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Screening and HPLC methods for carotenoids in sweetpotato, cassava and maize for plant breeding trials

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Abstract

Analytical methods for sweetpotato, cassava and maize were developed. In orange and salmon-fleshed sweetpotatoes, (all-*E*)-β-carotene predominated and results of spectrophotometric screening and HPLC quantification did not differ significantly. In yellow-fleshed sweetpotato and cassava, however, spectrophotometric screening overestimated the HPLC values because of the presence of several minor carotenoids. Aside from (all-*E*)-β-carotene, *Z*-isomers were present in cassava in appreciable amounts. For both crops, extraction with acetone or tetrahydrofuran:methanol (1:1), using a mortar and pestle or a Polytron homogenizer, gave equivalent results. Rehydration of dry maize at room temperature for 30 min or at 85 °C for 5, 10 or 15 min gave equivalent results. Concentrations obtained with the C18 and C30 columns did not differ significantly for zeaxanthin, lutein, β-cryptoxanthin and β-carotene in the all-*E*-configuration, but their *Z*-isomers were difficult to locate in the chromatogram obtained with the C30 column. Extraction with tetrahydrofuran:methanol (1:1) gave significantly lower results for zeaxanthin and lutein.

Keywords: Sweetpotato; Cassava; Maize; Analysis; Carotenoids; Provitamin A

1. Introduction

HarvestPlus is an international program that seeks to reduce micronutrient malnutrition by harnessing the power of plant breeding to develop staple food crops rich in micronutrients, a process called biofortification. The program involves a global alliance of research institutions and implementing agencies in developed and developing countries. Among the latter, national agricultural and nutrition-related institutions are key partners.

Under HarvestPlus, provitamin A-rich sweetpotato, cassava and maize will be identified and further bred using the best traditional breeding practices and modern biotechnology to achieve provitamin A concentrations that can have measurable effects on human health and nutritional status. Selection of the varieties or breeding lines begins with identifying those that are naturally rich in provitamins A at harvest. Additionally, to select both the promising varieties and set the target provitamin A levels, the amount and form of provitamin A carotenoids remaining in food after processing, storage and cooking must be known.

Fundamental to meeting the objectives of HarvestPlus is having reliable analytical data on the carotenoid composition of each crop. Thus, the present study was carried out to develop screening and high performance liquid chromatography (HPLC) methods for sweetpotato, cassava and maize. Because working conditions in laboratories collaborating with HarvestPlus differ, alternatives to key steps in the analytical procedures were tested so that each labora-

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tory can choose procedures that best suit its conditions, without sacrificing accuracy. Given that sweetpotato, cassava and maize are important staple foods in many countries of the world, the information herein provided will also be useful to food and nutrition scientists and technicians who are not part of HarvestPlus.

The qualitative and quantitative carotenoid composition of foods varies considerably. A given food will also have compositional variation due to factors such as variety/cultivar, geographic effects/climate, season, stage of maturity and part of the plant utilized. Thus, a single method for determining carotenoid composition cannot be developed for all crops. Indeed, the method needs to be adapted to the carotenoid composition and the food matrix.

In a plant breeding program, thousands of varieties and lines have to be screened and quantified. Fast, simple, low-cost screening that does not require sophisticated instrumentation, is needed to select those varieties/lines that meet or are close to the target provitamin level; only those samples that meet the screening criteria need to be submitted for accurate quantification. In developing countries, screening a large number of samples can be done in simple laboratories, preferably near the sites of production, after which the promising varieties or lines can be sent to a central, well-equipped laboratory for HPLC quantification.

2. Materials and methods

2.1. Sample preparation

Three types of sweetpotato, two types of cassava and three varieties of maize were included in the study. The sweetpotato samples included the orange-fleshed variety Resisto, salmon-fleshed EMBRAPA (Empresa Brasileira de Pesquisa Agropecuaria) breeding line CNPH 477-2 and yellow-fleshed variety IAC 60-M-3-Brasília that had salmon-colored specks. Both, a yellow and cream-fleshed cassava, variety BRA 005771 and variety IAC 576-70, respectively, were included. The maize varieties were Assum Preto, Asa Branca and BR-473.

At least 5 sweetpotato tubers or cassava roots were taken, peeled, quartered and opposite sections from each root were combined and homogenized in a food processor. About 250 g of dry maize was ground in an automatic grain grinder.

2.2. Identification of the carotenoids

The carotenoids were identified by the ultraviolet–visible absorption spectra, specifically the wavelengths of maximum absorption and the spectral fine structure expressed as %III/II, co-chromatography with authentic samples and chemical reactions (Rodriguez-Amaya, 1999). The %III/II is the ratio of the height of the longest-wavelength absorption peak, designated III, and that of the middle absorption peak, designated as II, taking the minimum between the two peaks as baseline, multiplied by 100 (Britton, 1995). The *Z*-isomers were identified by λ_{max} slightly lower than those of the all-*E*-carotenoids and by the presence of the "*cis*" peak at about 142 nm below the longest-wavelength absorption maximum of the all-*E*-form. The location of the *Z*-double bond was indicated by the $\%A_B/A_{II}$, which is the ratio of the height of the "*cis*" peak, designated A_B, and that of the middle main absorption peak, designated A_{II}, multiplied by 100 (Britton, 1995). This ratio is an indicator of the intensity of the "*cis*" peak, which is greater as the *Z*-double bond is closer to the center of the molecule. Methylation with acidified methanol was used to differentiate lutein and zeaxanthin, α-cryptoxanthin and zeinoxanthin, this reaction being positive to hydroxy substituent in allylic position, as in lutein and α-cryptoxanthin (Rodriguez-Amaya, 1999). The presence of secondary hydroxy groups in allylic or non-allylic positions was verified by acetylation with acetic anhydride.

2.3. Screening method for sweetpotato and cassava

The different steps in both the screening and HPLC methods were established through preliminary tests to choose the conditions for each step that gave the best results for the food sample being analyzed. Thus, the analytical procedures presented in this paper had been optimized before the final evaluation.

A portion (about 2–5 g of sweetpotato and 5–15 g of cassava, the actual weight depending on the carotenoid content of the sample) of the homogeneous sample was weighed. Three extraction procedures were evaluated. In the first procedure, the sample was ground with 50 ml cold acetone (acetone refrigerated for about 2 h) and about 3 g of Hyflosupercel with a mortar and pestle. In the second and third procedures, the sample was weighed in the extraction tube and then homogenized with 50 ml solvent for 1 min with a Polytron homogenizer, the solvent being acetone and tetrahydrofuran:methanol (THF:MeOH) (1:1), respectively. The extract was filtered through a glass sintered funnel. These operations resulted in a colorless residue for cassava. For sweetpotato, however, extraction and filtration had to be repeated because the residue after the first extraction–filtration was still colored.

The extract was transferred to a 500 ml separatory funnel with Teflon stop-cock that had about 40 ml petroleum ether for sweetpotato or 20 ml for cassava. Water (about 300 ml) was added slowly, letting it flow along the wall of the funnel. Adding the water slowly and not shaking the funnel prevented an emulsion forming. However, even with these precautions, an emulsion occasionally formed with the cassava extract. When this happened, the emulsion was broken with saturated NaCl solution. The phases were allowed to separate and the lower aqueous phase was discarded. The petroleum ether phase was washed 3–4 times with water (about 200 ml each time) and then collected in a 50 ml volumetric flask for sweetpotato or 25 ml for cassava, passing the solution through a small funnel with about 15 g anhydrous sodium sulfate to remove the resid-

ual water. The carotenoid solution was made up to volume with petroleum ether and the absorbance was taken at 450 nm. The total carotenoid content was calculated according to Davies (1976), using the absorption coefficient ($A^{1\%}_{1\,cm}$) of β-carotene in petroleum ether (i.e., 2592).

2.4. Screening method for dry maize

Dry maize is a difficult sample to extract; thus, room temperature or hot rehydration was tried to allow efficient penetration of the extracting solvent into the maize tissues. Acetone was used because it is less expensive and readily available, and it penetrates the food tissues well.

In room temperature rehydration, 3 g of ground maize was weighed in a beaker. Enough water to cover (about 10 ml) was added and the mixture allowed to stand at room temperature for 30 min. About 20 ml acetone was added and the mixture was again allowed to stand for 15 min. For hot rehydration, 3 g of ground maize was weighed, mixed with 20 ml of water, allowed to stand for 5, 10 or 15 min in a water bath at 85 °C, mixed at 5 min interval for the second and third time period, and cooled in a water bath.

The carotenoids were extracted from the rehydrated sample with 50 ml acetone using a mortar and pestle or the Polytron homogenizer. Partition to petroleum ether was carried out as described for sweetpotato and cassava, except that the extract was added to 20 ml petroleum ether in three portions. After each addition, water was added, the phases were allowed to separate and the lower aqueous phase was discarded. After the third portion had been transferred to petroleum ether, the petroleum ether phase was washed three times with water and collected in a 25 ml volumetric flask, passing through a funnel with anhydrous sodium sulfate. The carotenoid solution was made up to volume and a 20 ml aliquot was concentrated in a rotary evaporator (temperature $\leqslant$35 °C) to about 1 ml for the separation of the carotene and monohydroxycarotenoid fractions. The absorbance of the remaining extract was taken and the total carotenoid content was calculated according to Davies (1976), using the absorption coefficient of 2500 that is recommended for mixtures.

The concentrated solution was applied on a minicolumn consisting of a Pasteur pipette (with the tip cut to 2 cm) containing about 1 g of neutral alumina activity III held by a plug of glass wool at the bottom of the column, topped with a 0.5 cm layer of sodium sulfate. The light orange carotene fraction (fraction 1) was eluted by petroleum ether and collected directly in a 5 ml volumetric flask. The second yellow monohydroxycarotenoid fraction (fraction 2) was eluted with 20% ethyl ether in petroleum ether and collected directly in a 10 ml volumetric flask. The bright yellow band (lutein and zeaxanthin) was left in the column.

The carotene content was calculated using the absorbance of fraction 1 and the absorption coefficient of β-carotene in petroleum ether (i.e., 2592). The monohydroxycarotenoid content was calculated using the absorbance of fraction 2 and the absorption coefficient of β-cryptoxanthin in petroleum ether (i.e., 2386). Because fraction 2 normally had zeinoxanthin and β-cryptoxanthin at about the same proportion, β-cryptoxanthin was estimated by dividing the monohydroxycarotenoid content by two. The lutein + zeaxanthin content was estimated by subtracting the carotene and monohydroxycarotenoid contents from the total carotenoid.

2.5. Isolation of carotenoid standards for HPLC quantification

Because carotenoids are unstable and carotenoid standards are costly and difficult to obtain in developing countries, to have a constant supply of standards, a laboratory must be able to isolate them from natural sources.

β-Carotene standard was isolated from sweetpotato and standards of β-cryptoxanthin, lutein and zeaxanthin were isolated from green maize (freshly harvested mature maize) by open column chromatography. The carotenoids of about 20 g of homogenized sweetpotato or 120 g of homogenized green maize were extracted three times with acetone (50 ml each time) and partitioned, in portions, to 100 ml petroleum ether as described previously for maize.

Saponification of the carotenoids of maize was carried out to get better separation on the open column. This was done by adding 100 ml 10% methanolic KOH and 0.1% butylated hydroxytoluene to the extract and, after flushing with N_2, leaving the stoppered flask in the dark at room temperature overnight (about 16 h) (Kimura, Rodriguez-Amaya, & Godoy, 1990). The saponified extract was then washed five times with water, dried with anhydrous sodium sulfate and concentrated in a rotary evaporator.

Separation of the carotenoids was carried out on a 25 × 300 mm glass column packed to a height of about 150 mm with MgO:Hyflosupercel (1:1) activated for 4 h at 110 °C (Kimura & Rodriguez-Amaya, 2002). β-Carotene was eluted from the column with 2% acetone, β-cryptoxanthin with 25–30% acetone and zeaxanthin with 40–45% acetone, all in petroleum ether. Only the main portion of each band of desired carotenoid was collected to avoid contamination from other bands. The fractions were washed four times with water to remove acetone. The purity of the standards was determined by HPLC and the carotenoid standard solutions were corrected accordingly.

2.6. HPLC methods

In the comparison of screening and quantitative methods for sweetpotato and cassava, aliquots for HPLC analyses were taken from the same extract used for screening. For direct HPLC analysis, extraction, filtration, partition to petroleum ether and drying with anhydrous sodium sulfate were carried out as described for screening. The carotenoid solution was then concentrated in a rotary

evaporator and brought to dryness with N_2. Immediately before injection the carotenoids were dissolved in 1 ml HPLC grade acetone, filtered through a 0.22 μm PTFE syringe filter (Millipore) directly to sample vials, and 10 μL were injected into the chromatograph. To obtain representative samples, the minimum amount of sweetpotato

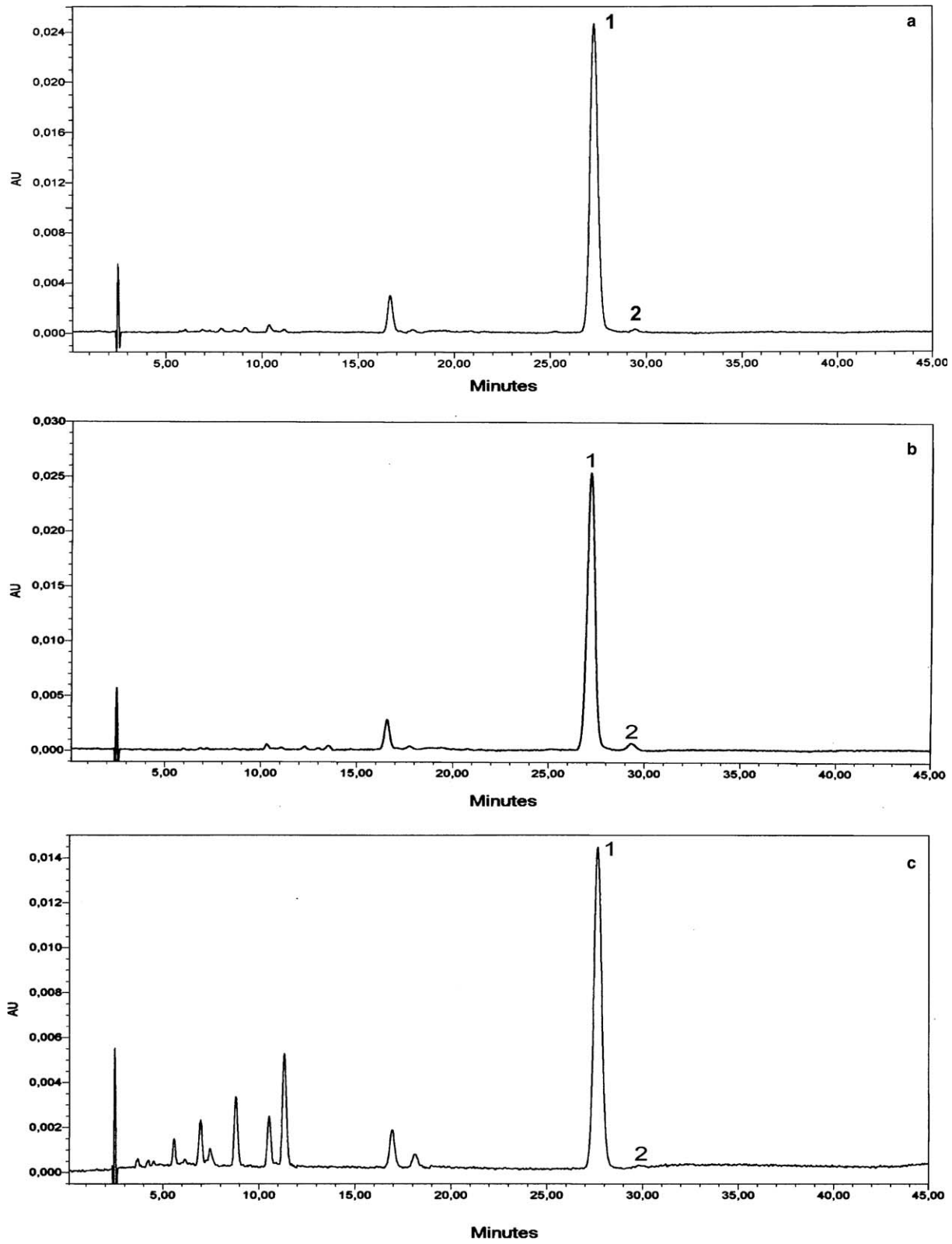

Fig. 1. HPLC chromatograms obtained with C18 Spherisorb monomeric ODS2 column of the carotenoids of (a) orange-fleshed Resisto, (b) salmon-fleshed EMBRAPA breeding line CNPH 477-2 and (c) yellow-fleshed IAC 60-M-3-Brasília sweetpotatoes. HPLC conditions are described in the text. Detection was at 450 nm. Peak identification: 1 – (all-*E*)-β-carotene; 2 – (13*Z*)-β-carotene.

weighed for analysis was 2 g. For sweetpotatoes that were very rich in β-carotene (e.g., orange-fleshed variety Resisto), dissolving in 1 ml acetone would result in a very concentrated solution, thus more solvent was used.

As with screening, 3 g of ground maize were first rehydrated cold or hot. The carotenoids were extracted with 50 ml acetone using a mortar and pestle or a Polytron homogenizer, partitioned to petroleum ether, dried with anhydrous sodium sulfate and concentrated in rotary evaporator as described previously. The extract was brought to dryness with N_2 and, immediately before injection, redissolved in 1 ml acetone and filtered; an aliquot of 10 μl was injected into the chromatograph. The standards were injected in the same volume.

The carotenoids of the three crops analyzed in the present study are mostly unesterified and both sweetpotato and cassava have low lipid content. Maize has more lipids, but the gradient elution employed was sufficient to remove the lipids from the column. Thus, the error-prone saponification step was not necessary.

For the C18 column, the analyses were carried out on a Waters Alliance separation module (model 2690) equipped with quaternary pump, in-line vacuum degasser, autosampler injector and photodiode array detector (model 996), controlled by Millenium 2010 workstation. For simultaneous comparison, another liquid chromatograph was used for the C30 column: Waters system consisting of quaternary pump (model 600), in-line vacuum degasser, rheodyne injector and photodiode array detector (model 996), controlled by Millenium 2010 workstation.

For sweetpotato and cassava the following chromatographic conditions were used: (a) monomeric C18 column, Waters Spherisorb ODS 2, 3 μm, 4.6 × 150 mm; mobile phase, acetonitrile:methanol:ethyl acetate (with 0.05% triethylamine), 80:10:10, isocratic elution, flow rate of 0.7 ml/min and (b) polymeric C30 column, YMC C30, 3 μm, 4.6 × 250 mm; mobile phase, methanol:methyl-*tert*-butyl ether, 80:20, isocratic elution, flow rate of 0.8 ml/min. The same two columns and mobile phases were used for maize, but gradient elution was employed. For the first column, the initial proportion of 95:5:0 was increased to 60:20:20 in 20 min (concave gradient), staying in this proportion until 40 min, then changing to 20:40:40 in 60 min (linear gradient) to remove the lipids. The flow rate was 0.5 ml/min and re-equilibration took 15 min. For the second column, the gradient was linear with the initial proportion of 90:10 changing to 40:60 in 60 min. The flow rate was 0.8 ml/min and re-equilibration also took 15 min.

Quantification was carried out by external standardization. Full standard curves were constructed with five different concentrations for each carotenoid in triplicate. The curves passed through or very near the origin, were linear and bracketed the concentrations expected in the samples. For sweetpotato and cassava, the purity of the β-carotene standard was 97%, the coefficient of correlation (r^2) was 0.9985 and the coefficient of variation (CV) was 0–2%. For maize, the purity of the standards was 98% for lutein, 97% for zeaxanthin, 96% for β-cryptoxanthin, and 98% for β-carotene. The r^2 values were 0.9998, 0.9995, 0.9998, and 0.9983, respectively. The corresponding ranges of the CV of the triplicate injections at each of five points were 0–2%, 0–1%, 1–2%, and 1–2%. These results demonstrated excellent linearity of the instruments' (liquid chromatograph and UV–visible spectrophotometer) response and accuracy of the standard solutions. Day-to-day quantification was done by one-point calibration, verifying any change in detector's response.

The values quoted above were those obtained with the C18 column. Similar results were obtained with the C30 column (data not presented).

2.7. Measures to prevent isomerization and degradation of carotenoids during analysis

Precautionary measures to prevent artifact formation and losses of carotenoids during analysis are standard practices in our laboratories and were rigorously followed in the present work. These include (Britton, 1991; Davies, 1976; Schiedt & Liaaen-Jensen, 1995): (1) completion of the analysis within the shortest possible time; (2) exclusion of oxygen; (3) protection from light; (4) avoiding high temperatures; (5) avoiding contact with acid; (6) use of high purity solvents, free from harmful impurities. Samples were analyzed immediately after collection.

Table 1
Comparison of variety, extraction procedure, screening and HPLC methods for determining the principal carotenoids (μg/g)[A] of sweetpotato

Variety	Extraction procedure	Screening total carotenoid	HPLC	
			Total-β-carotene	(all-*E*)-β-carotene
Resisto	Acetone – mortar	135 ± 3^{a}	127 ± 1^{ab}	123 ± 1^{b}
	Acetone – Polytron	132 ± 2^{a}	123 ± 1^{ab}	119 ± 1^{b}
	THF:MeOH – Polytron	129 ± 1^{a}	122 ± 1^{ab}	118 ± 1^{b}
IAC 60-M-3-Brasília	Acetone – mortar	18.4 ± 0.7^{a}	11.5 ± 0.3^{b}	11.2 ± 0.1^{b}
	Acetone – Polytron	17.9 ± 0.2^{a}	11.4 ± 0.1^{b}	11.1 ± 0.1^{b}
	THF:MeOH – Polytron	17.6 ± 0.5^{a}	11.2 ± 0.1^{b}	11.0 ± 0.1^{b}

Values in the same horizontal row with different superscript letters are significantly different ($p \leqslant 0.05$).
For each variety, values in the same column with different superscript letters are significantly different ($p \leqslant 0.05$).
[A] Means and SDs of triplicate analyses.

2.8. Validation of the methods developed using a certified reference material

Two-grams of the BCR (Community Bureau of Reference, Brussels, Belgium) certified reference material CRM 485 lyophilized mixed vegetables was weighed and rehydrated with 5 ml distilled water at room temperature for 5 min. The carotenoid extract was prepared and injected into the liquid chromatograph as described for maize. Because of the presence of lycopene, the linear gradient for the C30 column was from 90:10 to 10:90 in 80 min, instead of 40:60 in 60 min.

Since the certified values are given in mg/kg (μg/g) dry matter, the moisture content of the reference material

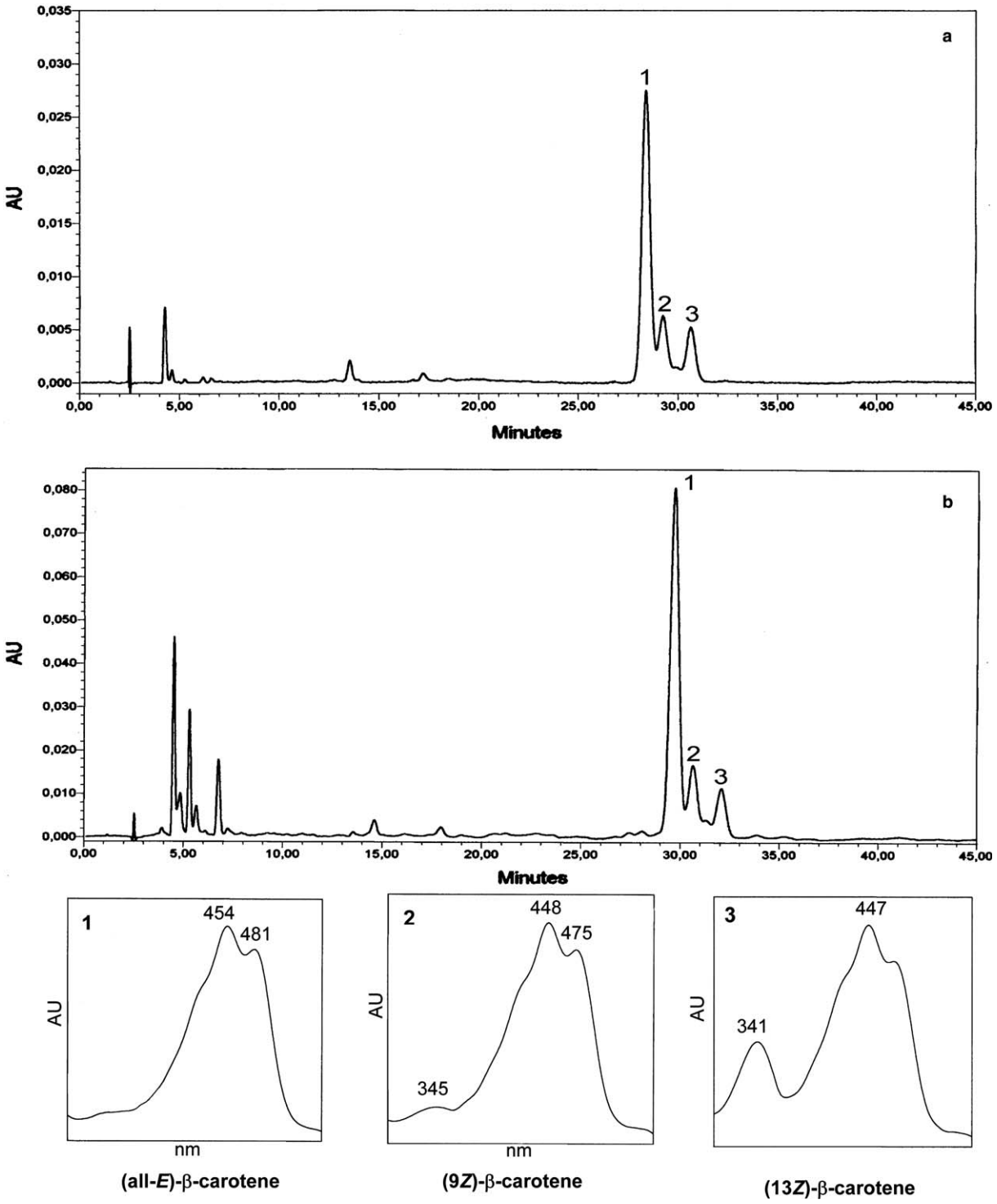

Fig. 2. HPLC chromatograms (detection at 450 nm) obtained with C18 monomeric Spherisorb ODS2 column and photodiode array spectra of the carotenoids of (a) cream-fleshed IAC 576-70 and (b) yellow-fleshed BRA 005771 cassavas. HPLC conditions are described in the text.

was determined and the carotenoid values obtained with the methods developed in the present study were transformed to the dry weight basis according to BCR recommendation.

2.9. Statistical analysis

The results obtained were submitted to analysis of variance ($p \leqslant 0.05$), the means being compared by the Tukey's test.

To assess the agreement between the results obtained with the developed methods and the certified values for the certified reference material, the *z*-score was used (ISO/IEC, 1996; Thompson & Wood, 1993).

3. Results and discussion

3.1. Identity of the carotenoids

The qualitative carotenoid compositions of the three agricultural crops investigated were very different, making conclusive identification critical for accurate quantitative results.

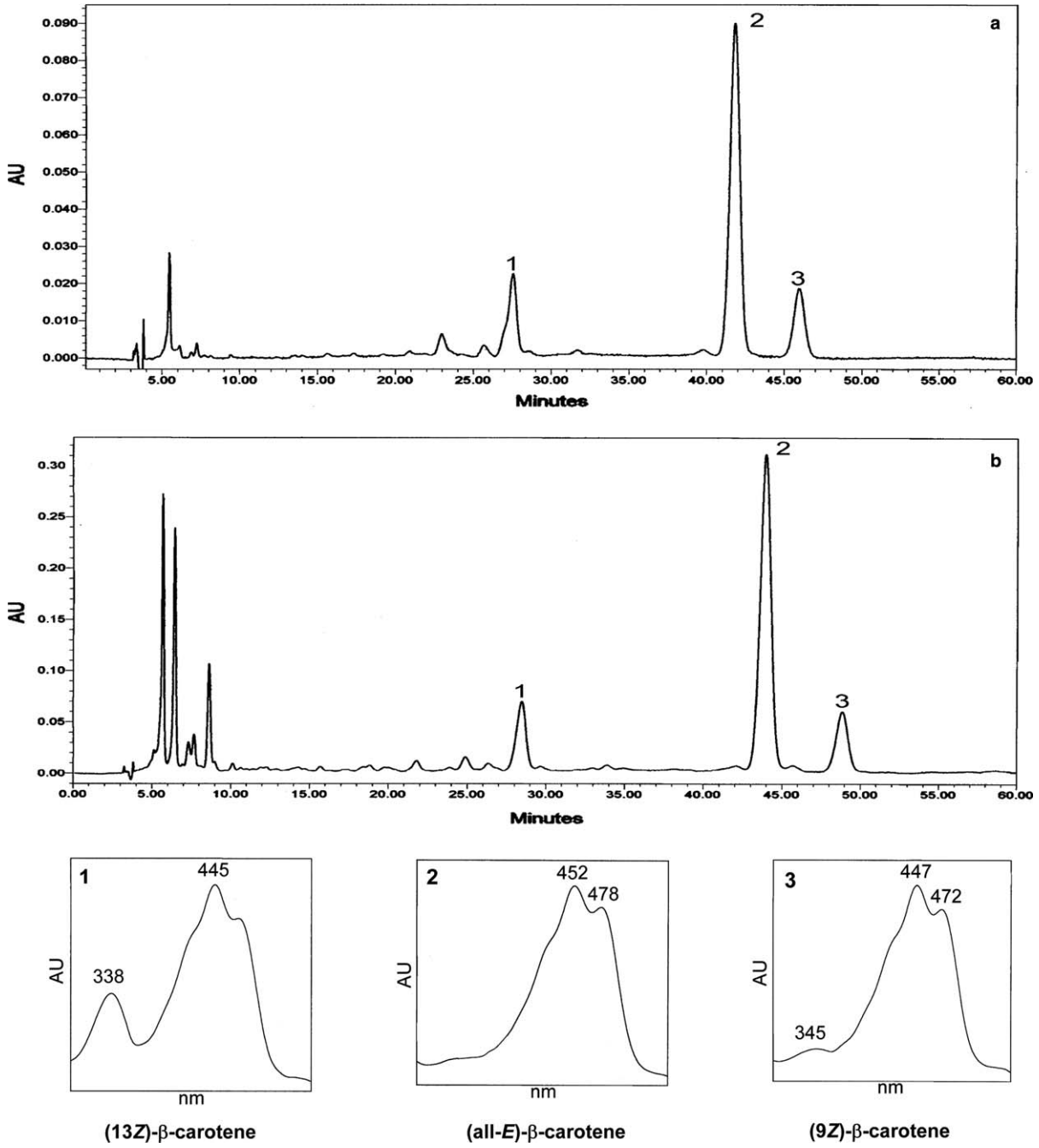

Fig. 3. HPLC chromatograms (detection at 450 nm) obtained with YMC polymeric C30 column and photodiode array spectra of the carotenoids of (a) cream-fleshed IAC 576-70 and (b) yellow-fleshed BRA 005771 cassavas. HPLC conditions are described in the text.

(All-*E*)-β-carotene (β,β-carotene) was identified by co-chromatography with authentic (all-*E*)-β-carotene and the visible absorption spectrum having λ_{max} at 425 (shoulder), 450 and 477 nm in petroleum ether and little fine structure (%III/II = 25), commensurate with a chromophore of 11 conjugated double bonds, two of which situated in β-rings.

The *Z*-isomers were first perceived by the visible absorption spectra having λ_{max} 2–6 nm lower than those of the (all-*E*)-carotenoid and the appearance of a "*cis*" peak. The $\%A_B/A_{II}$ of 10, 45 and 56 identified (9*Z*)-β-carotene, (13*Z*)-β-carotene and (15*Z*)-β-carotene, respectively (Mercadante, Steck, & Pfander, 1999).

Recommended measures to avoid geometric isomerization during analysis were carefully taken in this study. The fact that *Z*-isomers were practically absent in the sweetpotato extract and the presence of high amounts of *Z*-isomers in cassava extract, under the same analytical conditions, attested to the occurrence of these isomers in cassava and maize as natural constituents. Although heating was carried out with maize, the qualitative and quantitative carotenoid composition of the heated samples was compared and found not to be significantly different from that of the unheated sample.

Two monohydroxy carotenoids were detected in maize. One had the same absorption spectrum as β-carotene and was identified as β-cryptoxanthin (β,β-caroten-3-ol). The other, identified as zeinoxanthin (β,ε-caroten-3-ol), had the same spectrum as α-carotene (β,ε-carotene), having λ_{max} at 422, 444 and 472 nm in petroleum ether and more defined spectral fine structure compared to that of β-carotene (%III/II = 55), reflecting a chromophore of 10 conjugated double bonds, one of which located in a β-ring. The presence of a hydroxy substituent in both carotenoids was indicated by the retention times and confirmed by positive acetylation with acetic anhydride. The negative response to methylation with acidified methanol showed that this substituent was not in allylic position, identifying the second monohydroxy carotenoid as zeinoxanthin.

The dihydroxy carotenoids of maize were confirmed to be zeaxanthin (β,β-carotene-3,3′-diol) and lutein (β,ε-carotene-3,3′-diol), exhibiting the visible absorption spectra of β-carotene and α-carotene, respectively. The presence of the hydroxyl groups, reflected in the retention times, was confirmed by positive acetylation with acetic anhydride. The allylic position of one of the hydroxyls of lutein was shown by the positive response to methylation with acidified methanol, producing a monohydroxy compound. Zeaxanthin responded negatively to methylation.

3.2. Carotenoid concentrations in sweetpotato

The typical chromatograms of three types of sweetpotatoes in Fig. 1 show varietal difference in the carotenoid composition of this crop. In both the orange-fleshed Res-

Table 2
Comparison of extraction procedure, screening and HPLC methods for determining the principal carotenoids (μg/g)[A] of cream-fleshed cassava, variety IAC 576-70

Extraction procedure	Screening total carotenoid	HPLC				
		Column	β-Carotene			
			Total	(all-*E*)-	(9-*Z*)-	(13-*Z*)-
Acetone	3.11 ± 0.05^{a}	C18	2.69 ± 0.02^{b}	1.78 ± 0.06^{c}	0.43 ± 0.02^{d}	0.43 ± 0.02^{d}
Mortar		C30	2.61 ± 0.04^{b}	1.84 ± 0.00^{c}	0.39 ± 0.01^{d}	0.39 ± 0.02^{d}
Acetone	3.07 ± 0.02^{a}	C18	2.63 ± 0.06^{b}	1.78 ± 0.06^{c}	0.43 ± 0.01^{d}	0.42 ± 0.01^{d}
Polytron		C30	2.63 ± 0.10^{b}	1.82 ± 0.08^{c}	0.40 ± 0.01^{d}	0.41 ± 0.00^{d}
THF:MeOH	3.03 ± 0.01^{a}	C18	2.65 ± 0.02^{b}	1.79 ± 0.01^{c}	0.43 ± 0.00^{d}	0.42 ± 0.01^{d}
Polytron		C30	2.59 ± 0.01^{b}	1.80 ± 0.00^{c}	0.39 ± 0.01^{d}	0.40 ± 0.01^{d}

Values in the same row or column with different superscript letters are significantly different ($p \leqslant 0.05$).
[A] Means and SDs of triplicate analyses.

Table 3
Effect of storage on the carotenoid content (μg/g)[A] of cream-fleshed cassava, variety IAC 576-70

Storage time (h), condition	Screening total carotenoid	β-Carotene concentration obtained by HPLC			
		Total	(all-*E*)-	(9-*Z*)-	(13-*Z*)-
6, Ambient[B]	3.11 ± 0.05^{a}	2.69 ± 0.02^{a}	1.78 ± 0.06^{a}	0.43 ± 0.02^{a}	0.43 ± 0.02^{a}
24, Freezer[C]	2.75 ± 0.06^{b}	2.48 ± 0.01^{b}	1.60 ± 0.07^{b}	0.44 ± 0.02^{a}	0.42 ± 0.02^{a}
48, Freezer	2.63 ± 0.09^{bc}	2.30 ± 0.04^{c}	1.45 ± 0.08^{c}	0.42 ± 0.03^{a}	0.44 ± 0.02^{a}
24, Ambient[D]	2.56 ± 0.08^{cd}	2.38 ± 0.04^{d}	1.45 ± 0.03^{c}	0.44 ± 0.02^{a}	0.43 ± 0.00^{a}
48, Ambient	2.35 ± 0.10^{d}	2.25 ± 0.01^{e}	1.34 ± 0.01^{d}	0.47 ± 0.05^{a}	0.48 ± 0.02^{a}

Values in the same column with different superscript letters are significantly different ($p \leqslant 0.05$).
[A] Means and SDs of triplicate analyses.
[B] Intact cassava kept at ambient temperature.
[C] Cassava peeled and packed in polyethylene bags, kept in a freezer at −18 °C.
[D] Intact cassava kept at ambient temperature.

isto variety and the salmon-fleshed EMBRAPA breeding line CNPH 477-2, the predominance of (all-*E*)-β-carotene was evident. In a yellow-fleshed variety IAC 60-M-3-Brasília, with some salmon colored specks, (all-*E*)-β-carotene was still the principal carotenoid, but there were several small peaks that together summed up to about 37% of the total carotenoid content.

Particularly because the concentrations were at μg/g, the quantitative data obtained by screening and HPLC quantification of sweetpotato carotenoids showed excellent repeatability (Table 1), the CV for triplicate measurements varying from 0.5% to 3.7% with an average of 1.4%.

For the variety Resisto, because of the dominance of β-carotene, the difference between the total carotenoid content obtained by spectrophotometric screening and the β-carotene level obtained by HPLC was not statistically significant. Thus, the β-carotene concentration in Resisto and the salmon-fleshed sweetpotatoes can be determined spectrophotometrically, without resorting to HPLC.

The difference between the results obtained by screening and those by HPLC for the yellow variety, however, was statistically significant (Table 1). Thus, screening can still be used to select the β-carotene-rich yellow varieties but for accurate quantification, HPLC needs to be used or the β-carotene fraction separated by open column chromatography before spectrophotometric quantification.

For both the orange Resisto and yellow varieties analyzed, there was no significant difference between the total β-carotene and the (all-*E*)-β-carotene contents because the *Z*-isomer, (13*Z*)-β-carotene, was present in trace amounts. There was also no significant difference in the results obtained using acetone or THF:MeOH as extracting solvent nor by extraction with the mortar and pestle or the Polytron homogenizer.

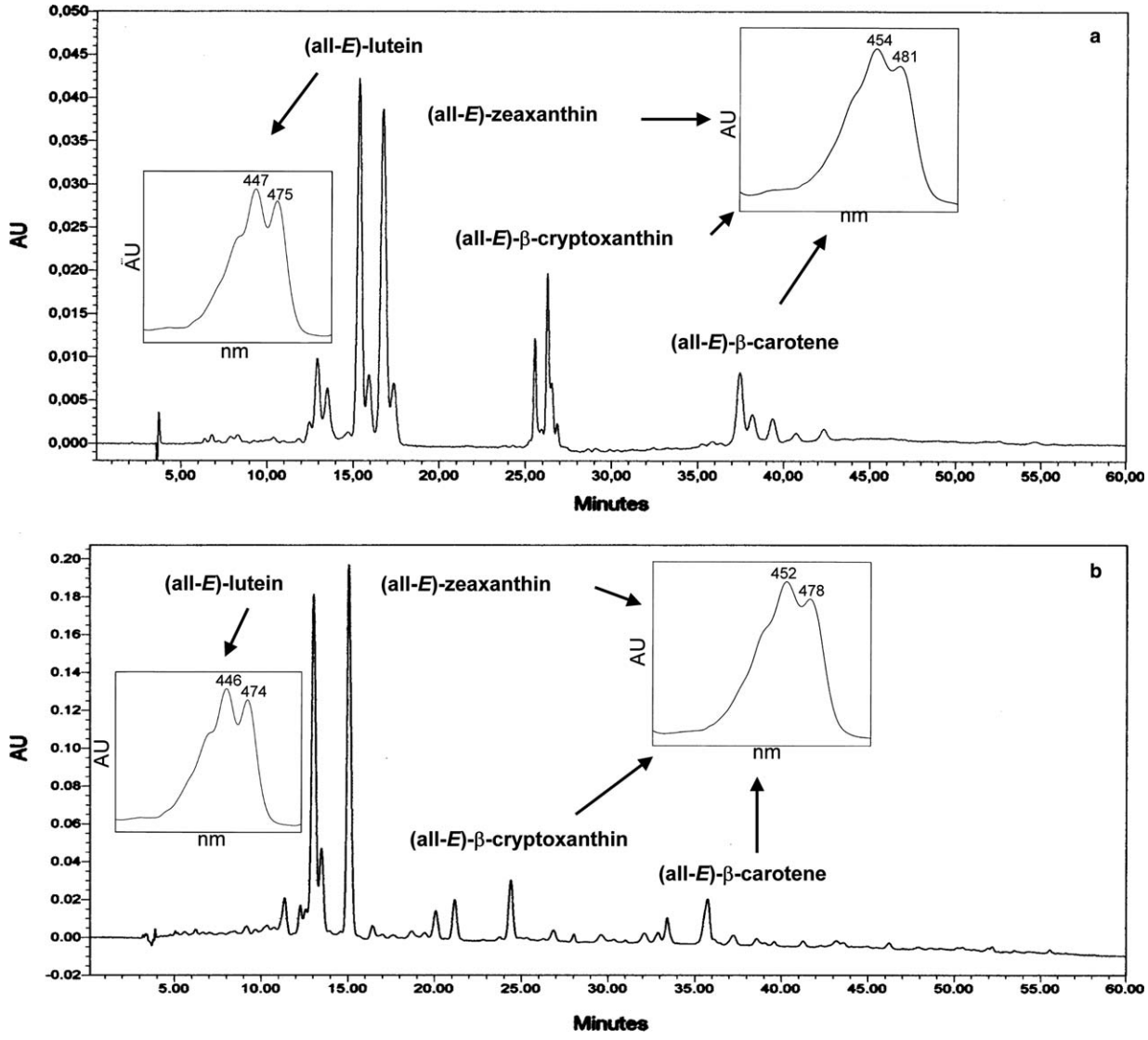

Fig. 4. HPLC chromatograms obtained with (a) C18 monomeric Spherisorb ODS2 column and (b) YMC C30 polymeric column, and photodiode array spectra of the carotenoids of dry maize Assum Preto. Detection at 450 nm. HPLC conditions are described in the text. Zeaxanthin, β-cryptoxanthin, and β-carotene have the same chromophore and therefore the same absorption spectrum.

3.3. Carotenoid concentrations in cassava

β-Carotene was also the principal carotenoid of the yellow BRA 005771 and cream-fleshed IAC 576-70 varieties of cassava analyzed, but the amounts of (9*Z*)-β-carotene and (13*Z*)-β-carotene were comparatively substantial (Figs. 2 and 3). In all varieties, several other small unidentified peaks were present, the levels of these minor carotenoids being greater in the yellow-fleshed cassava.

The data again show excellent repeatability, the CVs for triplicate measurements for total carotenoid, total β-carotene and (all-*E*)-β-carotene ranging from 0% to 3.8% with a mean of 1.7%. The CVs for the *Z*-isomers were slightly higher, the range being 0.1–5.1% with an average of 2.6%; these values are understandable considering that the levels were lower and these were estimates as the calculation was based on the (all-*E*)-β-carotene curve.

Because of the presence of the unidentified carotenoids, the total β-carotene content obtained by HPLC was 85–89% and 83–86% of the total carotenoid content obtained by screening, using the C18 and C30 columns, respectively. This difference would be even greater with the yellow-fleshed cassava, in which these other carotenoids were in greater amounts (Figs. 2 and 3). Notwithstanding the difference, the screening method can be used to analyze a large number of samples to identify those having provitamin A with levels close to the target, which would then be submitted to HPLC analyses.

Z-β-carotene accounted for 32–33% and 30–31% of the total β-carotene in the C18 and C30 columns, respectively.

The data presented in Table 2 demonstrate, as in sweetpotato, that there was no significant difference in the results obtained using acetone or THF:MeOH as the extracting solvent and between using a mortar or pestle or a Polytron homogenizer. Additionally, although there was overlapping of peaks in the chromatogram obtained with the C18 column, the results obtained with the C18 and C30 columns were equivalent.

Cassava is known to deteriorate rapidly. It is recommended that this crop be analyzed, cooked or processed

Table 4
Comparison of screening and HPLC methods for determining the principal carotenoids (μg/g)[A] of maize

Variety	Method	Dihydroxy-carotenoids	Lutein	Zeaxanthin	β-Cryptoxanthin	β-Carotene
Assum Preto	Screening	8.21 ± 0.25			1.43 ± 0.04	1.53 ± 0.07
	HPLC[B]		3.60 ± 0.12	4.01 ± 0.07	0.91 ± 0.03	0.53 ± 0.02
BR 473	Screening	6.83 ± 0.25			1.16 ± 0.05	1.18 ± 0.05
	HPLC[B]		1.48 ± 0.04	4.67 ± 0.25	0.82 ± 0.05	0.28 ± 0.01
Asa Branca	Screening	8.48 ± 0.35			1.70 ± 0.06	1.65 ± 0.07
	HPLC[B]		1.81 ± 0.07	5.65 ± 0.23	1.40 ± 0.04	0.77 ± 0.02

[A] Means and SDs of triplicate analyses.
[B] $E + Z$ isomers.

Table 5
Effect of rehydration conditions on the principal carotenoids (μg/g)[A] of maize variety Assum Preto

Carotenoid	Column	Rehydration conditions			
		25 °C[B], 30 min	85 °C, 5 min	85 °C, 10 min	85 °C, 15 min
Lutein					
Total	C18	3.60 ± 0.12[a]	3.92 ± 0.18[a]	4.25 ± 0.19[a]	3.94 ± 0.21[a]
All-*E*	C18	2.85 ± 0.09[b]	3.24 ± 0.15[b]	3.53 ± 0.20[b]	3.26 ± 0.16[b]
All-*E*	C30	2.75 ± 0.09[b]	2.73 ± 0.08[b]	3.24 ± 0.13[b]	2.93 ± 0.13[b]
Zeaxanthin					
Total	C18	4.01 ± 0.07[a]	4.28 ± 0.16[a]	4.65 ± 0.19[a]	4.45 ± 0.19[a]
All-*E*	C18	3.37 ± 0.07[b]	3.66 ± 0.11[b]	3.84 ± 0.16[b]	3.72 ± 0.15[b]
All-*E*	C30	3.10 ± 0.11[b]	3.58 ± 0.06[b]	3.62 ± 0.05[b]	3.53 ± 0.09[b]
β-Cryptoxanthin					
Total	C18	0.91 ± 0.03[a]	0.99 ± 0.01[a]	1.06 ± 0.05[a]	1.02 ± 0.04[a]
All-*E*	C18	0.71 ± 0.03[b]	0.73 ± 0.03[b]	0.78 ± 0.04[b]	0.76 ± 0.03[b]
All-*E*	C30	0.72 ± 0.03[b]	0.76 ± 0.02[b]	0.75 ± 0.03[b]	0.74 ± 0.02[b]
β-Carotene					
Total	C18	0.58 ± 0.02[a]	0.63 ± 0.03[a]	0.67 ± 0.04[a]	0.61 ± 0.04[a]
All-*E*	C18	0.33 ± 0.02[b]	0.33 ± 0.01[b]	0.34 ± 0.02[b]	0.35 ± 0.01[b]
All-*E*	C30	0.33 ± 0.02[b]	0.39 ± 0.01[b]	0.34 ± 0.03[b]	0.33 ± 0.02[b]

Values in the same horizontal row with different superscript letters are significantly different ($p \leqslant 0.05$).
For each carotenoid, values in the same column with different superscript letters are significantly different ($p \leqslant 0.05$).
[A] Means and SDs of triplicate analyses.
[B] Room temperature.

within 24 h after harvest. Thus, the possible effect of the time after harvest on the carotenoid content was investigated.

The total carotenoid obtained by screening and the total and (all-*E*)-β-carotene contents obtained by HPLC decreased significantly within 24 h after harvest, more so when stored at room temperature than at freezer temperature (−18 °C) (Table 3). The *Z*-isomers of β-carotene tended to increase slightly.

3.4. Carotenoid concentrations in dry maize

Dry maize was difficult to extract and the carotenoid composition was complex (Fig. 4). Lutein and zeaxanthin were the principal carotenoids with small amounts of β-cryptoxanthin and β-carotene; all these carotenoids were present in all-*E*- and *Z*-forms.

Because the objective of HarvestPlus is to breed high provitamin A-maize, the screening method needs to give an estimate of β-carotene and of β-cryptoxanthin. β-Cryptoxanthin has only one-half of the provitamin A activity of β-carotene, but in many varieties or breeding lines of maize, it is found in a higher concentration than β-carotene. The total carotenoid content of maize comprises mostly lutein and zeaxanthin and cannot be used as an index of the provitamin A content. Thus, a minicolumn was used in this study to separate the carotene and monohydroxycarotenoid fraction, which were then submitted to spectrophotometric measurement.

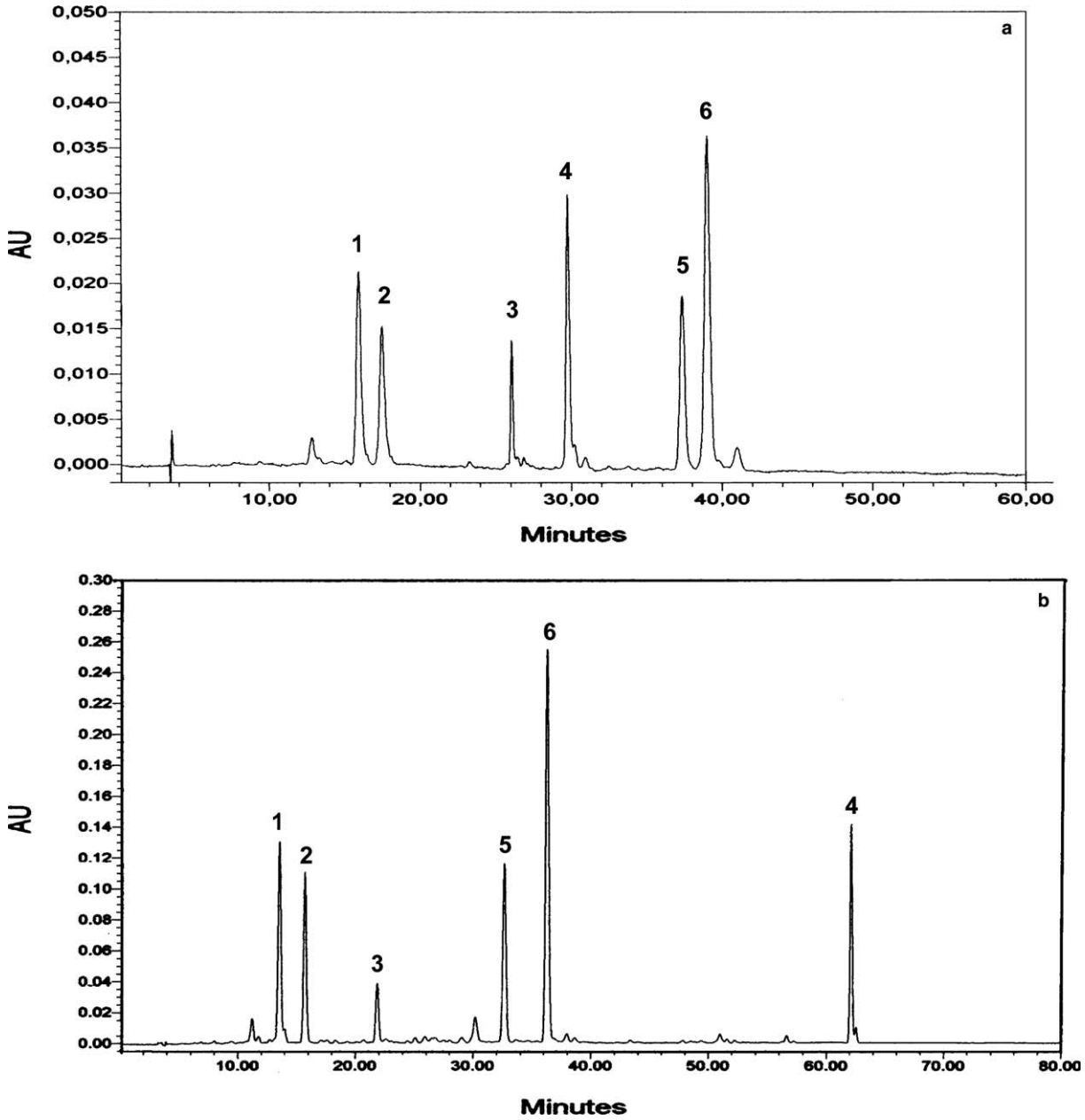

Fig. 5. HPLC chromatograms obtained with (a) C18 column and (b) C30 column of the carotenoids of CRM 485. Detection was at 450 nm. Peak identification: 1 – (all-*E*)-lutein; 2 – (all-*E*)-zeaxanthin; 3 – (all-*E*)-β-cryptoxanthin; 4 – (all-*E*)-lycopene; 5 – (all-*E*)-α-carotene; 6 – (all-*E*)-β-carotene.

Comparing the results from the screening and HPLC quantification with room temperature rehydration, the lutein + zeaxanthin contents were equivalent and those for β-cryptoxanthin close (Table 4). Screening overestimated the β-carotene content because the carotene fraction obtained included small amounts of α-carotene and carotenoid esters. Nevertheless, the screening method is valid, and the results for those samples close to the target levels can then be verified by HPLC.

Rehydration is necessary for dry maize to allow the extracting solvent to penetrate the tissues. The HPLC results obtained by hot rehydration tended to be higher than those obtained by ambient temperature rehydration, but were not statistically different (Table 5), thus either of these procedures can be used, but preference should be given to hot rehydration. Although not statistically different from those obtained with 10 min hot rehydration, the concentrations obtained by hot rehydration for 15 min were lower. Thus, hot rehydration for 10 min is recommended to diminish the possibility of thermal degradation of the carotenoids.

The concentrations of lutein, zeaxanthin, β-cryptoxanthin and β-carotene in the all-*E*-configuration obtained with both the C18 and C30 columns were equivalent. The total ($E + Z$) contents of these carotenoids could not be calculated with the C30 column because it was difficult to locate the *Z*-form of the different carotenoids (Fig. 4). In the C18 column the *Z*-isomers eluted close to the respective all-*E*-carotenoids.

Extraction with THF:MeOH as solvent with samples rehydrated for 30 min was also carried out to compare with extraction with acetone. The means in μg/g were: total lutein, 2.23 ± 0.12; (all-*E*)-lutein, 1.81 ± 0.07; total zeaxanthin, 2.61 ± 0.15; (all-*E*)-zeaxanthin, 2.17 ± 0.10; total β-cryptoxanthin, 0.94 ± 0.01; (all-*E*)-β-cryptoxanthin, 0.72 ± 0.00; total β -carotene, 0.5 ± 0.02; (all-*E*)-β-carotene, 0.34 ± 0.01. The values for both β-carotene and β-cryptoxanthin obtained with acetone and THF:MeOH as extracting solvents were equivalent. However, the values for lutein and zeaxanthin were significantly lower for THF:MeOH. This was not due to the extraction efficiency as this solvent mixture was very efficient in extracting the carotenoids. The problem was in the partition to petroleum ether. Transferring the dihydroxycarotenoids from THF:MeOH to the apolar solvent was more difficult than transferring from acetone, and some of these carotenoids were apparently lost with the aqueous phase.

The results discussed above refer to the maize variety Assum Preto. Similar data were obtained with the Asa Branca and BR-473 varieties. Data for the latter varieties are not shown for the sake of brevity.

3.5. Carotenoid concentrations in the certified reference material

Applicability of an analytical method depends on the matrix, the analytes present and their levels. Thus, CRM 485 (lyophilized vegetable mix) was chosen for validation of the methods developed. Typical chromatograms of the carotenoids of this material, obtained with the C18 and C30 columns, are shown in Fig. 5.

As with the sweetpotato, cassava and maize samples, the results obtained with the certified reference material showed that there was no significant difference in extrac-

Table 6
Values (μg/g dry weight)[A] obtained with the BCR reference material CRM 485 using the developed methods

Carotenoid	Certified values	Extraction procedures			
		Acetone – mortar		Acetone – Polytron	
		C18	C30	C18	C30
Lutein					
Total	12.5	11.9 ± 0.2^{a} (0.8)		11.6 ± 0.5^{a} (1.1)	
All-*E*		11.3 ± 0.3^{a}	11.0 ± 0.4^{a}	11.0 ± 0.1^{a}	10.7 ± 0.0^{a}
Zeaxanthin					
Total		11.3 ± 0.3^{a}		11.9 ± 0.2^{a}	
All-*E*	9.7[B]	10.3 ± 0.3^{a}	10.9 ± 0.1^{a}	10.9 ± 0.3^{a}	10.8 ± 0.0^{a}
Lycopene					
Total	14.2[B]	15.3 ± 0.7^{a}		16.6 ± 0.3^{a}	
All-*E*	13.8[B]	14.7 ± 0.4^{a}	13.9 ± 0.5^{a}	15.9 ± 0.4^{a}	14.7 ± 0.5^{a}
α-Carotene					
Total	9.8	10.6 ± 0.4^{a} (1.1)		10.7 ± 0.3^{a} (1.3)	
All-*E*	10.5	10.1 ± 0.3^{a} (0.7)	9.9 ± 0.0^{a} (1.0)	10.2 ± 0.4^{a} (1.2)	9.8 ± 0.1^{a}
β-Carotene					
Total	25.6	25.7 ± 0.4^{a} (0.1)		25.5 ± 0.7^{a} (0.1)	
All-*E*	23.7	23.4 ± 0.6^{a} (0.2)	22.8 ± 0.3^{a} (0.6)	23.5 ± 0.2^{a} (0.1)	22.9 ± 0.0^{a} (0.5)

Values in parenthesis are the *z*-scores.
Values in the same horizontal row with different superscript letters are significantly different ($p \leqslant 0.05$).
[A] Means and SDs of triplicate analyses.
[B] Indicated values.

tion using a mortar and pestle or a Polytron homogenizer, nor was there a significant difference between using a C18 or a C30 column (Table 6).

A z-score $\leqslant 2$ is considered satisfactory (ISO/IEC, 1996; Thompson & Wood, 1993). All of the z-scores presented in Table 6 ($\leqslant 1$) calculated for those carotenoids with certified values and total uncertainty (all-*E* and total α-carotene, all-*E*- and total β-carotene and total lutein) fell well within the satisfactory criterion (Table 6), attesting to the excellent performance of the methods. There was also good agreement between the results obtained in this work and the information values for (all-*E*)-zeaxanthin and for (all-*E*)- and total lycopene.

4. Conclusions

Because the qualitative and quantitative carotenoid composition and the nature of the food matrix differ, a single method cannot be established for the three crops. Although the general procedure is the same, the details differ. In sweetpotato varieties in which (all-*E*)-β-carotene is predominant, spectrophotometric quantification is sufficient without resorting to HPLC. When several other minor carotenoids are present screening can be done spectrophotometrically, but chromatographic separation of β-carotene is necessary for accurate quantification. For cassava there is a need to separate and quantify, aside from (all-*E*)-β-carotene, the *Z*-isomers that are present in appreciable amounts. Screening can be done spectrophotometrically, but quantification needs HPLC. In maize screening requires separation of the monohydroxycarotenoid and β-carotene fractions, which are completely masked by lutein and zeaxanthin when total carotenoid content is determined spectrophotometrically. For both sweetpotato and cassava extraction can be carried out with acetone or THF:MeOH (1:1), using a mortar and pestle or a Polytron homogenizer.

For maize, although THF:MeOH is an efficient extracting solvent, partition of lutein and zeaxanthin to petroleum ether is problematic. Rehydration of dry maize at room temperature for 30 min or preferably rehydration at 85 °C for 10 min is recommended to make the extraction efficient. Either a C18 or a C30 column can be used for cassava, although there is some overlapping of the isomers' peaks with the C18 column. These columns also give equivalent results for lutein, zeaxanthin, β-cryptoxanthin and β-carotene in the all-*E*-form, but the *Z*-isomers are difficult to locate in the chromatogram obtained with a C30 column.

The methods developed performed very well with a certified reference material.

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