

The attached protocol describes a procedure to allow an inexpensive and rapid estimation in maize. It has been designed to perform a pre-screening of carotenoids in maize to select the 10% (or so) of best candidate lines to be then subjected to more detailed analysis by HPLC.

Most of the lines in the protocols are annotated and relate to more detailed explanations given further below

A practical example using materials provided by Torbert Rocheford is given at the end.

The procedure given is for maize only. Do not use it for other crops. By experience, modifications are normally required in this case.

Maize quick carotenoid extraction protocol

All work to be carried out in dim daylight

Materials

Grinding mill, 15 ml Falcon tubes, water bath, TLC Plates, TLC Tank, calibration filter, centrifuge, photometer

Chemicals

Diethyl ether, acetone, petroleum benzene (40-60C-fraction) ethanol, BHT (2,6-Di-tert-butyl-4-methylphenol; Sigma B1378-100G), chloroform

Methods

1. Grind seeds to a very fine powder¹.
2. Weigh 0,5 g to a 15ml blue cap plastic tube (Falcon tube)²
3. Add 6ml EtOH:BHT to every sample and mix the sample by vortexing.³
4. Incubate the samples at 85°C in a water bath for 6 minutes, mix the sample after 3 minutes by vortexing for 10 seconds. (Make sure that the cap is not tightly closed during heating; close tightly when vortexing)
5. Add 120 µl KOH (1g/ml H₂O, prepare fresh daily) and vortex thoroughly for 20 seconds.
6. Saponification: Incubate the samples for 5 minutes at 85°C, vortex for 10 seconds and incubate another 5 minutes at 85°C.⁴
7. Cool down samples on ice. Add 4ml H₂O.
8. Add 3ml PE:DE (2+1,v,v).⁵
9. Mix sample by shaking or vortexing and centrifuge for 10 minutes at 1400 x g. A phase separation will be visible.⁶
10. Transfer upper phase to a fresh 15 ml tube (make sure not to carry over a portion of the aqueous hypophase, better leave a little behind which will be recovered in the subsequent steps)
11. Repeat step 8 to 10 another 2 times. Combine the organic epiphases in the new tubes introduced in 10. This should produce approximately a total of 8-9 ml of extract
12. Fill up the combined supernatants to the same volume (10ml) with PE:DE (2+1,v,v) and mix the samples

13. Take the following aliquots:
 - a. 1 ml for photometry,
 - b. 2 ml for TLC
 - c. 1 ml for HPLC (optional)⁷.
14. Go for photometry immediately to minimise solvent evaporation. Measure OD at 450nm in a spectrophotometer with the appropriate blank (PE:DE (2+1,v,v) and calculate the carotenoid amount using Lambert-Beer equation⁸
15. TLC: dry the 2ml aliquot, resuspend it in 50µl chloroform and load it to a silica TLC plate using an eppendorff pipette. Run the TLC plate in PE+DE+acetone (40+10+10 v,v,v) for only 4-5 cm (about 5 minutes). Take a picture by using a normal flat bed scanner attached to a PC (or Mac, if you so desire)⁹.

¹ The grains should be milled to a fine powder. We are using a micro-dismembrator (Braun Melsingen) for this purpose (a steel ball in a teflon capsule that is rotated in a shaker). But there are many other possibilities (maybe even a good electric coffee mill can do the job). Whatever is used, the procedure should be normalized with respect to time and intensity of the treatment.

A Stein mill can be used for larger quantities. This way you can grind from a larger sample and take an aliquot. Corn is usually not limiting, and some programs may have these.

² Falcon tubes are polypropylene tubes with a tightly closing cap and a ml graduation . The procedure requires 15 ml tubes.

³ 1mg BHT/ml EtOH prepare fresh daily. BHT is an antioxidant (butylated hydroxyl toluene, e.g. Sigma: B1378-100G).

⁴ Saponification is required to cope with the large amount of lipids present in maize extracts mostly stemming from the grain's outer layers and germ. These lipids may interfere with analyses. In addition, xanthophylls may occur in an esterified form. These would migrate together with β-carotene in the solvent front upon TLC (see below). Thus, false positives might occur using this analytical method.

⁵ We have compared several extraction methods especially with one based on acetone and a partition step (which we use normally with GoldenRice). All of these methods were found to be more or less equivalent. The method suggested is a combination of a protocol provided by the Rocheford lab and one of our extraction protocols.

We give this method a preference because of its rapidity.

PE; petroleum ether, often referred to as petroleum benzene, PE comes in different qualities distinguished by the boiling point. We are using PE with a boiling point of 40-60°C .

DE; diethyl ether

⁶ This phase separation should be very clearly visible after centrifugation. The epiphase (upper phase) is yellow colored but transparent and contains the carotenoids. The hypophase is yellowish in color as well. This color does NOT represent carotenoids so, do not worry.

⁷ To perform the HPLC analysis is a completely different matter and shall not be considered here.

All our comparisons on which the current protocol is based, relied on HPLC using a C30 RP HPLC column. No reliable quantification can be obtained without using internal and external standardization.

All of this is not part of this protocol. Thus, if you want to use this aliquot for HPLC, the only information you can reliably get is on the distribution of the carotenoids (in relative % given appropriate use of your detector considering λ_{max} and the molar extinction coefficients (ϵ)).

⁸ Photometers are instruments which are in many labs not properly looked after. **Please make sure that your photometer is in a good shape; e.g. the lamp is not over-aged. Check for performance.**

How to do this routinely:

Calibration sets consist of filters used for checking the absorbance accuracy, and a holmium oxide glass filter for checking the wavelength accuracy. The glass filters are mounted in precision aluminium frames. They are designed for use with the standard 10 mm cell holder provided with spectrophotometers. The set, along with an empty mount, is supplied in a sturdy wooden case. For identification purposes, the set number is engraved on each filter mount. The absorbance values and/or peak position wavelengths of each filter are quoted in the accompanying calibration certificate.

The cost for such a calibration filter should be around 200 \$.

See for instance <http://www.hellma-worldwide.com/en/default.asp> if such test filters are not supplied with your photometer

Cuvettes need to be clean, of course. You do not need quartz cuvettes, normal glass cuvettes are perfect.

With your calibrated photometer, any quantification of carotenoids is simple. We have attached an Excel file that calculates your values from your photometer readings. For those interested, see the explanations below:

Using Lambert-Beer equation carotenoid content of your sample is determined from the OD at 450nm.

$E = \epsilon \cdot c \cdot d$ (E: extinction ϵ : molar extinction coefficient, c: concentration, d: distance =1)

to determine concentration:

$$c = E / \epsilon$$

Lutein, zeaxanthin and beta carotene are the major carotenoids in maize. One can use an average value for ϵ and for the molecular mass.

ϵ lutein = 122688 l/mol cm; M=568 g/mol
 ϵ zeaxanthin = 133480 l/mol cm; M=568 g/mol
 ϵ beta carotene = 134000 l/mol cm; M=537 g/mol

This is $\epsilon_{\text{average}}$: 130056 l/molcm M_{average} =557,7 g/mol

Why can you use the average:

Extinction (your photometer reading) is a small number; maximally 1 (for reasons of accuracy, see below).

Divide 1 by 122688 (lutein) or 134000 (β -carotene).

The numbers produced are almost identical; the error is almost zero.

A similar rationale can be given for using an average molecular mass.

Example:

Dry mass: 0,5g

Total volume: 10ml

OD: 0,5

$$\Rightarrow 0,5 / 130056 * 557,7 * 1000 * 10 / 0,5 = \mathbf{42,88 \mu\text{g/g}}$$

(The OD is divided by ϵ , multiplied with the molecular mass, multiplied by 1000 to get from mg/ml to $\mu\text{g/ml}$, multiplied by 10 to correct for the total volume and divided by the dry mass.)

See Excel file “Maize photometry”. (Note: if OD is bigger than 1 the sample should be diluted !!! (see column “dilution” : putting 1 means not diluted)

⁹ Sample drying:

To dry down samples, we are using a Haake Buchler concentrator

Alternatively a normal Speed-Vac does this job perfectly well, as does a stream of nitrogen. PE/DE mixtures evaporate very quickly

TLC loading (dim light):

Samples in 50 μl chloroform are applied to the plates in the form of short lines (about 1,5 cm in length, see example below). Make sure to keep a 1cm distance from the margins to avoid band smiling and from the bottom of the plate to not submerge the carotenoids in the solvent. Put into the tank containing the solvent immediately thereafter.

TLC

Silica gel thin layer plates with the solvent system given separate carotenoids according to the number of their functional (-OH) groups.

- Carotenes, such as β -carotene have no retention and migrate with the solvent front
- Monohydroxylated compounds (cryptoxanthins) migrate at an intermediate distance (R_f about 0.5)

- Dihydroxylated compounds (lutein and zeaxanthin) remain close to the origin (See Figure 3)

Note: only the upper two bands represent provitamin A carotenoids. Here the uppermost is most effective yielding 2 molecules of retinal

Thin layer plates are commercially available. We are using Silica Gel 60 plates. These come in 25 packs and cost about 100 USD. 1 plate is used from both sides and can analyze about 16 samples.

See Figure 1 for the material required



Fig.1:
This still life shows 1. the chromatography tank (put 2 plates face-to-face) 2. the silica plate 3. the plastic tubes and a maize sample in powdered form

Here is an example using maize lines obtained from Torbert. Please note that some of these lines represent a selection of some of the best lines for b-carotene. You will quite rarely see maize lines that are so high in β -carotene

Carotenoid content by photometry (compared to HPLC)

We have been looking at 6 lines numbered consecutively (A-F, Figure 2). Using HPLC methodologies and photometry we obtained very comparable values with respect to amount. Moreover these values corresponded well to numbers produced independently in Torbert's lab.

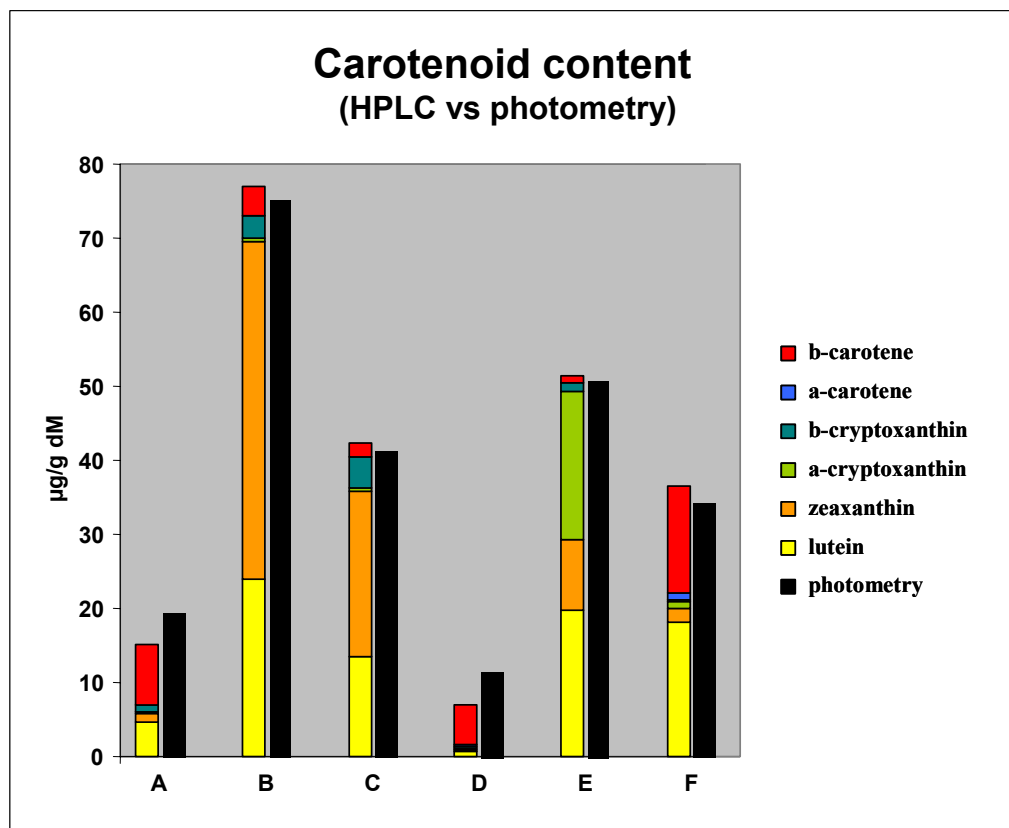
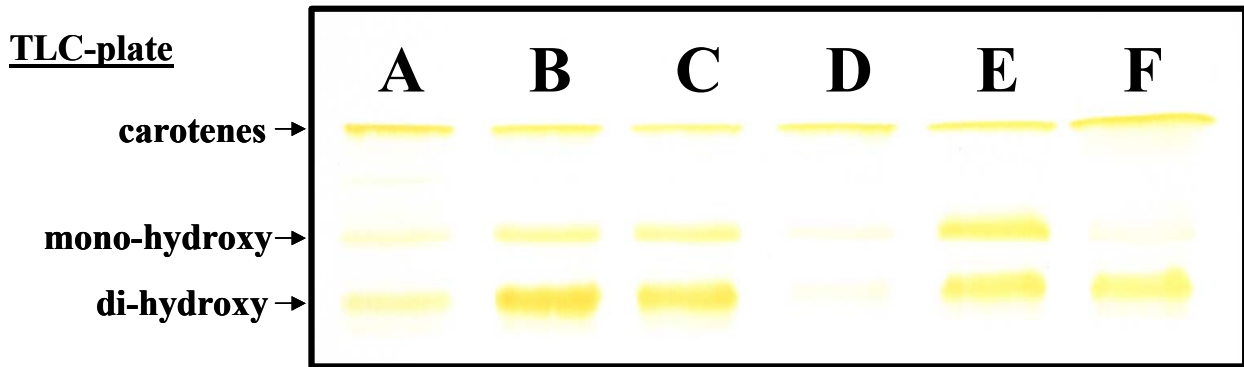


Fig.2: Carotenoid content by HPLC and photometry.

The distribution of carotenoids given is from HPLC integration. We did not consider precursor carotenoids such as phytoene and phytofluene, because they do not appear in the photometer at 450 nm.

Pattern Analysis by TLC (compared to HPLC)

TLC analysis reveals the following pattern (Fig. 3)



Some people like it better when the scanned picture is inverted

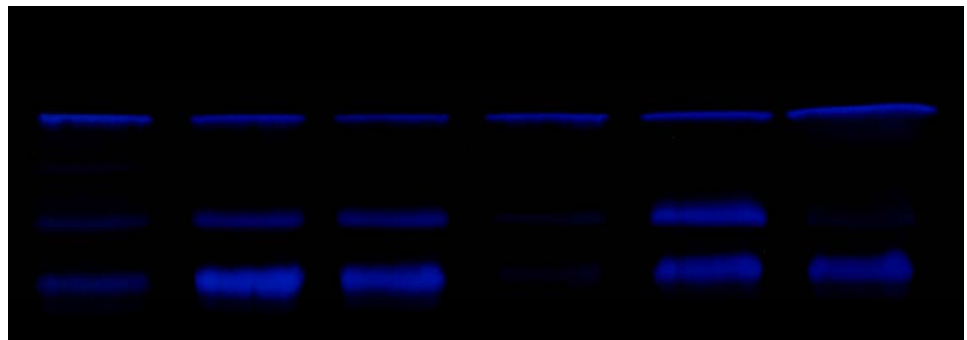


Fig.3: Pattern analysis by TLC.

What to look at/consider:

In this chromatogram, the **RATIO** of the two upper bands (provitamin A carotenoids) relative to the lowest band (no provitamin A carotenoids) are to be estimated visually. Do not try to estimate amount. Here you are having the photometer values already. Remember, we are looking at a positive selection of lines in which C seems pretty standards. Most of the lines will probably look like C.

A, D and F are pretty exceptional. They are characterized by a very large proportion of β -carotene; they are high in provitamin A. According to photometry, F is the most interesting. Please note that the proportion of β -carotene visible on the plate is well reflected in the HPLC analysis (see Fig. 2).

There is a false positive too. Line E shows a low β -carotene content on HPLC (Fig.2), but shows a strong β -carotene band in TLC. This is because this line contains large amounts of zeta-carotene which migrates in the front but was not integrated on HPLC. Such false positives may occur occasionally, but are most probably very seldom (we had only 1 in the 26 lines we examined in a total).

The selection of interesting lines would thus be by:

- 1. Carotenoid amount. Select the 10 or 15 % of top scoring lines according to the photometric values.**
- 2. Make a second independent estimation according to pattern. Screen your electronic pictures for the best occurrence of the top 2 bands; a strong β -carotene band would be especially valuable.**
- 3. If the top scoring 1) and 2) plot together in a matrix these are to be subjected to a detailed HPLC analysis.**
- 4. If you find top-scoring lines with respect to amount or pattern alone, subject the best 10 or 15% to HPLC analysis as well.**

Since all of these scans are in an electronic form it is possible to have the task of line selection by one experienced person, if this is deemed necessary.

Costs: The running costs are low, in the range of 50 cents per sample for extraction, TLC and photometry. The time for analysis is largely reduced as well, given that a large number of samples are analyzed simultaneously by TLC within only minutes.

Freiburg, 30.1 2004

**Patrick Schaub
Peter Beyer
Center for Applied Biosciences
University of Freiburg, Germany**

**Sultana Islam
Torbert Rocheford
Dept. of Crop Sciences
University of Illinois, Urbana-Champaign**