1)H_2O \xrightarrow{AGS} n \cdot D - glucose$$

II. D–Glucose is phosphorylated in the presence of the enzym HK (Hexokinase):

$$D - glucose + ATP \xrightarrow{HK} G - 6 - P + ADP$$

III. Oxidation of Glucose–6–Phosphate in the presence of the enzyme G6PDH (Glucose– 6–Phosphate Dehydrogenase):

$$G - 6 - P + NADP^+ \xrightarrow{G6PDH} gluconate - 6 - phosphate + NADPH + H^+$$

The mean operations of the methods are the following:

1. Sample preparation: mince, treatment with dimethylsulfoxide / 2% hydrochloric acid, 60 min incubation at 60°C, cooling, dilution, pH adjustment to 4-5 with sodium hydroxide, 100 ml volumetric flask transfer, filltration.
2. Buffer solution preparation with AGS (R4 kit) dissolved in 5 ml distilled water.
3. Pipetting AGS buffer solution into cuvettes (blanks and samples) (Fig. 2B), pipetting 100 µl sample.
4. 15 min incubation at 60 ° C.
5. Adding 100 µl buffer solution (R1), 100 µl ATP and $NADP^+$ (R2) and 2 ml distilled water.
6. 3 min homogenization.
7. Reading absorbance value A_1.
8. Adding 20 µl solution HK and G6PDH (R3), reading absorbances (A_2) after 10 minutes.
9. Calculation of results: Determine the absorbance difference between sample and blank (A_2-A_1). The difference between the absorbances obtained from the blank samples is reduced from the difference between the absorbance valuesobtained from the samples being calculated as ΔA:

$$\Delta A = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{blank} \quad (1)$$

Sample concentration is calculated as follows:

$$C = \frac{V \cdot MW}{\varepsilon \cdot D \cdot V_s \cdot 1000} \cdot \Delta A(g/L) \quad (2)$$

where V – Final Volume (mL);
V_s – Sample Volume (mL);
MW – Molecular weight of Starch ($MW_{Glucose} - MW_{Water}$);
ε – extinction coefficient of NADPH at 340nm=6.3 (L/(mmol·cm));
d – light path (cm)

2.2. Determination of vitamin C in potato tubers

Biological material used for analysis: romanian varieties of potato tubers from the National Research Institute for Potato and Sugar Beet Brasov (Christian, Roclas, Sevastia, Castrum, Blue Congo).
The kits from the "*L ascorbic acid EnzyPlus*" set (Biocontrol company, www.biocontrolsys.com) used for the determination of vitamin C are made up of the following supplies:

- ✓ 2 x R1 Phosphate/Citrate Buffer pH 3.5 MTT
- ✓ R2 PMS
- ✓ 2 x R3 (Powder) Ascorbate Oxidase (700U) (Figure 2C)
- ✓ R4 L-Ascorbic Acid

The principle of the method is based on the fact that L-Ascorbic Acid and some reducing compounds (x-H_2) reduce the Tetrazolium salt MTT [3–(4,5dimethylthyazolyl–2)–2,5– diphenyltetrazolium bromide] in the presence of an electron carrier PMS (5–methylphenazinium methosulfate) at pH 3.5 to a formazan.[4,5]. In the test the amount of reducing substances was estimated.

$$L-Ascorbate(x-H_2)+MTT \xrightarrow{PMS} Dehydroascorbate(x)+MTT-formazan+H^+$$

For the specific determination of L – Ascorbate, in a sample blank assay only the L-Ascorbate fraction as part of all reducing substances from the sample, is oxidized by Ascorbate Oxidase (AAO) in the presence of oxygen from the air. The Dehydroascorbate formed does not react with MTT / PMS.

$$L-Ascorbate+\frac{1}{2}O_2 \xrightarrow{AAO} Dehydroascorbate+H_2O$$

The absorbance difference between the sample and the absorbance difference of the sample blank is equivalent to the quantity of L–Ascorbate in the sample. The obtained MTT is the measuring parameter and its concentration is estimated by determining the absorbance values in the visible range at 586nm wavelength.
The mean operations of this method were the following:

1. Sample preparation: 50g sample is minced in 50 mL of 1M potassium phosphate buffer solution. Adjusting to pH 3.5 – 4.0 with 2M potassium hydroxide solution. Quantitatively transfer the mixture into a volumetric flask of 500 ml with distilled water, fill up to mark with distilled water then is mixed and filtered.
2. Reagents preparation: enzyme solution R3 (Ascorbate Oxidase) is dissolved in 0.5 ml distilled water.
3. Pipetting the enzyme solution R3 in cuvettes: blanks and samples.
4. Pipetting sample (filtered) 100 µl test tube only.
5. Mixing and 15 min incubation to 37ºC.
6. Reading blank and sample absorbance values (A_1).
7. Adding 100 µl of R2 (PMS) to trigger the oxidation reaction.
8. Mixing, then the solutions are allowed to stand for 10 minutes at 37ºC and then read the absorbance of the sample and blank solutions (A_2).
9. Calculation of results: Determine the absorbance difference between sample and blank (A_2-A_1). The difference between the absorbances obtained from the blank samples is reduced from the difference between the absorbances obtained from the samples being calculated as $\Delta A_{L\text{-Ascorbic Acid}}$:

$$\Delta A_{L-\text{Ascorbic Acid}} = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{blank} \quad (1)$$

The concentration of L – Ascorbic Acid can be calculated as it follows:

$$C = \frac{V \cdot MW}{\varepsilon \cdot d \cdot V_s \cdot 1000} \cdot \Delta A(g/L) \quad (2)$$

where V – Final Volume;
V_s – Sample Volume;
MW – Molecular weight of L–Ascorbic Acid [176.13 g/mol];
ε – extinction coefficient of MTT – Formazan at 578 nm=16.9 (l /(mmol·cm));
d – light path (cm);

3. RESULTS AND DISCUSSIONS

3.1. Starch in potato tubers

Preliminary results obtained are presented in Table 1. It can be noted that the highest starch content was recorded in Roclas variety which is suitable also for industrialization (starch production).
The poorest varieties in starch were Sevastia and Blue Congo. The obtained results were verified by other methods (polarimetric and gravimetric), recording the same hierarchy of varieties in terms of the starch content of potato tubers.

Table 1: Experimental data obtained in the determination of starch from different potato varieties (mean of four repetitions)

Variety	Sample		Blank		ΔA	Starch content (g/100g)
	A_2	A_1	A_2	A_1		
Castrum	1,252	0,352	1,097	0,326	0,129	16,066
Christian	1,269	0,353	1,11	0,328	0,134	16,688
Sevastia	1,271	0,392	1,089	0,329	0,119	14,820
Roclas	1,263	0,344	1,101	0,325	0,143	17,809
Blue Congo	1,291	0,382	1,099	0,339	0,120	14,822

3.1. Vitamin C in potato tubers

Preliminary results obtained are presented in Table 2. It can be noted that the highest content of vitamin C has been recorded to the variety Sevastia. The lowest L-ascorbic acid content was recorded in Zamolxis and Blue Congo varieties. The results obtained were verified also by chemical methods (Palladin method), recording the same hierarchy of varieties in terms of vitamin C content of samples (potato tubers).

Table 2: Experimental data obtained in the determination of vitamin C from different potato varieties (mean of four repetitions)

Variety	Absorbance (578nm)				L–Ascorbic Acid content (mg/100g)
	Sample A_2	Sample A_1	Blank A_2	Blank A_1	
Castrum	0,348	0,112	0,229	0,094	22,1
Christian	0,435	0,099	0,293	0,055	21,4
Sevastia	0,252	0,081	0,108	0,048	24,2
Roclas	0,406	0,118	0,243	0,052	22,7
Blue Congo	0,445	0,099	0,303	0,055	21,5

Vitamin C is the most abundant vitamin in potato and it is estimated that about 18% of the recommended daily allowance (RDA) of vitamin C in Australia and 21% in the UK are provided by potatoes [6,7]. Three main biological functions have been identified for L ascorbic acid enzyme cofactor, free radical scavenger and donor/acceptor of electrons al the plasma membrane. Humans have lose ability to synthesize L ascorbic acid and depend on the diet to acquire the necessary amounts required to maintain good health. Deficiency of the vitamin C cause the disease scurvy characterized by spots on the skin, spongy gums and bleeding from mucous membranes. Is caused by deficient synthesis of collagen in which L ascorbic acid is cofactor [7]. Although, nowadays, scurvy is considered rare in developed nations, the vitamin C intake of s significant part of the population of some of these countries may be below RDA (80mg per day in European Union EC) [8].

4. CONCLUSION

The practical applications of enzymatic kits used to estimate Starch and L–Ascorbic Acid content in some samples of potato tuber (Romanian varieties) have provided similar results to other common methods. Thus, there was the same hierarchy of varieties in terms of starch and vitamin C content of the samples analyzed. The essential condition for safety compliance testing is to respect the conditions imposed by the providing company of the kits. Starting from the fact that the methods are based on the use of enzymes, for the safety of the tests, the pH and the temperature of the solution must be strictly observed.

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