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A Simple Chromatographic (HPLC) Method for the Determination of β- Carotene Contents in Inbred Maize Genotypes

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The paper presents a method for determination of β-carotene concentration from 50 maize genotypes carotenoidic extracts using the high performance liquid chromatography (HPLC). The analysed genotypes were cultivated at the Vivekanand Parvatiya Krishi Anusandhan Sansthan, Almora (Uttarakhand). In view of a more complete carotenoidic pigments liberation from the raw material, it was used an improved carotenoids extraction technique. In this purpose the maize flour was moisten with distilled water and then treated with ethanol and let at rest 50 minutes for starch hydrolysis and advanced liberation of the carotenoids from plants cells. Carotenoidic pigments extraction was achieved with an organic solvents mixture of Petroleum ether: Diethyl etherto colorless. β-Carotene was identified in all the maize genotypes. Selected three analyzed maize lines (V391, V351 and V345) presents higher β–carotene content, compared with the literature data concerning β–carotene content in other maize hybrids.

Introduction

Carotenoids comprise a large group of natural pigments widely distributed in the plant and animal kingdoms. They are yellow-orange in colour, insoluble in water but soluble in organic solvents. They are present as pigments in many vegetables and fruits and are associated with chlorophyll in higher plants, playing important role during photosynthesis by passing on the light energy they absorb to chlorophyll, they also protect the chlorophyll from excess light and oxidation. Carotenoids are of two types: carotenes and xanthophylls. The most widespread and important carotene is β-carotene which is found abundantly in some plants. The essential role of β-carotene as a

dietary source of vitamin A has been known for many years (Cooper 2004). Among the provitamin A carotenoids in food namely beta-carotene, alpha-carotene, gamma-carotene and beta-cryptoxanthin, beta-carotene is the one that is most efficiently converted to retinol (Olson *et al.,* 2000). Vitamin A is essential for a variety of biological processes, many of which are related to growth cellular differentiation and interactions of cells with each other or with extracellular matrix (Javeria *et al.,* 2013; Bhatnagar *et al.,* 2015). Its deficiency, even in its relatively early stage, results in impairments in linear growth, cartilage and bone development and epithelial cell

differentiation and function (Deluca, 1991). There is an obvious need for a rapid simple and accurate method for routine determination of pro - vitamin A carotenoid content of food crops. Hence an accurate method for determination of β-carotene to provide reproducible and rapid results has immense importance in maize.

Although, much work has been done in optimizing methods for the extraction and estimation of carotenoids from maize, little attention has been paid to the development of improved and reproducible methods for β-carotene using HPLC. It is well known that to increase the maize nutritive value, there exists a lot of maize varieties from which were obtained numerous hybrids with different physical-chemical characteristics has been developed for superior utilization in human and animal food. In the present study, we tried to obtain few maize genotypes with high β-carotene content. Keeping in view the challenge of estimation of carotenoids using HPLC, the present study was aimed to standardize and develop an efficient and high throughput extraction method. It is envisaged that this findings would provide additional information on the nutritional status of the maize.

Materials and Methods

Maize genotypes (Fifty) were collected from the maize breeding programme of the Vivekananda Institute of Hill Agriculture, Almora, Uttarakhand, India and were evaluated for β-carotene contents. Samples were provided from the field trials in triplicates maintained in the same environment.

Sample preparation

Fifty grams seeds of 5 maize genotypes were taken from germplasm maintained by Maize

Breeding programme in a similar environment at the VPKAS, Almora. Seeds of each genotype were visually screened and uniform representative seeds were taken for grinding. About 10 g maize grains from each line were milled to fine flour in laboratory miller and further the maize flour were sieved through the fine muslin cloth. The sieved fine flour of each genotype was packed in water-proof butter paper bag and kept at 4 °C before the processing for extraction of carotenoids.

Extraction of β-carotenoids

Ground maize (2 g) was soaked overnight in 5 mL water at 4 °C and then extracted twice with 15 ml cold acetone and once with 25 ml cold acetone: hexane (65:35 v/v) until the hexane phase developed fully. Acetone is used in this method because it is inexpensive and readily available and it penetrates food tissue well. The hexane fraction was transferred into a screw cap centrifuge tube and centrifuged for 5 min at 1800 g.

An aliquot of 1.0 ml of the clear hexane extract was transferred to rotary evaporators and evaporated to dryness and the residue was dissolved in1.0 ml of 15% alkaline methanol KOH. KOH was used as an alkaline electrolyte. To this extract, 3 ml of distilled water and 3 ml of 3:1 of Petroleum ether: Diethyl ether was added. This was subjected to centrifugation at 5000 rpm for 10 min at 4ºC. Upper colored organic phase containing β-carotene was transferred to a fresh tube filtered through a 0.45 µm syringe filter (Millipore) into an HPLC sample vial. Only two extractions or filtrations are usually enough but the procedure could be repeated until the residue becomes colorless.

HPLC determination

For quantitative determination of carotenoidic pigments and their metabolites, the most used

method is high performance liquid chromatography (HPLC) (Dumbrava *et al.,* 2007; Sharma *et al.,* 2017). The methanolic extracts of the samples were filtered using pore size 0.45 µm Millipore filters. 20 µl of the samples were injected into a loop injection valve of HPLC (Waters HPLC system) equipped with Photodiode detector and analog pump connected to controller. Running conditions included mobile phase acetonitrile: Methanol (70:30, v/v), flow rate 1.0 ml/min, injection volume 5 µl and detection at 450 nm. β-carotene content present in the sample was identified by comparing retention time (Rt) of standard β-carotenoids. During the run, a flow rate of 1 ml/min was maintained using isocratic mode for 10 min. The separation was carried out on a C18, 5µm (125 x 4.0mm) at 40 °C oven temperature and equipped with a Diode Array Detector monitoring at 450 nm. β- carotene content in the samples was expressed as µg g-1 of dry matter. For quantitative determination of the peak, the integration area value of the standard β-carotenoids with known concentration was compared with the sample peak and the β-carotene content was calculated accordingly.

Results and Discussion

β carotene functions both as an antioxidant and as an essential nutrient. Results show that these residues could be a significant source of vitamin A in both conventional foods and dietary supplements. People with high dietary intake of beta- carotene or high blood levels of this nutrient have a reduced risk of various diseases, including cancer and heart diseases (Chandrika *et al.,* 2005).

The external standard (ESTD) quantitation procedure is the basic quantification procedure in which both calibration and unknown samples are analysed under the same conditions. The response factor is normally calculated as amount/area of the analyte in the calibration sample. The Single point external standard method requires the analysis of more than just the sample of interest. Here sample is analysed containing a known amount of analyte and peak area is recorded. Then a response factor (Response factor=peak area/sample amount) is calculated (Graph 1). To perform this, sample is injected in the machine with the unknown analyte concentration and record the peak area. Subsequently, component amounts are calculated by applying the response factor to the measured sample area (Amount of analyte= peak area/response factor).

In this study, response factor is 1988.54 units, which can be used as analyst to calculate the amount of β-carotene in the unknown genotypes.

Fig.1 HPLC chromatogram of standard and genotypes
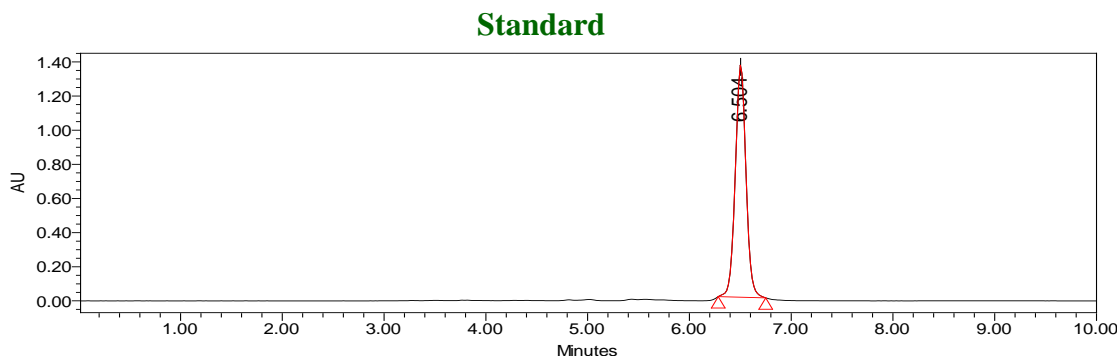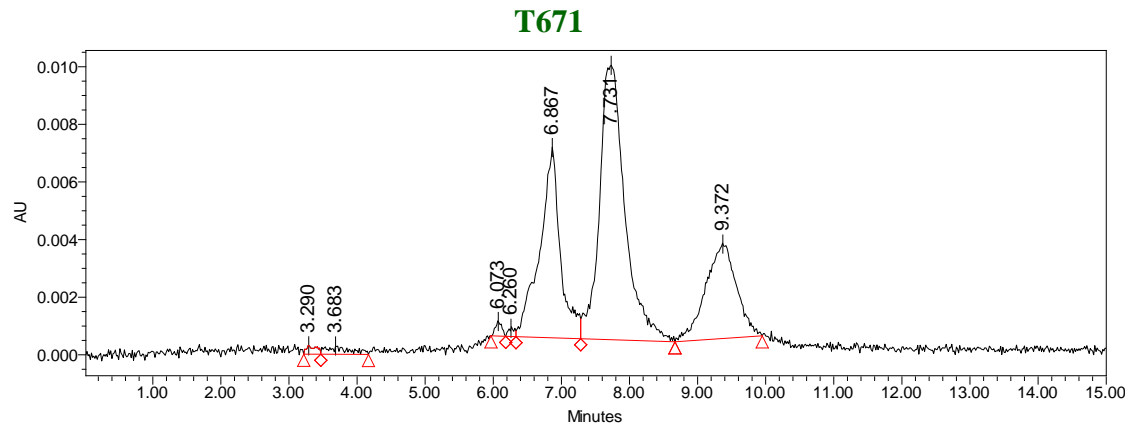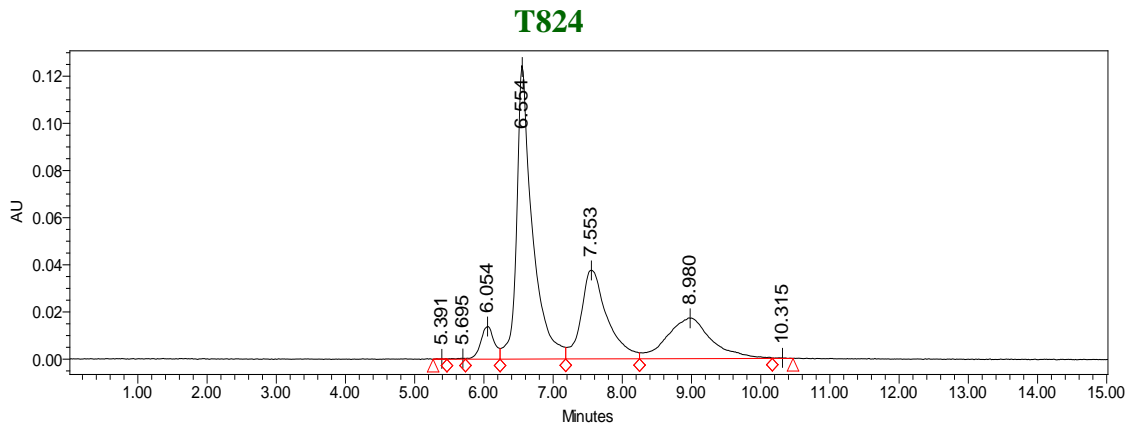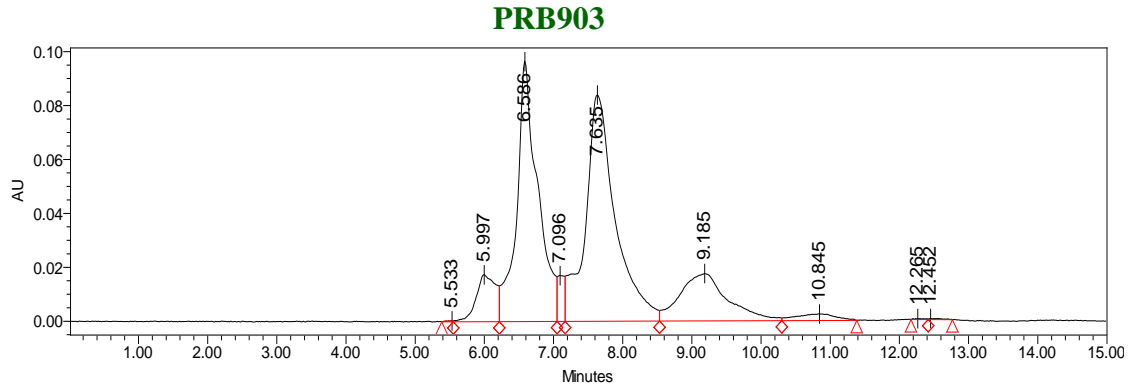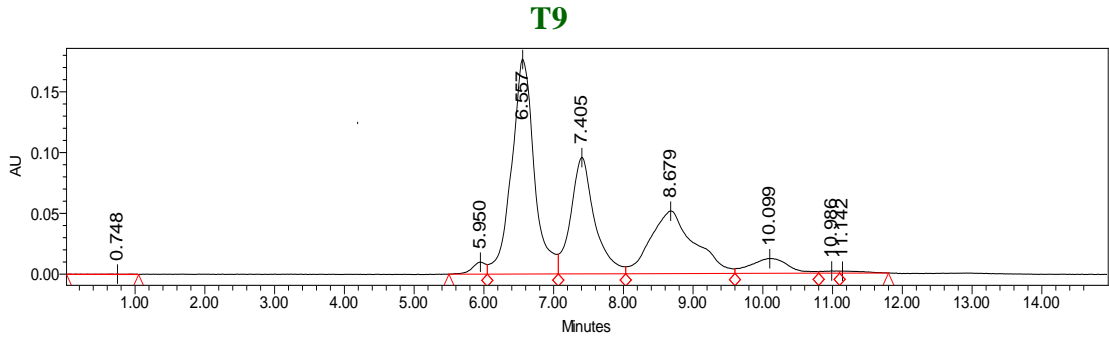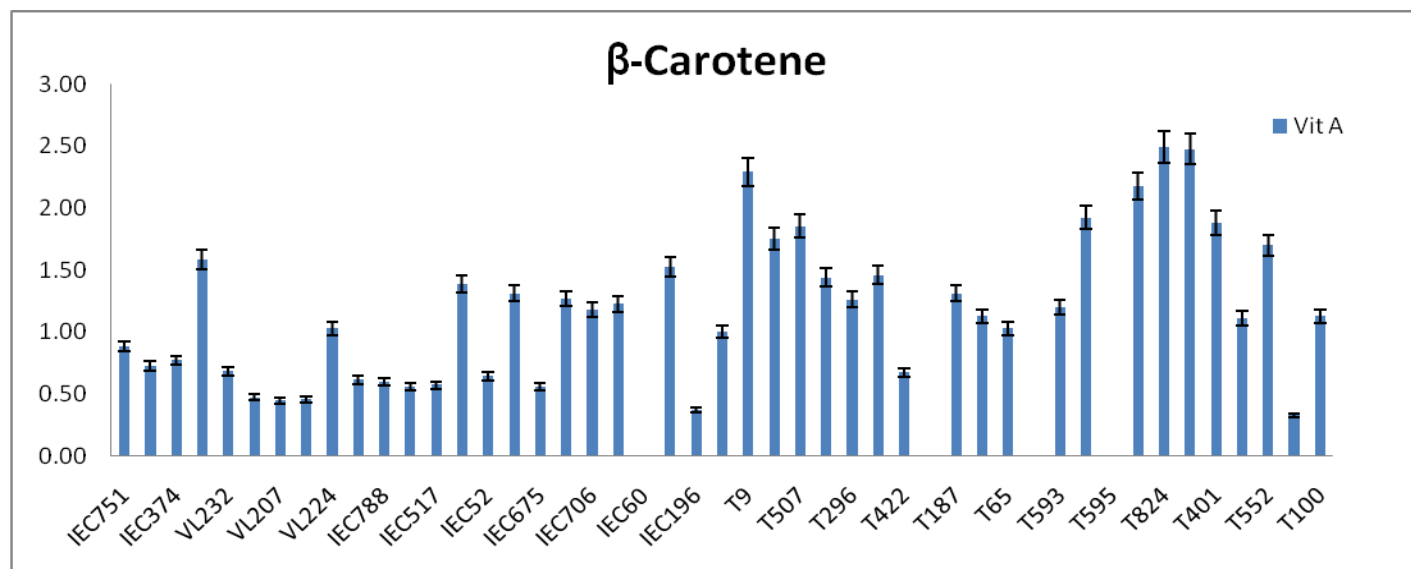

Standard

Graph.1 Basic quantified maize genotypes

Table.1 HPLC data of selected genotypes

Genotypes	Retention Time	Area	% Area	Height
Standard	6.504	9942679	100.00	1356952
T9	6.557	3979286	43.42	176993
PRB903	6.586	2045300	34.18	96609
T824	6.554	1951864	50.02	124570
T671	6.867	138183	27.31	6507

The RP-HPLC superposed chromatograms obtained for standard β-carotene and selected genotypes are presented in figure 1.

The chromatographic peaks area and the β-carotene concentration as per response factor in the raw material are presented in tables 1. From the Figure 1 it is observed that the all maize genotypes have a high β-carotene content, the T9 have the best content (40.2μg/ml), followed by the T824 (20.57μg/ml) and PRB903 (19.63μg/ml).

The obtained values, greater than some literature data concerning β-carotene content in others maize hybrids (2-18 μg/g) (Egesel *et al.,* 2003), could be used towards developing some new maize hybrids with a high carotenoidic pigments content, especially β-carotene and other provitaminic carotenoids. Also, in this

result, the used extraction method has a great importance, because this method determined the complete discolouring of the maize flour (that in the case of other literature methods utilization doesn't happened). Thus, the maize flour maceration with water and methanol, before the extraction, allowed the starch hydrolysis and advanced liberation of carotenoids from cells. Similar results concerning β-carotene content in maize were obtained by Dumbrava *et al.,* (2007) for others high β-carotene maize hybrids.

As a result of the achieved researches it could take out the following conclusions:

HPLC method is very adequate for β-carotene determination in maize flour because this method is sensitive, selective, fast, reproducible and reliable.

All analyzed maize genotypes are very rich in β-carotene, V391 presents the best β-carotene content (40.2 μg/ml), followed by the V351 (20.57 μg/ml) and V345 (19.63 μg/ml).

The obtained values are higher than that from some literature data for others maize hybrids.

This protocol has a potential to be not only used for large scale screening of carotenoids, but also for its use in evaluation of biofortified carotene technologies. From this point of view, these maize genotypes have a high nutritive value.

Acknowledgement

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