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Application of Visible and Near-Infrared Reflectance Spectroscopy (Vis/NIRS) to Determine Carotenoid Contents in Banana (*Musa* spp.) Fruit Pulp

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The analysis of carotenoids is complicated by the tendency of these compounds to react with radical species, leading to oxidative breakdown and isomerization during extraction. Therefore, protocols should be rapid and avoid unnecessary exposure to heat, acids, and so forth. Here, we evaluate the use of visible and near infrared reflectance spectroscopy (Vis/NIRS) to measure carotenoid contents in fruit from 28 *Musa* (banana and plantain) varieties. Carotenoid contents were first quantified using standardized RP-HPLC protocols, and these results were then used to develop algorithms to predict carotenoid contents from Vis/NIR spectra of the same samples. Cross-validation of the predictive algorithms across a genetically diverse group of varieties demonstrated that correlation coefficients between the HPLC measurements and the Vis/NIRS predictions varied from good for the total carotenoids and β-carotene fractions (r^2_{cv}, 0.84, 0.89) to reasonable for α-carotene and *cis*-carotenes (r^2_{cv}, 0.61, 0.66), but there was only a poor correlation (r^2_{cv}, 0.30) for the minor lutein component. Nonetheless, since ~90% of the *Musa* carotenoids consist of only α- and β-carotene, results indicate that Vis/NIRS can be used for the high-throughput screening of fruit pulp samples for vitamin A nutritional content on the basis of their total carotenoids content.

KEYWORDS: Banana; biofortification; HPLC, Vis/NIRS, micronutrients; *Musa*; nutrition; plantain; provitamin A carotenoids; vitamin A; screening

INTRODUCTION

Bananas and plantains (*Musa* spp.) are the world's fourth most important food crop, with an annual production of about 100 Mt. They are also a staple part of the diet across some of the poorest parts of the world, including Africa, Latin America, and South East Asia, where the fruits are not only consumed raw but are also processed in a wide variety of ways. This means that in these areas, *Musa* fruits and fruit-products represent an important source of essential dietary micronutrients. Recent reports from the World Health Organisation and the World Bank indicate that micronutrient deficiencies and in particular vitamin A (vit A, retinol), iron (Fe), and zinc (Zn) affect literally billions of people in developing countries so that the introduction or promotion of micronutrient-rich *Musa* varieties could have a significant long-term beneficial impact on the health of populations in these regions.

Most dietary vit A is obtained from plants, and there are about 50 naturally occurring carotenoid compounds having vit A biological activity (*1*). These are the so-called provitamin A carotenoids (pVACs), which are broken down in the body to yield retinol, the active form of vit A (*1*, *2*). The widely perceived health benefits of carotenoids have stimulated much interest in the development of food crops with enhanced carotenoid contents (biofortification) (*3*−*7*). However, while biotechnological approaches have had some spectacular and high-profile successes in this regard, e.g. refs *3* and *8*, numerous horticultural crops are already good sources of carotenoids (*9*). Therefore, introgression of these traits into existing elite *Musa* breeding lines from wild-type or exotic germplasm by classical or molecular breeding would seem to be a feasible strategy. However, such an approach depends on the presence of a sufficiently large degree of diversity for the trait in question within the *Musa* germplasm pool. Despite the economic and social importance of bananas and plantains (*Musa* spp.), there

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has to our knowledge been only one large-scale, systematic survey of *Musa* germplasm collections for fruit micronutrient contents to date (*10*).

Germplasm screening relies on the accurate phenotyping of the trait in question. The most popular and widespread approach for carotenoid quantification is based on RP-HPLC using C_{18} or C_{30} columns coupled to diode-array detection (DAD). RP-HPLC allows the separation and quantification of all individual carotenoid compounds including the pVACs that have dietary vit A activity (*11*−*13*). Alternative methods include spectrophotometry in the visible range to determine the total carotenoids content. For crops such as *Musa*, cassava, and sweet potato in which the carotenoids content of fruit tissue consists primarily of pVACs (*11, 14*), Vis-spectroscopy can provide an estimate of tissue vit A nutritional contents. However, both HPLC and spectrophotometric analyses involve lengthy and labor-intensive extraction protocols with large volumes of organic solvents, solvent partitioning, and/or saponification steps (*15, 16*). This greatly increases the chance of the formation of carotenoid byproducts, increases running costs, and decreases sample throughput. Typical byproducts result from *cis*−*trans* isomerization events, oxidative cleavage, and/or epoxidation of the carotenoid backbone leading to products that can no longer be detected by RP-HPLC (*17*−*19*). Recently, we have described modifications to simplify extraction, minimize handling steps, and to allow direct on-column injection of *Musa* fruit extracts without sample cleanup (*14*). Nonetheless, RP-HPLC remains relatively expensive and requires well-equipped laboratories, which may not always be available in the regions of the developing countries where these crops are primarily cultivated and consumed.

In this work, we were interested in developing methodologies for the high-throughput analysis of fruit pVAC contents and vit A nutritional contents as encountered in breeding and germplasm-screening programs. For this, we have carried out a relatively small-scale trial to evaluate the potential of visible and near-infrared reflectance spectroscopy (Vis/NIRS) to screen for carotenoid contents in fruit from a wide variety of *Musa* genotypes. Vis/NIRS is a nondestructive procedure that has found a wide variety of applications within the food industry, including, for example, the control of fruit and vegetable quality (for recent reviews see refs *20*−*22*), and more recently, it has been applied to the analysis of carotenoid contents in maize (*23*). Here, we have developed predictive models for Vis/NIR spectra to determine the carotenoid levels in lyophilized fruit pulp samples obtained from a wide selection of *Musa* varieties using standardized RP-HPLC protocols to calibrate the methodology.

MATERIALS AND METHODS

Most cultivated bananas and plantains are natural hybrids, derived from inter- and intraspecific crosses between two diploid wild species: *Musa acuminata* (designated by genome A) and *M. balbisiana* (designated by genome B) (*24*). These hybrids are most usually triploid, but may also be diploid or tetraploid. In this study, a total of 66 fruit pulp samples obtained from 28 individual *Musa* genotypes including representatives of all the major genome groups were analyzed by both HPLC and Vis/NIRS (see **Table 1**).

***Musa* Varieties and Cultivation Conditions.** Samples were obtained from the *Musa* germplasm collection maintained by CARBAP (Centre Africain Régional de Recherches sur Bananiers et Plantains) at Njombé in Cameroon, from individual registered farms in Eastern Uganda, from collections on the islands of Maui and Haiku, USA, and from the Banana Genebank Collection, Davao City, Philippines. All fruits were healthy and undamaged unless specifically indicated otherwise.

Fruit Sampling. As far as possible, standardized sampling protocols were used to harvest fruit (*25*). This involved collecting fruit from the middle of hands situated at the top (proximal), middle and bottom (distal) end of each bunch, and where possible from three individual bunches (plants) at the same time. The fruit maturity stage was estimated according to the peel color essentially as described by Dadzie and Orchard (*26*). According to this scale, stage 1 is unripe/immature, stage 3 is starting to ripen, stage 5 is ripe, stage 7 is fully ripe, and stage 9 is overripe. Fruits obtained from CARBAP were harvested at the immature green stage (stage 1), and other samples were delivered fresh to the laboratory and the ripening stage noted on arrival, as determined by the peel color (*26*). An overview of the varieties analyzed, the number of samples, the maturity stage of the fruit, and other important descriptors is given in **Table 1**.

Sample Transport. Fruits obtained from CARBAP were frozen immediately after harvest and lyophilized before shipping to the laboratory in Leuven in sealed polyethylene bags in the dark. All other fruits were transported fresh in padded boxes with free air circulation at temperatures of around ± 8 °C.

Sample Processing. Upon arrival, fresh fruit samples were photographed, weighed, frozen in liquid nitrogen, and stored lyophilized in sealed plastic bags at −20 C. For analysis by either HPLC or Vis/NIRS, lyophilized fruit samples were crushed to a fine powder in a pestle and mortar in liquid nitrogen under reduced light.

RP-HPLC Analysis. Carotenoids were extracted from lyophilised powders under reduced light, using ice-cooled tetrahydrofuran/methanol (THF/MeOH) 1:1 (v/v), containing 0.1% butylated hydroxytoluene (BHT) (w/v), essentially as previously described (*14*). Briefly, 50−100 mg aliquots of powdered *Musa* fruit pulp were extracted with 5−10 volumes/gdw of ice-cooled THF/MeOH, 1:1 (v/v), containing 0.1% BHT and 2% insoluble PVPP. Following centrifugation (14,000*g* × 12 min), the residue was extracted twice more with 10 volumes of extraction buffer, but without PVPP. All supernatants were then combined, filtered, and analyzed by HPLC within 24 h. All chromatographic analyses were carried out on a Waters Alliance, 2690 Separations System, fitted with a thermostatted autosampler, a pulse dampener, a 996 UV−vis photodiode array detector, and a column heater (Waters, Massachusetts, USA). Detection was carried out in the range 300−600 nm, at a frequency of 2 Hz and a spectral resolution of 2 nm. The system was controlled and data collected and integrated using the Millenium 4.0 software package. Individual carotenoids species were resolved by C_{18} RP-HPLC using a 150 × 4.6 mm, Waters ODS-2 3-μm particle size column (Millipore, Brussels, Belgium), according to refs *14* and *25*. Quantification was carried out at 450 nm, and peaks were identified on the basis of their characteristic absorption spectra and their retention times relative to known standards (*15, 27, 28*). *trans*-β-apo-8′-carotenal (8-apo-carotene) at a final concentration of 0.004 μg/mL was used as an internal standard. The concentrations of all-*trans*-α-carotene (α-carotene) were calculated using molar absorption coefficients calculated from the all-*trans*-β-carotene (β-carotene) standard curve at 450 nm. Since the molar absorption coefficient of α-carotene is slightly different from that of β-carotene at this wavelength and commercial standards are not available, a compensation factor of 0.925 was used to correct α-carotene values, as previously described (*14*). Concentrations of lutein were calculated using a standard curve constructed with commercial standards in extraction buffer.

Vis/NIRS Analysis of Carotenoids. Vis/NIR spectra from powdered, lyophilized *Musa* fruit pulp samples were obtained using a Labspec Pro Vis-NIR spectrophotometer (Analytical Spectral Devices Inc., Boulder, CO). Approximately 0.5−1 g of each sample was packed into a black sample cup, and the diffuse reflectance spectrum was then recorded using a ProTec DR7 + 1 diffuse reflectance probe (Prozess-Labor- and Sensortechnik GmbH, Jena, Germany) connected to an internal light source and the detector port of the Labspec Pro spectrophotometer. Each recorded spectrum represented the average of 50 scans in the wavelength range 367−2388 nm collected at 1 nm intervals over a period of 100 ms. In total, 2151 data points were collected per scan. For each sample, 3 spectra were recorded with samples being gently shaken between scans to average out the effect of inserting the diffuse reflectance probe into the powder and to avoid

Table 1. Overview of the Different *Musa* Varieties Analyzed and General Descriptors

source	variety	genome group[a]	subgroup/related type[a]	type	no of fruit analyzed	ripening stage[d]	vis/NIRS #[b]	HPLC #[c]
Hawaii	Iholena Lele sub var. 'Long Peduncular'	AAB	Pacific Plantain - Iholena	n/a	3	7	9	6
	Maoli 'Ctahr-kona'	AAB	Pacific Plantain - Maoli-Popo'ulu (Maoli subdivision)	n/a	2	9	6	4
	Maoli 'Ele'ele subvar 'Hinpua'a'	AAB	Pacific Plantain - Maoli-Popo'ulu (Maoli subdivision)	n/a	1	7	3	2
	Popo'ulu Aulena	AAB	Pacific Plantain - Maoli-Popo'ulu (Popo'ulu subdivision)	n/a	3	7	9	6
	Popo'ulu Lahi	AAB	Pacific Plantain - Maoli-Popo'ulu (Popo'ulu subdivision)	n/a	2	7	6	4
Uganda	Mbwazirume	AAA	Mutika/Lujugira (Nakitembe)	cooking	6	7	18	12
	Mukubakkonde	AAA	Mutika/Lujugira (Nakabululu)	cooking	1	7	3	2
	Nakabululu	AAA	Mutika/Lujugira (Nakabululu)	cooking	3	7	9	6
	Nakitembe	AAA	Mutika/Lujugira (Nakitembe)	cooking	3	7	9	6
BPI, Philippines	Bantol red	n/a	n/a	n/a	3	6	9	6
	Henderneyargh	AAS	n/a	n/a	2	6	6	4
	Katimor	AAB	n/a	dessert	3	6	9	6
	Kluai kai boran	AA	n/a	dessert	4	6	12	8
	Oonoonoo kengoa	AA	n/a	dessert	3	6	9	6
	Pisang talas	AA	n/a	dessert	4	6	12	8
	Pusit	n/a	n/a	n/a	4	6	12	8
	Senorita	AA	n/a	dessert	3	6	9	6
CARBAP, Cameroon	Batard	AAB	Plantain (French Horn)	cooking/frying	1	1	3	2
	Cachaco	ABB	Bluggoe	cooking	1	1	3	2
	Espermo	ABB	Bluggoe	cooking	1	1	3	2
	Highgate	AAA	Gros Michel (Highgate)	dessert	1	1	3	2
	Kontrike	AAA	Cavendish	dessert	1	1	3	2
	Mbeta 2	AAB	Plantain (Horn)	cooking/frying	1	1	3	2
	Mbouroukou-1	AAB	Plantain (False Horn)	cooking/frying	6	1	18	12
	Mbouroukou-3	AAB	Plantain (False Horn)	cooking/frying	2	1	6	4
	Px2	AAB	Plantain	cooking/frying	1	1	3	2
	Valery	AAA	Cavendish (Giant Cavendish)	dessert	1	1	3	2
	Williams	AAA	Cavendish (Giant Cavendish)	dessert	1	1	3	2

[a] Information obtained from the MGIS database http://bananas.bioversityinternational.org/. [b] 3 scans per sample. # = total number of analyses carried out per variety. [c] 2 extractions per sample. # = total number of analyses carried out per variety. [d] The fruit maturity stage was estimated according to the peel color as described by Dadzie and Orchard (*26*). According to this scale, stage 1 is unripe/immature, stage 3 is starting to ripen, stage 5 is ripe, stage 7 is fully ripe, and stage 9 is overripe.

differences in powder compaction. The spectra were recorded using the Indico Pro software package (Analytical Spectral Devices Inc., Boulder, CO) and stored on an IBM-compatible PC.

Data Processing/Statistics. A multivariate statistical analysis was performed on the results using the Unscrambler 9 software package (Camo Process AS). Here, data from the HPLC analysis of aliquots of the same samples was used to develop calibration models for the prediction of the carotenoids content from the Vis/NIR spectral data using a partial least-squares regression approach. From the plot of the root mean squared errors of cross-validation for increasing number of latent variables (LVs), the number of LVs corresponding to the first local minimum as suggested by the software was chosen. A full multiplicative scatter correction (MSC) and the first and second derivative obtained with the Savitzky−Golay algorithm were tested as preprocessing techniques to reduce the effects of light scattering on the spectra and to improve the quality of the results. Best results were obtained using the first derivative derived from the Savitzky−Golay algorithm with a polynomial order of 2 fitted over a 21 nm interval.

A calibration set consisting of 49 samples was used to build predictive models for carotenoid concentrations from the Vis/NIR spectra of the samples, which had previously been analyzed with standardized HPLC procedures (*14*). The complexity of the predictive models, i.e., the number of LVs, was optimized on the basis of the estimated prediction error obtained in cross-validation. This cross-validation consisted of 24 iterations, where each time, all samples of one variety were excluded from the calibration set and used to cross-validate the models with different numbers of LVs built on the others. The prediction error (RMSECV) was then averaged over the iterations and plotted against the number of LVs used by each model. The model corresponding to the first local minimum of this average prediction error curve was then selected by the software to build a model based on the entire calibration set. These models were then used to predict the carotenoids content for the remaining 17 samples (test sample set), which had been excluded from the calibration set.

The choice of samples used to generate the test set was based on the need to create a group as far as possible containing representative values for each of the carotenoid species measured (α-carotene, β-carotene, c-carotene, lutein, and total carotenoid contents), within the limits of the ranges found in the calibration set for each of these components. Since none of the 17 varieties present in the second group (test sample set) are present in the first calibration sample set, the test set can then be used to determine the power of the developed models to predict the carotenoid contents of (new) varieties, which were not included in the calibration set. These results were then evaluated on the basis of the coefficients of determination for cross-validation (r^2_{cv}) and/or prediction (r^2_{pred}), calculated as the square of the Pearson correlation coefficients between the predicted and observed values, and with the ratio (RPD) of the standard deviation to root-mean-square error of cross-validation (RMSECV) or prediction (RMSEP).

The usefulness and accuracy of the developed models were evaluated on the basis of the r^2_{cv}, r^2_{pred}, and RPD values (*29, 30*). The r^2 values give an indication of the percentage variation in the *Y* variable that is accounted for by the *X* variable. Therefore r^2 values above 0.50 indicate that over 50% of the variation in *Y* is attributable to variation in *X*, and this allows discriminations between high and low concentrations to be made. Higher r^2 values improve discrimination, and models with an r^2 of 0.66−0.81 can be used for screening and approximate quantitative predictions, models with r^2 values between 0.83−0.90 can be used for many applications, while models

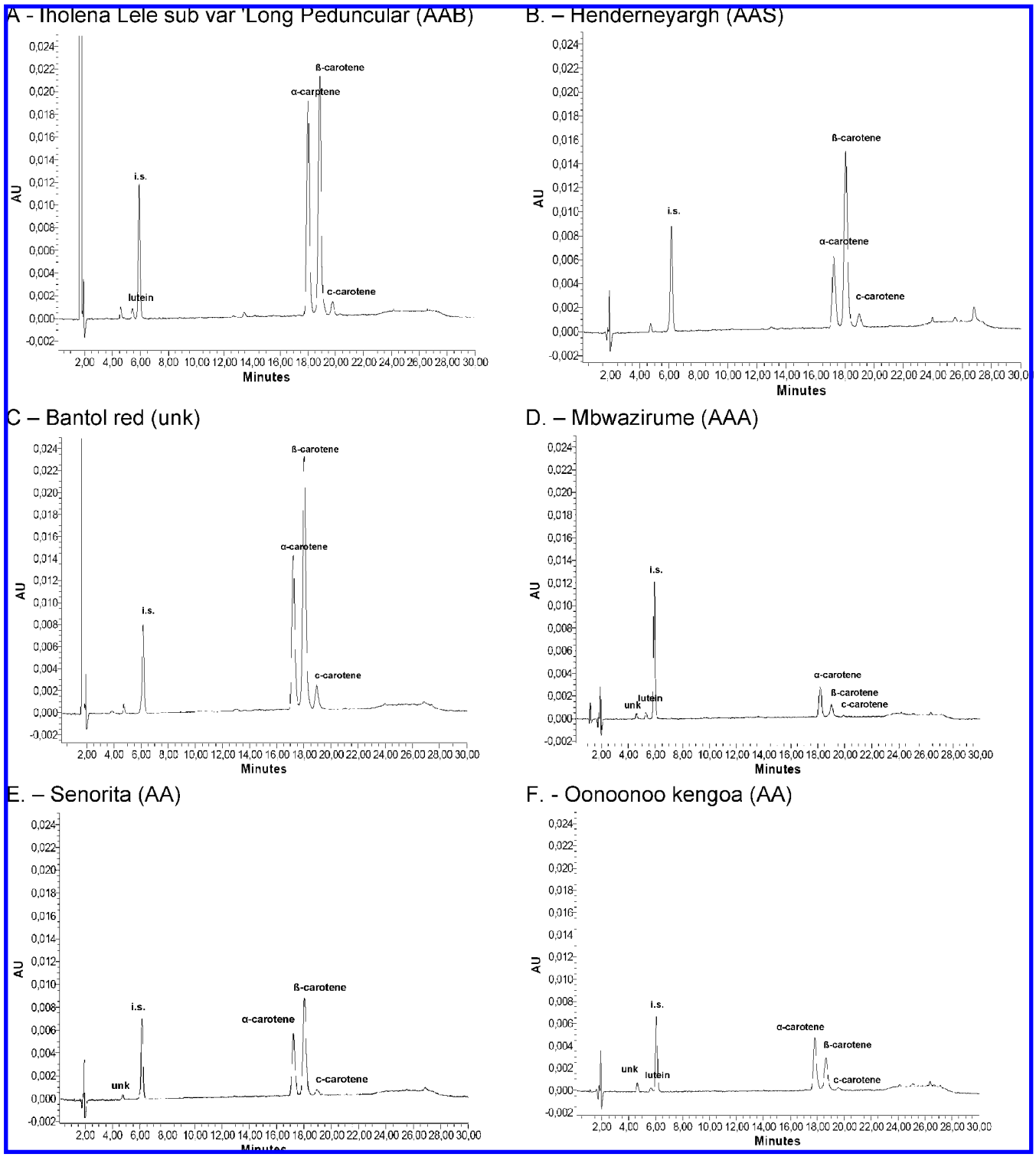

Figure 1. Typical C_{18} RP-HPLC chromatograms for the quantification of carotenoid compounds of banana pulp in a range of *Musa* genotypes. α-carotene = all-*trans*-α-carotene; *β*-carotene = all-*trans*-*β*-carotene; c-carotene = 13-*cis*-*β*-carotene; i.s. = internal standard, *trans*-apo-8′-*β*-carotenal; unk = unknown.

with values of 0.92−0.96 are suitable for most applications including quality assurance, and those above 0.98 for all applications (*29*). Although r^2 values give good information about the quality of the calibration, they do not provide information on the prediction accuracy. For this, we used the RPD classifications defined by Saeys et al. (*30*). Here, RPD values below 1.5 are considered unusable, values of between 1.5−2.0 can be used for rough predictions, those between 2.0 and 2.5 allow approximate quantitative predictions to be made, while values above 2.5 and 3.0 are considered to be good and excellent predictive models, respectively (*30*).

RESULTS AND DISCUSSION

The *Musa* varieties used in this work were chosen on the basis of their known genome compositions as well the need to cover as wide a range of fruit carotenoid contents as possible.

HPLC Analysis of Fruit Carotenoid Contents. HPLC analysis of *Musa* fruit pulp indicates that over 90% of the provitamin A carotenoids (pVACs) present consist of all-*trans*-

Table 2. Summary of *Musa* Fruit Individual pVACs, Lutein, and Total Carotenoid Contents as Determined by HPLC[a]

variety	#[c]	α-carotene	β-carotene	c-carotene	lutein	total carotenoids	% β-carotene	% α-carotene	% c-carotene	% lutein
Mukubakkonde	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Valery	4	0.0	0.0	0.0	0.7	0.7	0.0	0.0	0.0	100.0
Kontrike	3	3.3	0.0	0.0	0.0	3.3	100.0	0.0	0.0	0.0
Williams	3	3.2	0.0	0.0	0.5	3.7	86.1	0.0	0.0	13.9
Espermo	1	0.7	4.0	0.0	0.0	4.7	14.0	86.0	0.0	0.0
Highgate	1	5.3	0.0	0.0	0.0	5.3	100.0	0.0	0.0	0.0
Cachaco	1	1.6	6.8	0.0	0.0	8.4	19.5	80.5	0.0	0.0
Mbouroukou-3	3	7.1	6.8	0.0	0.2	14.1	50.3	48.5	0.0	1.1
Px2	2	12.1	14.4	0.0	0.0	26.5	45.7	54.3	0.0	0.0
Nakitembe	1	12.7	9.6	0.0	4.6	26.9	47.3	35.6	0.0	17.1
Mbwazirume[b]	2	14.8	11.8	0.0	2.4	29.0	51.1	40.7	0.0	8.2
Mbeta 2	1	14.9	15.1	0.0	0.0	30.0	49.6	50.4	0.0	0.0
Popo'ulu Aulena	2	7.3	32.9	1.4	0.9	42.6	17.2	77.2	3.4	2.2
Batard	1	23.9	22.0	0.0	0.3	46.2	51.6	47.6	0.0	0.7
Nakabululu	3	28.6	20.7	0.0	8.4	57.7	49.5	35.9	0.0	14.6
Maoli 'Ctahr-kona'	1	17.7	46.0	4.5	6.0	74.1	23.8	62.0	6.1	8.1
Mbouroukou-1	2	37.4	37.7	2.3	3.0	80.4	46.5	46.9	2.9	3.7
Pisang Talas[b]	4	31.0	36.6	4.0	11.6	83.2	37.3	44.0	4.8	13.9
Oonoonoo kengoa	4	52.1	40.5	3.3	3.0	99.0	52.7	41.0	3.4	3.0
Popo'ulu Lahi	3	35.8	62.5	5.4	3.6	107.2	33.4	58.3	5.0	3.3
Maoli 'Ele'ele subvar 'Hinpua'a'	6	26.2	83.5	7.4	2.7	119.7	21.8	69.7	6.2	2.2
Kluai Kai Boran	6	42.8	69.9	10.1	5.6	128.5	33.3	54.4	7.9	4.4
Katimor[b]	3	0.3	119.8	10.8	14.0	144.9	0.2	82.7	7.4	9.7
Senorita	1	49.4	90.5	7.9	6.2	154.1	32.1	58.8	5.1	4.0
Henderneyargh	1	47.6	137.9	8.7	3.1	197.3	24.1	69.9	4.4	1.6
Pusit[b]	3	99.1	101.1	8.4	4.1	212.7	46.6	47.5	3.9	1.9
Bantol Red	1	82.0	144.8	14.0	3.9	244.7	33.5	59.2	5.7	1.6
Iholena Lele sub var. 'Long Peduncular'[b]	3	110.0	144.1	8.1	9.3	271.6	40.5	53.1	3.0	3.4
mean calibration set		22.2	36.8	2.8	2.3	64.1	40.5	45.1	2.2	7.9
S.D.		21.7	43.0	4.1	2.5	68.1	26.9	27.5	2.7	20.7
range		0.0−82.0	0.0−144.8	0.0−14.0	0.0−8.4	0.0−244.7	0.0−100.0	0.0−86.0	0.0−7.9	0.0−100.0
mean training set		51.0	82.7	6.3	8.3	148.3	35.1	53.6	3.8	7.4
S.D.		50.2	56.2	4.3	4.9	97.3	20.3	16.9	2.7	4.9
range		0.3−110.0	11.8−144.1	0.0−10.8	2.4−14.0	29.0−271.6	0.2−51.1	40.7−82.7	0.0−7.4	1.9−13.9
mean total		27.4	45.0	3.4	3.4	79.2	39.6	46.6	2.5	7.8
S.D.		29.7	47.9	4.3	3.8	79.1	25.6	25.9	2.7	18.8
range		0.0−110.0	0.0−144.8	0.0−14.0	0.0−14.0	0.0−271.6	0.0−100.0	0.0−86.0	0.0−7.9	0.0−100.0

[a] Values represent the means of all analyses carried out per variety and are ordered according to increasing total carotenoids content. All concentrations are expressed in nmol/g dry weight. α-carotene, all-*trans*-α-carotene; β-carotene, all-*trans*-β-carotene; c-carotene,13-*cis*-β-carotene. Total carotenoids = sum of α-, β-, and c-carotene and lutein. Means = means of all analyses carried out per sample set. S.D. = standard deviations. *N* = number of samples analyzed. [b] - varieties used for calibration model testing (test set). [c] # = number of plants from which samples were derived.

β-carotene (β-carotene), and all-*trans*-α-carotene (α-carotene), with only small additional quantities of the 13-*cis*-isomer of β-carotene (c-carotene). As reported previously, the only other major carotenoid compounds detected in fruit pulp extracts were small amounts of lutein (**Figure 1**) (14).

An overview of the mean pVACs and lutein contents for the varieties analyzed using reference HPLC methods is given in **Table 2**. Total carotenoid contents were calculated as the sum of the concentrations of the individual carotenoid species.

Overall, the mean total carotenoids content per variety ranged from 0 (undetectable) to 271.6 nmol/gdw with a mean for all analyses carried out of 83.6 nmol/gdw (**Table 2**). In 14 of the samples analyzed, there were no detectable levels of *cis*-carotenoids. Although *cis*-carotenoids are generally present in tissues, they tend to be formed by nonenzymatic isomerization events following exposure to light and/or acids and are generally not major products of metabolism. The results also show that α- and β-carotene contents are correlated with each other (r^2 = 0.778) but that correlations of both compounds are much higher with the total carotenoid contents of each variety (r^2 = 0.952 and 0.919, respectively) (**Table 3**). This is because α- and β-carotene are the two major carotenoid compounds present in *Musa* extracts and because the % proportion of α- and β-carotene in the total carotenoids extract is genetically determined and differs between genotypes. The high degree of correlation between the individual carotenoids is probably due to the fact that these compounds share a common biosynthetic pathway, a fact that complicates the analysis of individual carotenoids by Vis/NIRS.

Vis/NIRS for Carotenoid Determination. Figure 2a shows typical Vis/NIR spectra obtained for lyophilized *Musa* fruit pulp samples. These include the spectra from the varieties with the lowest and highest measured carotenoid concentrations ('Valery' and 'Iholena Lele sub var.' "Long Peduncular", respectively), together with spectra from three varieties with intermediate carotenoid levels and differing genome classifications. As is usually the case for Vis/NIRS, preprocessing techniques are required to improve spectral quality and to remove light-scattering effects (*20, 22*). Of the preprocessing techniques tested, the first derivatives of the spectra, calculated using the Savitzky−Golay algorithm with a second order polynomial fitted over a 21 nm interval, provided the best results (**Figure 2b**). Subsequently, this approach was always used to preprocess spectra prior to the development of the predictive models and model validation, and is analogous to procedures adopted by other workers using Vis/NIRS (*31*).

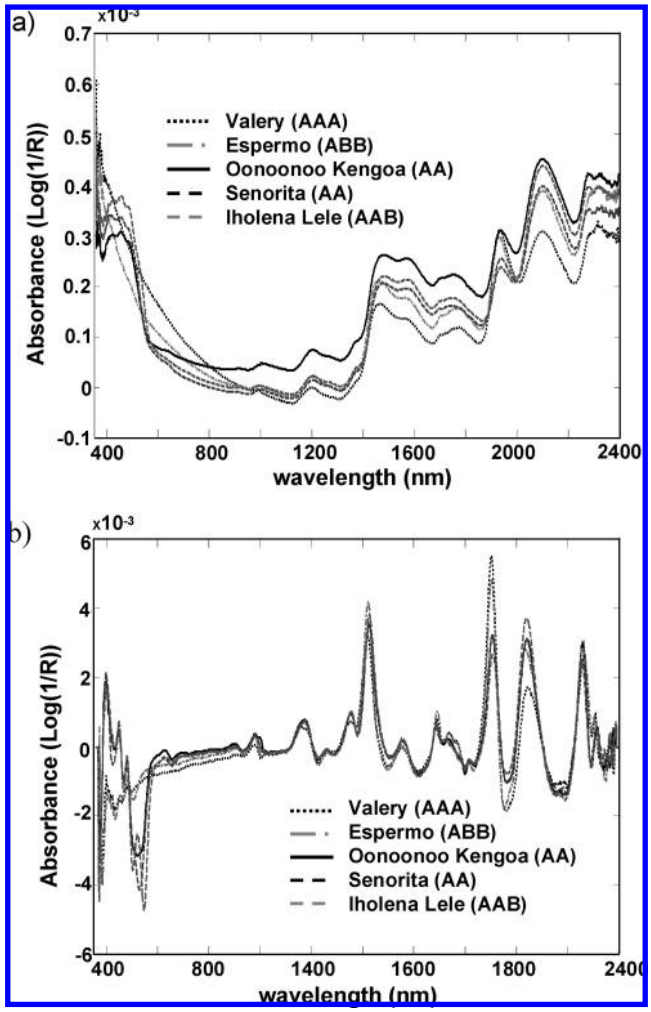

Figure 2. Typical Vis/NIR spectra for *Musa* fruit pulp samples from varieties of different genome groups (**a**) without preprocessing and (**b**) Savitzky−Golay smoothed first derivative of the spectra.

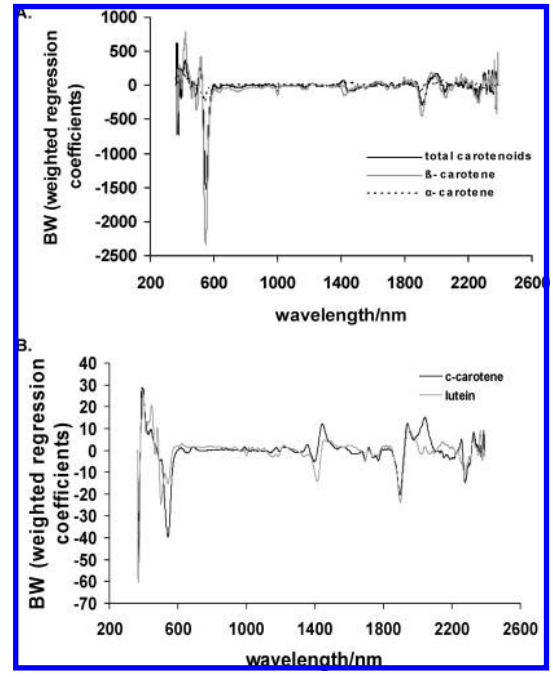

Figure 3. Plots of the weighted regression coefficients for the prediction of the different carotenoid concentrations from the first derivatives of the Vis/NIR spectral absorption data. (**A**) Total carotenoids, α- and β-carotene. (**B**) c-carotene and lutein.

Table 3. Correlation Coefficients (r^2) between Concentrations of Individual Carotenoid Fractions in *Musa* Fruit Samples ($N = 66$) as Determined by HPLC[a]

	total carotenoids	α-carotene	β-carotene	c-carotene	lutein
total carotenoids	1	0.9194	0.9523	0.7503	0.2113
α-carotene		1	0.7784	0.5823	0.2064
β-carotene			1	0.7568	0.1218
c-carotene				1	0.1587
lutein					1

[a] α-carotene, all-*trans*-α-carotene; β-carotene, all-*trans*-β-carotene; c-carotene, 13-*cis*-β-carotene. Total carotenoids = sum of α-, β-, and c-carotene and lutein.

Similar to the results obtained with carotenoid analysis in maize (*23*), a comparison of the processed Vis/NIR spectra obtained from different *Musa* genotypes indicates that the absorption profiles of samples yield most information in the visible region between 400 and 600 nm where carotenoids absorb strongly. This is demonstrated by plots of the weighted regression coefficients for the prediction of the different carotenoid concentrations from the first derivatives of the Vis/NIR spectral absorption data (**Figure 3**). These plots show that the models developed for the different carotenoid species mostly utilize the same spectral information. In particular, a clear negative peak around 540 nm corresponding to the absorption peak of around 520 nm in the original spectra (**Figure 2a**) seems to be particularly important.

The equations used to calculate the concentrations of the individual carotenoid species in the samples were derived by partial least-squares regression on a calibration set consisting of 49 fruit samples derived from 24 different *Musa* varieties. An overview of the statistics obtained during calibration and cross-validation for the individual *Musa* carotenoid species is given in Table **4**. Standard errors for calibration and validation (RMSEC and RMSECV, respectively) and coefficients of determination for calibration and cross-validation (r^2_c and r^2_{cv}, respectively) are also shown. Additional treatments such as truncating the lower end of the spectrum (350−380nm) and building the models using only the samples with high carotenoid concentrations had little impact on the results and certainly did not improve the power of the models (data not shown).

A statistical analysis of the cross-validation study for each carotenoid compound generated r^2_{cv} values ranging from 0.3 for lutein to 0.89 for β-carotene contents, and RMSECV values ranging from 2.8 nmol/g dry weight for c-carotene to 32.8 nmol/gdw for total carotenoids. Although low RMSECV values are desirable, the low RMSECV values for lutein and c-carotene have to be interpreted in light of the low actual concentrations and low % proportions of these components in tissues (**Table 2**). Because of these low concentrations, lutein and c-carotene both have quite low coefficients of determination, due to the proportionally greater technical errors associated with the HPLC and Vis/NIRS measurements of these two compounds.

As discussed in the Materials and Methods section, the correlation coefficients of cross-validation r^2_{cv} give an indication of the quality of the calibration models developed. Good model performance was obtained for β-carotene and total carotenoids with r^2_{cv} values of 0.89 and 0.84, respectively. The model for c-carotene performed moderately with an r^2_{cv} value of 0.66, while for α-carotene, only identification of high and low concentrations is possible ($r^2_{cv} = 0.61$). According to these criteria, Vis/NIRS cannot be used to estimate lutein contents ($r^2_{cv} = 0.30$). The accuracy of the cross-validation models was

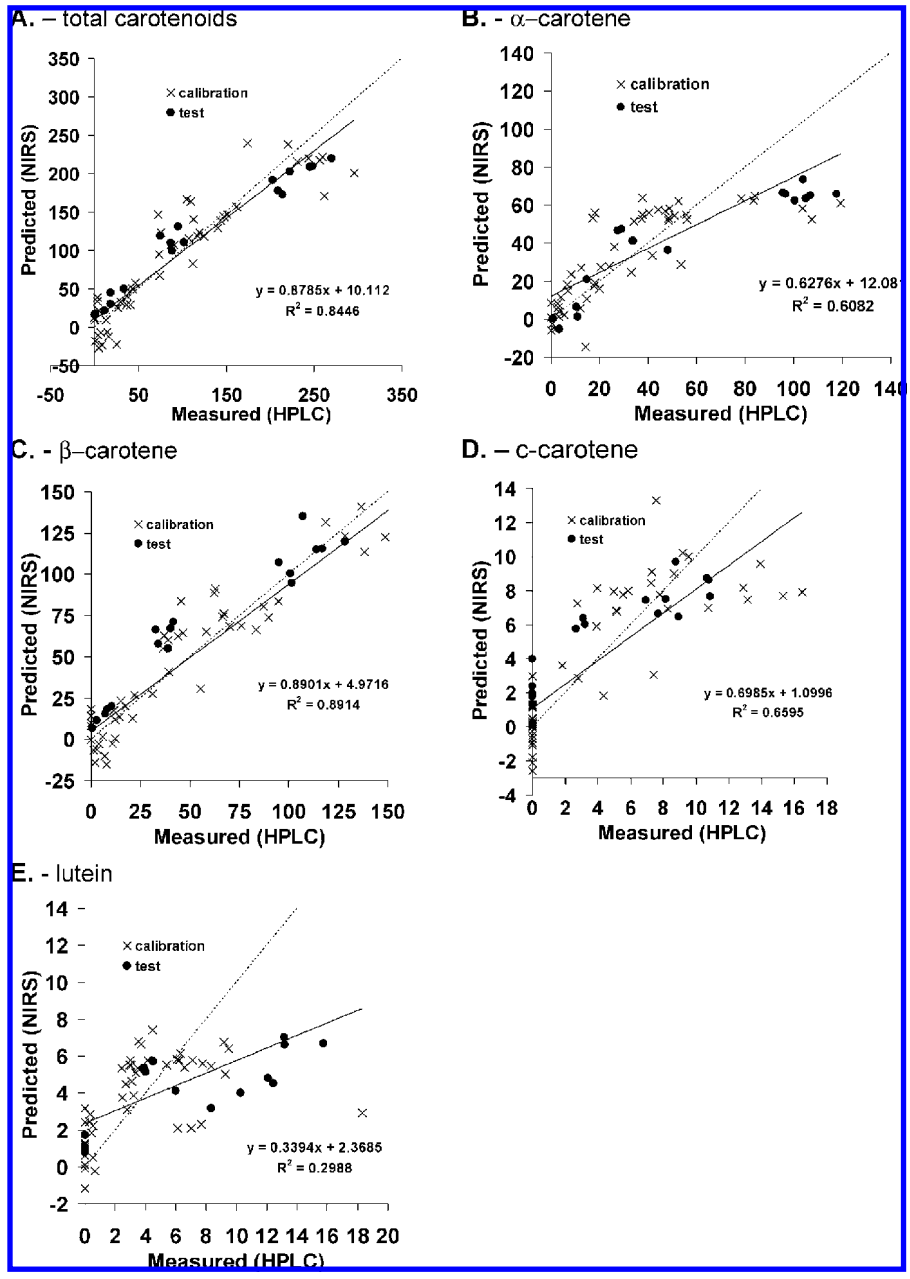

Figure 4. Plots of predicted versus measured carotenoid contents for both calibration and test sample sets, based on the first derivatives of the NIR spectra of lyophilized *Musa* fruit pulp in the range 350−2500 nm. All results are expressed in nmol/gdw. r^2_{cv} = regression coefficients for cross-validation results.

also evaluated on the basis of the RPD values (*30*). These ratios confirm that the accuracies of the developed Vis/NIRS models are good (RPD = 2.55) and excellent (RPD = 3.06) for total carotenoids and *β*-carotene, respectively, and are suitable for the rough screening of of c-carotene and α-carotene contents (1.5 > RPD < 2.0) but are unsuitable for measuring lutein content (RPD < 1.5).

The predictive power of the developed models was further tested on processed Vis/NIR spectra obtained from a separate group of 17 test fruit samples derived from 4 different *Musa* varieties. Since these test set samples were not part of the calibration set and have thus not been used for the selection of the preprocessing method or the number of latent variables, they can be used to estimate the power of the model to predict the carotenoid contents of untested varieties and fruits. An overview of the statistics obtained for this independent test sample set is shown in **Table 5**.

As summarized in **Table 5**, r^2_{pred} values ranged from 0.56 for lutein to 0.96 for total carotenoids with RMSEP values ranging from 2.25 nmol/gdw for c-carotene to 28.7 nmol/gdw for total carotenoids. Again, the low prediction error (RMSEP) value for lutein is due to the low concentrations of lutein in all *Musa* pulp samples. Using the categories identified by Williams (*29*) and Saeys (*30*), we can conclude again that Vis/NIRS cannot be used to predict lutein contents because while lutein r^2_{pred} values are at best suitable for the detection of outliers (r^2_{pred} = 0.56), a RPD value for lutein of only 1.16 indicates that the accuracy of the model is too poor. Although the models for α-, *β*-, c-, and total carotenoid contents all gave r^2_{pred} values >0.80, indicating good or excellent correlation between the measured and predicted concentrations, a systematic error (bias) is present. This impacts the RPD values, which show that for α- and c-carotene, Vis/NIRS can only be used for rough screening of outliers (PRD 1.5−2.0), while *β*-carotene and total carotenoid

Table 4. Overview of the Calibration and Cross-Validation Statistics for the Carotenoids and Total Carotenoid Contents of the Calibration Sample Set[a]

	HPLC		Vis/NIRS					
carotenoid species	mean	S.D.	r^2_c	RMSEC	r^2_{cv}	RMSECV	RPD	LVs
total carotenoids	91.4	83.8	0.95	18.89	0.84	32.84	2.55	4
α-carotene	32.7	30.3	0.72	15.87	0.61	18.76	1.62	1
β-carotene	51.1	49.5	0.95	10.65	0.89	16.16	3.06	4
c-carotene	4.12	4.86	0.77	2.30	0.66	2.82	1.72	2
lutein	3.97	3.73	0.41	2.84	0.30	3.10	1.20	1

[a] Predictive model was developed using 49 fruit samples obtained from 24 individual *Musa* varieties ($N = 49$) in a leave-one-variety-out cross-validation model with 24 segments. All results are expressed in nmol/gdw. S.D., standard deviation; r^2_c, determination coefficient for calibration; RMSEC, standard error of calibration; r^2_{cv}, determination coefficient for cross-validation; RMSECV, standard error of cross-validation; LVs, number of latent variables; α-carotene, all-*trans*-α-carotene; β-carotene, all-*trans*-β-carotene; c-carotene, 13-*cis*-β-carotene. Total carotenoids = sum of α-, β-, and c-carotene and lutein.

Table 5. Overview of the Statistics for the Carotenoids and Total Carotenoid Contents of the Test Sample Set Used for the Testing and Validation of the Calibration Models[a]

	HPLC		Vis/NIRS			
carotenoid species	mean	S.D.	r^2_{pred}	RMSEP	bias	RPD
total carotenoids	126.2	95.9	0.96	28.70	−1.92	3.34
β-carotene	57.7	46.7	0.92	17.07	11.52	2.74
α-carotene	55.3	43.6	0.82	26.00	−14.12	1.68
c-carotene	4.81	4.42	0.80	2.25	0.63	1.96
lutein[b]	6.72	5.49	0.56	4.75	−2.54	1.16

[a] Results were developed using 17 fruit samples obtained from 4 individual *Musa* varieties ($N = 17$). All results are expressed in nmol/gdw. Bias = difference between mean HPLC and mean Vis/NIRS measurements per compound. r^2_{pred} = coefficient of prediction. α-carotene, all-*trans*-α-carotene; β-carotene, all-*trans*-β-carotene; c-carotene,13-*cis*-β-carotene. Total carotenoids = sum of α-, β-, and c-carotene and lutein. Means and S.D.'s calculated on the basis of all analyses carried out. [b] For lutein, one compositional outlier (30.0 nmol/gdw) has been removed from the data set (so $N = 16$).

contents can be predicted with good and excellent accuracy, respectively (RPD values of 2.74 and 3.34, repectively). The observed bias is probably a result of the fact that the mean proportions of α- and β-carotene differed in the samples making up the calibration and the test sets (**Table 2**), a factor that has previously been shown to cause considerable bias in the predictions by a PLS model (*32*). The test set also contains a proportionally larger set of samples in which c-carotene contents were undetectable. These problems can be corrected for by increasing the size of the calibration and test sample sets to obtain two sets in which the distribution of carotenoid contents better represents the distribution present in the *Musa* germplasm pool and population, and it is expected that this will reduce the bias. However, since the relative proportions of each carotenoid species are also stable for each variety (*10*), the most accurate predicted value, the total carotenoids content can still be used to back-calculate the concentrations of individual carotenoid compounds once RP-HPLC analyses for that variety have been carried out.

Accuracy and Precision of HPLC and NIRS. HPLC values were calculated from duplicate extractions and analyses, and from three individual Vis/NIR spectral scans per sample. The mean variation in measured total carotenoid contents between duplicate HPLC analyses was ± 11.4%, while the mean variation in total carotenoid contents based on predictions from the Vis/NIR spectra was about twice as high at ± 27.6% (data not shown). The higher variation encountered with Vis/NIRS is probably related to the relatively low detection sensitivity of Vis/NIRS relative to HPLC. This means that it is more difficult to accurately measure carotenoid concentrations in those *Musa* varieties with inherently low carotenoids content (**Table 2**). An additional source of error can also be the nonhomogeneity of the lyophilized fruit powder, although experiments involving regrinding and relyophilization of samples indicated that additional grinding and lyophilization did not have a significant effect on Vis/NIRS results (data not shown). Plots of the predicted versus the measured concentrations of each of the carotenoid components are shown in **Figure 4**. These plots demonstrate that there is systematic bias to the Vis/NIRS-predicted values. As discussed, RPD values are still high enough to enable the use of the developed models for germplasm screening where the aim is to identify outliers, but application of the models will lead to errors in the estimation of the absolute concentrations of individual carotenoid compounds. The reasons for this bias are currently unclear, but as mentioned above, it could be due to differences in the mean proportions of the individual carotenoid compounds in the calibration and test sample sets. Alternatively, this effect could arise as a consequence of correlative effects with other matrix components or the fact that the carotenoids examined here are structurally very closely related, and all have very similar Vis/NIR absorption characteristics. Regardless of this, it is expected that the calibrations will stabilize for these effects, and the precision of the method wil improve when more samples of the same variety and more varieties are analyzed and incorporated into the models.

Applications of Vis/NIRS. Vis/NIRS has been shown to be a rapid and flexible technique that has found many applications within the fields of food and crop analysis (*20, 21, 33, 34*). The results from this trial study clearly demonstrate that Vis/NIRS can also be used to accurately predict the total carotenoid contents of samples of *Musa* fruits once the fruits are available in a lyophilized and powdered form. It still remains to be seen whether Vis/NIRS can also be used for the analysis of carotenoids in fresh fruit samples. While this would clearly represent a further saving in sample preparation time, the strong absorption due to the presence of water in tissues might override or at best reduce the sensitivity of Vis/NIRS analyses compared to analyses on lyophilized tissues. The models developed here had the highest predictive correlation coefficients (r^2_{pred}) for the total carotenoids and β-carotene contents, but within the framework of a screening program, r^2_{pred} values as low as 0.80 (as observed for c-carotene) are likely to be acceptable due to

the substantial savings in time and effort despite the significant bias in the model (**Table 5**).

The advantages of Vis/NIRS are that it is a nondestructive procedure and that the entire sample remains available for additional analyses if necessary. In addition, the operating costs are very low, and no (toxic) chemicals and/or waste products are produced. Even though Vis/NIRS is less-sensitive and less-reproducible than HPLC, and generally requires larger sample sizes, the fact that no extraction steps are involved helps to eliminate operator errors and will improve transferability of methods between laboratories. Despite the lower sensitivity and discriminatory power of Vis/NIRS compared to HPLC, the ability to rapidly and efficiently identify genotypes within a sample pool is a major advantage, and in practice, the carotenoid contents of interesting varieties would always be confirmed using a high-resolution technique such as HPLC. However, the major difficulty with Vis/NIRS is without doubt the need to build a stable and reliable calibration model, which in itself is dependent on robust and accurate reference procedures and a suitably large and diverse sample calibration set. In addition, models will have to be tested over different seasons to test robustness.

Conclusions. The results from this trial study demonstrate that Vis/NIRS has good potential for the high-throughput screening of carotenoid contents and in particular for the total carotenoid contents of lyophilized *Musa* fruit samples. Despite the relatively small sample group used to develop the predictive models, the procedure shows good accuracy for total carotenoids and β-carotene contents, but it remains to be seen whether larger sample sets will improve models sufficiently to enable the reliable prediction of the concentrations of other carotenoid compounds present. Importantly, however, the models were developed using a set of 28 genetically diverse *Musa* varieties obtained from a wide variety of growing environments, suggesting that the method could be applied to the analysis of fruit carotenoids in *Musa* varieties of all genome groups and across different seasons. Clearly, the fact that Vis/NIRS is a nondestructive analytical method and only requires minimal sample preparation will help prevent sample degradation during analysis. The disadvantages of Vis/NIRS are the low discriminatory power with respect to minor carotenoid species and the lower sensitivity compared to high-resolution chromatographic procedures. However, the relatively simple and stable carotenoids profile of *Musa* fruit pulp per variety (*10*, *25*) suggests that the method can provide a good estimate of the concentrations of individual carotenoids and thus the vit A nutritional content of *Musa* fruit samples from the total carotenoids content once the carotenoids profile has been determined by RP-HPLC. Further work will concentrate on validating the results of these findings across a wider range of varieties, over different production years, and on building more stable predictive models.

ABBREVIATIONS USED

Vis/NIRS, visible and near infrared reflectance spectroscopy; pVACs, provitamin A carotenoids; α-carotene, all-*trans*-α-carotene; β-carotene, all-*trans*-β-carotene; c-carotene, 13-*cis*-β-carotene; vit A, vitamin A; r^2_c, determination coefficient for calibration; r^2_{cv}, determination coefficient for cross-validation; r^2_{pred}, determination coefficient for prediction; RMSEC, root-mean-square error of calibration; RMSECV, root-mean-square error of cross-validation; RMSEP, root-mean-square error of prediction; RP-HPLC; reversed-phase high performance liquid chromatography.

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