Short communication

Considerations to prevent the breakdown and loss of fruit carotenoids during extraction and analysis in *Musa*

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ABSTRACT

The impact of treatments aimed at improving the robustness of protocols for the analysis of carotenoids in fruit of banana and plantain were examined. Neither the inclusion of polyvinylpolypyrrolidone in the extraction buffer, nor vigorous homogenisation with glass beads influenced recoveries or chromatographic profiles. By contrast, heating lead to losses of up to 53% and to the formation of degradation products that are no longer detectable on our RP-HPLC system. Carotenoid extracts are unstable and most sensitive to exposure to light. However, even in the dark at -20°C and in the presence of antioxidants breakdown rates of around 5% per day were observed.

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1. Introduction

Plants are the major dietary source of carotenoids for humans and have many health-related properties. However, the provitamin A carotenoids (pVACs) which are broken down in the body to yield retinol, the active form of vitamin A [1,2], are of particular interest due to the widespread prevalence of vitamin A deficiencies worldwide [3]. Understandably therefore there is much interest in developing crops biofortified for pVACs contents to help combat these problems [4,5]. Quantitatively, the major pVACs are α - and β -carotene, β -cryptoxanthin and *cis*-isomers of α - and β -carotene. Recent studies from our lab indicate that there is a huge range of diversity in *Musa* fruit carotenoids and pVACs contents [6,7] and that existing pVACs-rich *Musa* varieties could have significant long-term beneficial health impacts for vitamin A deficient populations, particularly in regions where *Musa* spp. are a staple part of the diet [4,6,8]. Within the framework of a widescale screening program we have already established protocols for the sampling, extraction and direct HPLC analysis of *Musa* fruit carotenoids [7,9]. However, plant carotenoids are particularly sensitive to degradation during extraction and this can lead to *cis-trans* isomerisation, oxidative cleavage and/or epoxidation of the carotenoid backbone [10–13]. While the deleterious effects of heat, acids, light, etc., on carotenoids have

regularly been cited, the extent of the impact of these factors has rarely been determined. Here we quantify the effect of several post-harvest procedures reported to influence carotenoid stability and recoveries, including the effects of heat and light during sample preparation and report on updates and modifications to protocols for carotenoid analysis in *Musa* fruit to improve robustness and reliability.

2. Experimental

2.1. *Musa* fruit samples

Experiments were carried out using the varieties “Batard”, “Mbouroukou-1”, “Oonoonoo kengoa” and “Henderneyargh”. Fruits were harvested immature at ripening stage 1 [14], and then peeled, sliced, frozen and lyophilised before shipping to the laboratory in Leuven. Prior to analysis, the lyophilised samples were crushed to a fine powder in a pestle and mortar in liquid nitrogen under reduced light and stored in sealed 50 mL Falcon tubes at -20°C in the dark [9].

2.2. Materials

HPLC-grade tetrahydrofuran (THF) and *tert*-butyl methyl ether (t-BME) were obtained from Sigma–Aldrich (Bornem, Belgium), HPLC-grade methanol (MeOH) from LabScan (Dublin, Ireland). Lutein, all-*trans* β -carotene (t-BC), *trans* β -8-apocarotenal (apocarotenal), butylated hydroxytoluene (BHT), triethylamine and

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insoluble polyvinylpolypyrrolidone (PVPP) were all obtained from Sigma–Aldrich.

2.3. Extraction

50–100 mg aliquots of powdered *Musa* fruit pulp were homogenised for 30 s at maximum speed with 3–5 glass beads in a 'FastPrep' reciprocal shaker (Savant Instruments, New York, USA) in 5–10 volumes/g dry weight of ice-cooled extraction buffer consisting of THF:MeOH, 1:1 (v/v), 0.25% BHT and 2% insoluble PVPP [6,9]. Following centrifugation ($14,000 \times g$ for 20 min at 4 °C), the pellet was re-extracted twice more with 5–10 volumes of THF:MeOH, 1:1 (v/v), 0.25% BHT without PVPP.

2.4. RP-HPLC analysis

The combined supernatants were directly analysed by RP-HPLC using a Waters Alliance, 2690 Separations System fitted with an autosampler at 8 °C, a pulse dampener and a 996 UV–vis photodiode array detector (Waters, MA, USA). The entire system was controlled and data collected and integrated using the Millennium 4.0 software package. Extracts were resolved on a 150×4.6 mm, YMC C₃₀ 3- μ m particle size HPLC column (Achrom, Zulte, Belgium), using an optimised 24 min linear gradient of 2–50% t-BME in MeOH at 1.0 mL/min and regenerated with 95% t-BME in MeOH. All buffers contained 0.05% BHT and 0.05% triethylamine. Peaks were quantified at 450 nm using a freshly prepared standard curve of t-BC in extraction buffer as previously described [9] and identified on the basis of their characteristic absorption spectra and retention times relative to known standards where available [9,15–18]. Apocarotenal at a final concentration of 0.002 μ g/mL was added as an internal standard to correct for differences in extraction volumes.

2.5. Impact of PVPP during extraction

Triplicate aliquots of lyophilised fruit pulp from the plantain Mbourokou-1 were homogenised in cooled extraction buffer either with or without 2% insoluble PVPP. In both cases two additional extractions of the pellet were carried out in extraction buffer without PVPP as described above. The combined extracts were directly analysed by RP-HPLC.

2.6. Impact of heat during extraction

Replicate aliquots of a lyophilised sample of Batard fruit pulp were extracted with ice-cold extraction buffer containing PVPP followed by a single heating step in a heating block at 85 °C for between 10 and 90 min in a closed screw-capped polypropylene container. After cooling, samples were centrifuged at $14,000 \times g$ for 20 min at 4 °C and the pellets re-extracted twice more with ice-cold extraction buffer before RP-HPLC analysis.

2.7. Impact of homogenisation on recoveries and oxidation

Replicate aliquots of powdered, lyophilised fruit pulp from the cultivars Oonoonoo kengoa and Henderneyargh were subjected either to vigorous shaking with glass beads in cold extraction buffer using a 'FastPrep' reciprocal shaker (30 s at maximum speed) during each of the 3 rounds of solvent extraction, for 2 rounds, during only the first extraction or not shaken at all.

2.8. Stability of carotenoid solutions and extracts

Freshly prepared 1 mL aliquots of 0.002 μ g/mL solutions of t-BC and apocarotenal standards in THF:MeOH, 1:1 (v/v), 0.1% BHT were stored for up to 10 days in either clear glass sample vials at room

temperature in direct daylight, in amber glass vials in the dark at room temperature or in amber vials in the dark at 4 °C. Samples were periodically analysed by C₃₀ RP-HPLC to monitor the impact of storage conditions on sample stability.

1 mg/mL stock solutions of t-BC and apocarotenal standards in THF:MeOH, 1:1 (v/v), 0.1% BHT, were prepared and aliquots of these solutions stored at –20 °C in the dark. These aliquots were used daily to prepare standard curves over the range 0.002–0.1 mg/mL and the response curves compared over time.

3. Results and discussion

The literature contains frequent references to procedures that impact on carotenoid stability and extraction efficiencies [19–23]. However, the extent of these effects has generally not been quantified, and there are no reports for similar studies in *Musa*.

3.1. Tissue sample storage

It has been reported that lyophilisation of tissues leads to carotenoid degradation, due to the increased porosity of samples and an increased exposure to air [23]. We previously demonstrated that there are no significant differences in the recoveries of total pVACs from either lyophilised or fresh *Musa* fruit pulp tissues [9]. There was further no significant loss or breakdown of carotenoids during storage of lyophilised tissue in the dark at –20 °C, whereas with frozen pulp tissues variable losses and a gradual browning (oxidation) indicative of residual peroxidase and polyphenoloxidase activities were observed. We therefore recommend storing tissues at –20 °C in lyophilised form in sealed containers in the dark.

3.2. Impact of PVPP during extraction

Musa tissues are known to be particularly rich in phenolic compounds which can potentially interfere with carotenoid extraction and/or analysis [24]. The inclusion of 2% insoluble PVPP in the extraction buffer to complex phenolics had no significant influence on absolute recoveries (66.0 ± 3.5 and 63.8 ± 5.0 nmol total pVACs/g dry weight for samples of Mbourokou-1 prepared with and without PVPP, respectively). There were further no differences in the RP-HPLC profiles demonstrating that PVPP has no significant effect on carotenoid analysis in *Musa*. However, PVPP did help to prevent occasional fouling of the HPLC columns and was therefore included as standard in the extraction buffer for the first extraction.

3.3. Impact of heat during extraction

The literature contains reports indicating that carotenoid contents are higher from tissue that has been boiled or heated [19,25] suggesting that a heating step during extraction could improve carotenoid yields. We previously reported that a short heat treatment had no significant influence on recoveries of pVACs from *Musa* when using ethanol as an extraction solvent, although there was a slight increase in the proportion of 13-*cis*- β -carotene [9]. Here, however, results clearly show that a longer period of heating significantly reduces the recoveries of all carotenoid compounds present, despite the inclusion of 0.25% BHT in the extraction buffer (Fig. 1).

At no point are extraction efficiencies improved and after a single 90 min heating step at 85 °C only 52–53% of the initial t-AC and t-BC concentrations remained. Heating has also been reported to selectively promote the formation of 13-*cis* isomers [10,19], but we observed only a minor increase in the levels of 13-*cis* isomers of α - (c-AC) and β -carotene (c-BC) and only after extended heating. Importantly, despite large decreases in pVACs concentrations, heat-

Fig. 1. Impact of a single heating step at 85 °C during extraction on the recovery of carotenoids from the *Musa* variety 'Batard'. Results represent the mean following extraction of duplicate aliquots of powdered, lyophilised *Musa* fruit pulp and analysis by C_{30} RP-HPLC.

ing had little impact on chromatographic profiles and additional peaks due to carotenoid degradation were not observed (Fig. 2).

3.4. Aeration during sample homogenisation

Reciprocal shakers are widely used in the routine extraction of nucleotides, proteins, etc., from plant tissues. Since the strong aeration that takes place could also lead to oxidative degradation we investigated the impact of this homogenisation step on pVACs recoveries and profiles in *Musa* fruit. For both cultivars there was no significant difference in recoveries with a total variation across analyses for all conditions of 133.6 ± 3.5 and 58.0 ± 4.6 nmol/gdw for Henderneyarh and Oonoonoo kengoa, respectively. The proportions of individual carotenoids and RP-HPLC profiles were also unaffected. Therefore as standard a single homogenisation step only was included to ensure uniform homogenisation of samples.

3.5. Stability of carotenoid extracts

When analysing large numbers of samples, it is important to determine the stability of extracts to avoid possible losses prior to analysis. Analysis of aliquots of 0.004 mg/mL solutions of t-BC and apocarotenal standards stored either at room temperature in the light, at room temperature in the dark or at 8 °C in the dark clearly demonstrated that solutions stored at room temperature in

Fig. 2. Impact of a single heating step at 85 °C for various lengths of time during extraction on the C_{30} RP-HPLC chromatographic profiles of carotenoid extracts from aliquots of fruit of the plantain variety 'Batard'. Eluates monitored at 450 nm as according to Section 2.

the light are the least stable. After 10 days in the light at room temperature, apocarotenal and t-BC solutions had lost 50% and 95% of their initial concentrations, respectively. By comparison, solutions kept in the dark retained around 90% of the initial concentrations after 10 days, with lower temperatures improving stabilities further (Fig. 3). Again, the observed losses were not mirrored by the appearance of comparable levels of carotenoid isomers or degradation products.

We also examined the stability of 1 mg/mL stock solutions of t-BC and apocarotenal stored at –20 °C in the dark. Surprisingly, the gradients of standard curves prepared from the same stock decreased significantly with time, such that after 5 days storage, gradients were only 88.4% and 89.4%, respectively, of the initial values.

3.6. Chromatographic separation methods

RP-HPLC profiles of *Musa* fruit pulp carotenoids consist primarily of t-AC, t-BC, c-AC and c-BC with only small amounts of lutein [7,9]. Consequently, adequate separations can be obtained using both C_{18} and C_{30} columns. However, the greater hydrophobicity of

Fig. 3. Stability of solutions of (A) all-trans-β-carotene and (B) apocarotenal stock solutions dissolved in THF:MeOH, (1:1, v/v), containing 0.25% BHT. Light Rt = samples stored in clear glass at room temperature, dark Rt = samples stored in amber vials in the dark at room temperature, dark 4 °C = samples stored in amber vials in the dark at 4 °C.

C₃₀ columns allows twice as much extract to be loaded, improving detection sensitivities as well as the resolution of minor carotenoid species. To minimise analysis times on the C₃₀ column though it is recommended to use a gradient separation and for *Musa* extracts excellent resolution is obtained with a 24 min linear gradient as described in Section 2.4. Minimum detection limits are in the region of 0.5 pmol with linearity of detector response observed at least over the range 2–9000 nmol injected.

4. Conclusions

The chemical structures of carotenoids and pVACs makes them inherently unstable and sensitive to isomerisation and oxidation during extraction and storage. Results here indicate that many of these breakdown products are not detected by RP-HPLC so that it cannot be determined whether losses have already occurred or not during sample preparation. Consequently each step of the extraction protocol has to be carefully optimised. Here we show that neither the inclusion of PVPP to complex phenolics compounds, nor the use of a reciprocal shaker to homogenise samples during extraction had any influence on carotenoid and pVACs recoveries. By contrast the use of heat to improve extraction efficiencies resulted in losses of over 50% of all carotenoid compounds after 90 min. Stability studies further show that exposure of extracts to light should be avoided at all costs, with t-BC being more sensitive than the internal standard apocarotenal even in the presence of BHT. However, while extracts are reasonably stable for short periods when stored in the dark at 4 °C, even at –20 °C in the dark, significant breakdown of concentrated stock solutions of standards occurs at a rate of about 2%/day occurs. Standard curves therefore should always be prepared fresh daily.

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