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BRIEF COMMUNICATION

Total carotenoid amount in crude twig extracts may be overestimated due to interference by high contents of co-extracted phenolics

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Abstract

Total carotenoids assessed spectrophotometrically in crude extracts may be considerably overestimated when high contents of phenolic compounds are co-extracted. In this case, the absorbance tails of phenolics extend well into the blue part of the spectrum, interfering with carotenoid estimation. Extracts of phenolic-rich organs, with a low ratio of photosynthetic to heterotrophic and/or supportive cells (for example, stems or twigs) are vulnerable to such pitfalls and may need chromatographic separation of carotenoids.

Additional key words: *Arbutus*; HPLC; *Pistacia*; *Populus*; *Prunus*; *Quercus*.

Chlorophylls (Chls) of green plant parts extracted in organic solvents are estimated by conventional spectrophotometry in the red region of the spectrum using equations of Lichtenthaler and Wellburn (1983) and Wellburn (1994). The same equations allow an assessment of total carotenoids through their absorbance at *ca.* 470 nm after removing the corresponding absorbance contribution of Chls at this wavelength. The method has a wide application, being a routine step in many physiological and ecological studies. Apparently, it is based on the high abundance and the high absorption coefficients of photosynthetic pigments, compared to other plant cell constituents that absorb in the same bands. Even when anthocyanins are present, their intense visible absorbance fades considerably at the slightly alkaline pH of the media used for Chl extraction (Swain 1976).

In a recent attempt to measure photosynthetic pigments in twigs of woody species, we observed that the spectral profiles of crude twig extracts compared to the corresponding leaf extracts showed disproportionately high absorbance in the blue and especially in the ultraviolet spectral region. We therefore assumed that twigs contain extremely large amounts of co-extracted UV-absorbing compounds, whose absorbance tails extend into the blue part of the spectrum and interfere with carotenoid estimation in crude extracts. Therefore, we designed experiments in order to verify this assumption.

Leaves and twigs from the following species were used: *Populus deltoides* Bartr. *ex* Marsh. (eastern cottonwood), *Quercus coccifera* L. (kermes oak), *Pistacia lentiscus* L. (mastic tree), *Prunus cerasus* L. (sour cherry), and *Arbutus unedo* L. (strawberry tree). Five individuals from each species were sampled during autumn 2002. On each sampling date, an adequate number of leaves and twigs from each individual were harvested. Pigment extraction and analysis were run in duplicate (with at least 6 leaves/twig extracted) and the mean values were considered characteristic for the individual. Twigs and leaves initiated during the previous (spring) growth period (*i.e.* 7–8 months old) were used.

Since a mortar-and-pestle homogenisation of twigs was not possible, we adopted the hot DMSO (dimethylsulphoxide) method proposed by Wittmann *et al.* (2001), modified as follows: twig segments were considered as cylinders and the projected cylindrical surface area was estimated geometrically from length and diameter measurements by means of a friction-stop caliper (*Mitutoyo*, Tokyo, Japan). Then, the twigs were cut in small pieces and immersed in $CaCO_3$-saturated DMSO for 2 h at 65 °C (Wittman *et al.* 2001). However, the extraction of some twigs was not complete in DMSO and therefore a second step was applied by immersing the plant material in methanol at 4 °C in the dark for 12 h in all cases. Under the same conditions, leaf discs of known areas were punched out and extracted. After centrifugation, total carotenoids in the two extracts were determined

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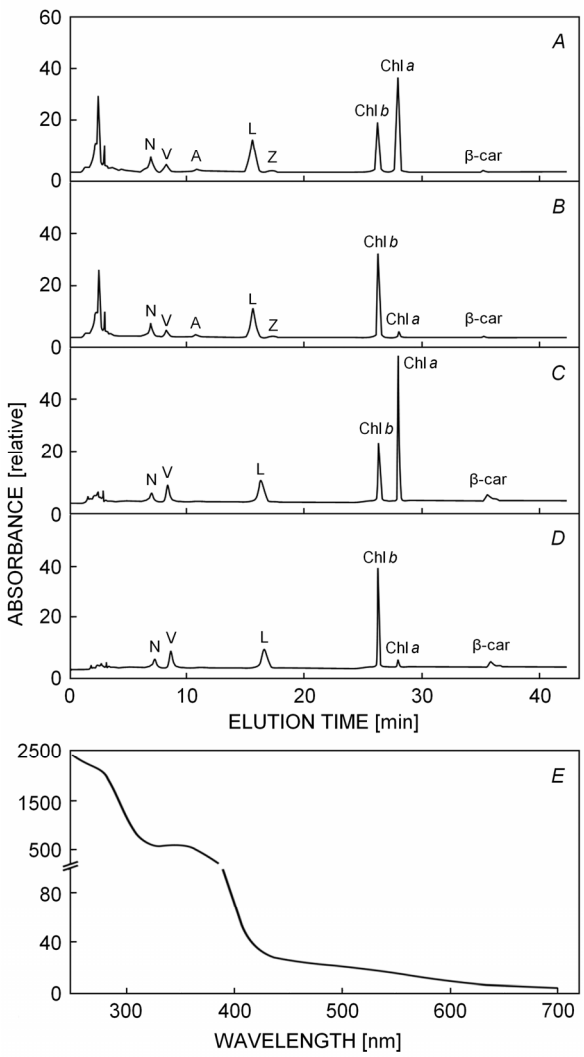

Fig. 1. Characteristic chromatographic profiles of photosynthetic pigments in twigs (*A, B*) or leaves (*C, D*) of *A. unedo* at 445 (*A, C*) and 470 (*B, D*) nm. *The letters* indicate the position of individual pigments. N, neoxanthin, V, violaxanthin, A, antheraxanthin, L, lutein, Z, zeaxanthin, Chl-*b*, chlorophyll *b*, Chl-*a*, chlorophyll *a*, β-car, β-carotene. (*E*) Absorbance spectrum of the compounds eluted at 2.6 min after chromatography of crude extract of an *A. unedo* twig.

spectrophotometrically using a *Shimadzu UV-160A* double beam spectrophotometer and the equations of Wellburn (1994). For further carotenoid analysis, the two extracts were combined, cleared by passing through 0.45 μm filters, and pigment separation was performed with a *Shimadzu LC-10 AD* HPL chromatography, equipped with a non-endcapped *Zorbax ODS* (4.6×25.0 mm) column (*Rockland Technologies*, Chadds Ford, PA, USA), calibrated against purified β-carotene (*Sigma Chemical*, St. Louis, MO, USA) and freshly prepared xanthophylls by TLC as described by Kyparissis *et al.* (1995). Development was performed isocratically at 1 cm^3 per min (20 min with acetonitrile : methanol, 85 : 15 v/v, and 20 min with methanol : ethyl acetate, 68 : 32 v/v), according to Thayer and Björkman (1990). Pigments were detected by a *Shimadzu SPD-M10A$_{VP}$* UV-VIS photodiode array detector and further analysed by a *Shimadzu Class-VP* version *6.1* software package. Significance of differences in carotenoid estimations between crude extracts and after chromatography were assessed by Student *t*-tests (*SPSS 9.0* statistical package).

Estimations of content of total carotenoids either from crude extracts (by applying published equations and corresponding spectral absorption data) or after chromatographic analysis (by summing up individual carotenoids) always gave the same results in the case of leaves (Table 1). In twigs, however, the values obtained from crude extracts were generally higher (Table 1). A paired *t*-test comparing the differences in total carotenoids obtained with the two methods indicates a systematic overestimation in crude extracts or, alternatively, an underestimation after chromatography in twigs of three species, *i.e. A. unedo, P. lentiscus*, and *Q. coccifera* (Table 1). The reasons for this discrepancy are discernible in the chromatographic profiles shown in Fig. 1. When detector wavelength was set at 445 nm (which is optimum for carotenoid detection), considerable absorbances were found at *ca.* 2–4 min after sample injection, especially in the case of twigs (Fig. 1*A,C*). The compounds corresponding to this elution time displayed phenolic-type absorbance spectra (Fig. 1*E*). When chromatography was run at 470 nm (*i.e.* at the wavelength used in crude extracts for total carotenoid estimation), elution of absorbance at 2–4 min persisted (although with lower intensity) in twigs (Fig. 1*B*) but almost disappeared in leaf samples (Fig. 1*D*). Analogous results were obtained with the rest of tested species, confirming the need for removing the UV-absorbing compounds in order to accurately estimate twig carotenoids in crude extracts.

Attempts were made for a quick removal of UV-absorbing compounds without having to recourse on time consuming and costly chromatographic methods. This would be especially useful when just the total carotenoid concentration is needed and the number of samples to be scanned is large. Yet, polyvinylpolypyrrolidone up to 6 % or polythyleneglycol up to 6 %, which precipitate phenolic substances in water extracts (Baldry *et al.* 1970), were not active in DMSO when put alone or in combination and either during or after pigment extraction. They did precipitate phenolic substances in methanol extracts (together with an appreciable amount of Chls), yet methanol is not recommended for a complete extraction of twig photosynthetic pigments (Wittmann *et al.* 2001).

Hence the estimation of total twig carotenoids in crude extracts may be misleading, at least with some plants, due to the interference of co-extracted large amounts of phenolics. This interference may be wide spread, since twigs are designed for long lasting function, needing enhanced mechanical and chemical defences based on phenolic compounds and their derivatives

Table 1. Contents of total carotenoids [g m^{-3}] in leaves and twigs estimated from crude extracts or after chromatography. Means ± SE from five independent replications for each species. The results of paired *t*-tests (P) comparing carotenoid contents estimated on the same sample before and after chromatography indicate significant differences (*bold figures*) in twigs of *A. unedo*, *P. lentiscus*, and *Q. coccifera*.

		Crude	HPLC	P
Arbutus unedo	twigs	2.90 ± 0.55	1.73± 0.30	**0.048**
	leaves	8.73 ± 0.78	8.97 ± 0.38	0.630
Pistacia lentiscus	twigs	2.38 ± 0.26	1.92± 0.17	**0.017**
	leaves	9.07 ± 0.57	9.04 ± 0.80	0.890
Populus deltoides	twigs	5.19 ± 0.22	4.80± 0.90	0.681
	leaves	7.10 ± 0.08	6.99 ± 1.11	0.929
Prunus cerasus	twigs	2.94 ± 0.14	2.65± 0.36	0.449
	leaves	7.86 ± 0.50	7.06 ± 0.94	0.187
Quercus coccifera	twigs	4.84 ± 0.19	4.22± 0.32	**0.009**
	leaves	10.38 ± 0.73	10.59 ± 0.91	0.716

(Shain 1995). No such interferences were observed with leaves and we may assume that in their case using crude extracts is safe, given also the fact that the evergreen sclerophyll leaves used in this study do contain large amounts of phenolic substances (Levizou and Manetas 2002).

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