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SPECTROPHOTOMETRIC DETERMINATION OF CHLOROPHYLLS AND CAROTENOIDS. AN EFFECT OF SONICATION AND SAMPLE PROCESSING

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ABSTRACT

Chlorophylls and carotenoids are abundant pigments in plants, algae and cyanobacteria. In this study we verified the applicability of two previously developed UV-vis spectrophotometric methods for simultaneous quantitative determination of chlorophylls (a, b) and carotenoids (lycopene, β-carotene or total carotenoids). The pigments were extracted from the strawberries, apricots and raspberries in both the acetone-water and acetone-hexane mixtures. Based on the statistical evaluation of the results the combination of mechanical disruption and sonication of fruit samples seems to be a suitable way to improve the pigment extraction efficiency from fruits in both types of solvents. In the case of apricot and raspberry fruit extracts the amount of chlorophylls and carotenoids calculated from the proposed equations was comparable to those published by other authors. However, the spectrophotometric determination of β-carotene content in strawberry acetone-hexane extract appeared to be problematic mainly due to the fact that carotenoids exhibited overlapping chlorophyll absorption bands. Overlap of bands leads to the negative values calculated from the proposed equation for the β-carotene content. The results indicate the limitations in use of the proposed set of equations for plant samples with comparable amounts of studied pigments.

Keywords: Carotenoids, chlorophylls, sonication

INTRODUCTION

Many of epidemiological studies have shown that the consumption of diets rich in fruit and vegetables helps to prevent a wide range of diseases associated with long-term oxidative stress, such as cardio-vascular diseases, cancer and illnesses related to the aging (**Kohlmeier and Hastings, 1995; Halliwell, 1997; Nishino *et al.*, 2009; Bystrická *et al.*, 2011; Jomová and Valko, 2011; Papaioannou *et al.*, 2011; Tanaka *et al.*, 2012**). Well-known biologically beneficial natural substances are carotenoids endowed either with antioxidant activity or with pro-vitamin A activity (**Bohm *et al.*, 2012**). Carotenoids are one of the most important groups of natural pigments abundant in many fruits and vegetables. Dietary carotenoid intake from vegetable and fruit sources has been correlated with a reduced cancer risk (**Sporn and Suh 2002; Tanaka *et al.*, 2012**). However, recent studies have shown that also chlorophyll (Chl) consumption might be linked to a chemoprotective effect (**Ferruzzi and Blakeslee, 2007**).
Beneficial effects of natural pigments on human health have led to increased interest in the study of these substances. Current research is oriented on identification and quantification of components in plant material and determination of their activity. The various sets of experimental data concerning the abundance as well as biological activity of effective substances in natural plant resources are required to develop the useful food and nutraceutical supplements. Identification and quantification of plant bioactive substances is influenced by various factors, the most significant being the sample preparation procedure, the type of extraction reagent and the method of their determination. The most commonly used techniques for identification and quantification of natural substances are either spectrophotometry or high-performance liquid chromatography (HPLC) (**Jeffrey *et al.*, 1997**). Each of the methods has different advantages and limitations (**Mantoura *et al.*, 1997**).
In our work we verified applicability of two developed spectrophotometric methods for simultaneous determination of chlorophylls (*a*, *b*) and carotenoids in strawberry, apricot and raspberry fruits. The pigments were extracted in two organic mixtures according to the used method and ultrasonication was applied with the aim to enhance the extraction yields.

MATERIAL AND METHODS

Chemicals

The used solvents (acetone and hexane) were purchased from Sigma Aldrich and were of analytical grade purity.

Processing of raw fruits

The fruits of strawberry, apricot and raspberry were obtained from market in a stage of full ripeness. The clean and dry fruit (free of stems or pits) was homogenized in household food blender fitted with a sieve with 2 mm perforations. The waste fraction was returned to blender one more time to assure complete homogenization. The whole isolation procedure was performed under dark conditions to avoid light degradation of the pigments.

Assay for chlorophylls, β-carotene and lycopene

In general, the samples prepared from raw fruits intended for pigment extraction were initially processed by two methods. In the first approach the fruit was processed only mechanically, the second approach involved mechanical processing plus sonication of the samples. The pigments were determined according to the method of **Nagata and Yamashita (1992)**. 1g of fresh weight of each fruit slurry was separately homogenized (IKA-WERKE T10 Basic) with 10 mL of an acetone–hexane mixture (2:3) for 2 minutes to uniform mass. In parallel experiments under the same conditions, in order to ascertain the effect of sonication on extraction yield, the samples were sonicated (sonicator Bandelin HD3100 Sonopuls) for 3 minutes (5 cycles: puls 30 s, pause 10 s). Samples were maintained in an ice-water bath to prevent over-heating of the samples. Homogenates were centrifuged (Eppendorf) at 5000 rpm for 10 minutes at 20 °C. The absorbance spectrum of each supernatant was measured and the absorption maxima were read at 453, 505, 645 and 663 nm (UV/VIS spectrophotometer Cary 50 Scan). Chl *a*, Chl *b*, β-carotene and lycopene content was calculated from the following equations:

$$Chlorophyll\ a\ (mg/100ml) = 0.999A_{663} - 0.0989\ A_{645}$$

$$Chlorophyll\ b\ (mg/100ml) = -0.328A_{663} + 1.77A_{645}$$

$$Lycopene\ (mg/100ml) = -0.0485A_{663} + 0.204A_{645} + 0.372A_{505} - 0.0806A_{453}$$

$$\beta - carotene\ \left(\frac{mg}{100ml}\right) = 0.216A_{663} - 1.22A_{645} - 0.304A_{505} + 0.452A_{453}$$

where A = absorbance.

Assay for Chlorophylls and Total Carotenoids

The Chl *a*, Chl *b* and total carotenoids were determined by the method of **Yang *et al.* (1998)**. Extract preparation procedure was identical to the previously described method. The acetone-water mixture (4:1) was used as a solvent. The absorbance maxima were read at 663.6 nm for Chl *a*, 646.6 nm for Chl *b* and 470.0 nm for carotenoids. Contents of Chl *a*, Chl *b* and total carotenoids were calculated from the following equations:

$$Chlorophyll\ a\ (\mu g/ml) = 12.25A_{663.6} - 2.25\ A_{646.6}$$

$$Chlorophyll\ b\ (\mu g/ml) = 20.31\ A_{646.6} - 4.91A_{663.6}$$

$$Total\ carotenoids\ (\mu g/ml) = \frac{1000\ A_{470} - 2.27\ (Chl\ a) - 81.4\ (Chl\ b)}{227}$$

The results were expressed as micrograms per gram fresh weight of sample

Statistical Analysis

All measurements were carried out for four independent samples (n=4) and the results are expressed as mean values ± standard deviation (SD). The data were statistically analysed using a common procedure based on calculating Student´s t criterion according to formula:

$$t = \frac{(\bar{x}_A - \bar{x}_B).\sqrt{n-1}}{\sqrt{s_A^2 + s_B^2}}$$

and comparing calculated *t* with critical $t_{crit.\ (n=4)}$ =3.182, where x_A and x_B are arithmetic averages of two sets under consideration, s_A and s_B are relating standard deviations and n is number of parallel experiments.

RESULTS AND DISCUSSION

Carotenoids have been extensively studied in a large number of fruits and vegetables because of their beneficial effect on human health. On the other hand, only little attention has been paid to the content of chlorophylls that undergo degradation during ripening, while the total carotenoid content increases throughout fruit ripening process. In this study we applied two previously described spectrophotometric methods (**Nagata and Yamashita, 1992; Yang *et al.*, 1998**) for simultaneous determination of chlorophylls (*a*, *b*) and carotenoids (lycopene, β-carotene or total carotenoids) with the aim to verify applicability of these methods for other fruit (strawberry, apricot, raspberry) in a stage of full ripeness. Based on the obtained spectral characteristics we calculated the content of carotenoids and chlorophylls in extracts of the individual fruits. Table 1 shows content of the pigments extracted in two organic mixtures (acetone-hexane, acetone-water) which enables us to compare the solvent effects on the extraction yield. Sample sonication followed by homogenization was used to evaluate the impact of ultrasonication on the pigments extraction yield.

Chlorophylls

Table 1 presents the pigment contents of the fruit samples as determined by UV-VIS spectrophotometry. It can be seen that chlorophyll pigments were extracted using both organic mixtures with the exception of raspberry fruit, where acetone-hexane organic mixture seems to be ineffective. Our results showed greater concentration of Chl *b* than Chl *a* unlike **Martinez *et al.* (2001)** who observed higher content of Chl *a*. The results obtained (Table 2) have shown that character of solvent was statistically significant for the extracted amount of Chl *a* in strawberry under the conditions of mechanical processing of samples plus sonication in acetone-hexane solvent. Under these conditions the yield of Chl *a* was double of that obtained from mechanical processing while the acetone-water yields under both disruption means (solely mechanical and mechanical plus sonication) did not exceed the critical value of Student´s t criterion. That means the extraction of Chl *a* by acetone-water gives lower yield and reflects that disruption mode is less efficient. These results could be consistent with the observation that acetone solvent does not extract polar pigments very well and chlorophyllase activity increases with water content (**Zhengyi *et al.*, 1998**). However, our results have not confirmed higher efficiency of acetone-hexane mixture solvent in the extraction of chlorophylls in all samples. In the case of strawberry extraction efficiency of Chl *b*, no significant difference was found for both solvent mixtures under the same conditions of sample processing (mechanical vs. mechanical plus sonication). Extraction of Chl *a* in apricot gave consistent results regardless of the solvent used or sample processing. On the other hand, amount of Chl *b* determined using acetone-water mixture was statistically significantly higher than that of acetone-hexane mixture.
Effect of disruption treatment can be seen in Table 3. Being extracted by both solvent mixtures, Chl *a* in strawberry reached significantly higher yields using the mechanical disruption followed by sonication. Although Chl *b* content is significantly higher by applying the mechanical processing plus sonication using acetone-water mixture, comparison of both ways of disruption and extraction by acetone-hexane mixture is very close to critical value of Student´s criterion (3.182 *versus* 3.013) in favour of mechanical processing plus sonication in this solvent mixture, too.

Table 1 Chlorophylls and carotenoids composition (μg.g^{-1} fresh weight) of fruit extracts in two organic mixtures. The results are expressed as mean ±SD (n = 4)

	Solvent	**Acetone-hexane 2:3**		**Acetone-water 4:1**	
	Homogenization	Mechanical disruption	Mech. disruption + sonication	Mechanical disruption	Mech. disruption + sonication
Strawberry	Chlorophyll a	2.76 ±0.69	5.68 ±0.98	2.03±0.12	3.67±0.11
	Chlorophyll b	3.09 ±1.03	6.49 ±1.66	3.89 ±0.30	5.92± 0.55
	Lycopene	1.24±0.33	2.63±0.25	----	----
	β - carotene	ND	ND	----	----
	T Carotenoids	----	----	2.10±0.65	2.41±0.96
Apricot	Chlorophyll a	0.92 ± 0.12	1.24±0.64	1.25± 0.17	1.51± 0.37
	Chlorophyll b	0.97±0.07	1.36±0.14	1.98± 0.13	2.30 ±0.62
	Lycopene	0.75±0.13	1.03±0.22	----	----
	β - carotene	30.63±3.81	33.22±2.97	----	----
	T Carotenoids	----	----	16.27± 1.44	16.23±1.67
Raspberry	Chlorophyll a	ND	ND	1.38± 0.15	2.37±0.23
	Chlorophyll b	ND	ND	4.06± 0.23	5.39±1.25
	Lycopene	ND	ND	----	----
	β - carotene	5.12±0.77	5.67±0.64	----	----
	T Carotenoids	----	----	10.40± 0.44	10.06± 0.34

Legend:ND - not detected, T - total

Analysing chlorophyll a in apricot showed that treatment of the sample brought no difference between disruption modes. However, extraction of Chl *b* by acetone-hexane reached significantly higher level using mechanical disruption plus sonication, while the results from acetone-water extraction were not distinct with respect to the method of disruption. In the case of raspberry, level of Chl *a* in acetone-water extract was significantly higher using mechanical processing plus sonication. Even though differences of Chl *b* results in acetone-water mixture were not over critical t-value, the yield was higher after mechanical disruption plus sonication.

Carotenoids

Because Chl *a* and Chl *b* contents are measured simultaneously with the content of some carotenoids, an additional objective of this study was to determine carotenoid concentrations. The obtained data (Table 1) have shown the high content of β-carotene in apricot (30 $\mu g.g^{-1}$) in acetone-hexane mixture. It is interesting to note that the content of lycopene in apricot was significantly lower (0.75 $\mu g.g^{-1}$) compared to β-carotene content and even lower than lycopene content in strawberry (1.24 $\mu g.g^{-1}$. The high level of β-carotene is consistent with observations of other authors who quantified the β – carotene content in apricot using HPLC method (**Hart and Scott, 1995**) or spectrophotometrically (**Radi *et al.*, 2004; Sass-Kiss *et al.*, 2005**). Despite the large number of existing data on the content of carotenoids it is difficult to compare them. Small differences in carotenoid contents can be attributed to the different solvents used for extraction. The results of **Lugasi *et al.* (2003)** showed significantly higher lycopene content in apricot purchased in summer compared to that purchased in winter indicating the effect of season on level of natural pigments.

Table 2 Statistical testing of solvent effect on the amount of extracted pigments. Compared couples are the sets treated by the same disruption and extracted with acetone-hexane versus extracted with acetone

	Acetone-hexane(2:3) *vs.* acetone-water (4:1) Student´s criterion t calculated; $t_{crit\ (n=4)} = 3.182$ Mechanical disruption		**Acetone-hexane(4:6) *vs.* acetone-water (4:1)** Mechanical disruption + sonication	
	Strawberry	Apricot	Strawberry	Apricot
Chlorophyll a	1.81	2.75	3.53> t_{crit}	0.63
Chlorophyll b	1.30	11.85 > t_{crit}	0.56	2.56

Though the acetone-hexane mixture is considered to be effective in carotenoid extraction, our results (Table 1) obtained in strawberry extract appeared to be problematic in terms of determination of β-carotene content, giving negative values. The similar results were obtained in case of raspberry fruits for lycopene. This was most probably due to the similarity of the absorbance values of studied pigments**. Rao *et al.* (1998)** have demonstrated that the results obtained by HPLC and by spectrophotometry are comparable and practically the same in case of those foods in which lycopene is the predominant carotenoid. In contrast to our findings **Barros *et al.* (2010)** have observed β-carotene in strawberry fruits (10.70 $\mu g.g^{-1}$) using the same method, but no lycopene has been detected. Based on the spectrophotometrical data **Singh (2011)** have found lower level of β-carotene in strawberry fruits (from 0.56 to 1.00 $\mu g.g^{-1}$) using a 3% acetone in petroleum ether as a solvent.

Taking into account the use of sonication, it can be seen (Tables 1, 3) that the extraction of lycopene by acetone-hexane mixture from strawberry gave significantly higher yield when disruption was carried out mechanically plus sonication. However, yields of lycopene and β-carotene in apricot extract can be considered to be the same regardless of disruption treatment. Similarly to this observation, yields of total carotenoids in acetone-water extract have not been significantly affected by the disruption process (Table 3).

Though our results have confirmed positive impact of mechanical disruption followed by sonication on pigment extraction quantity, determination procedures need to be optimized individually for every sort of fruit.

Table 3 Statistical testing of sample disruption effect on the amount of extracted pigments. Compared couples are the sets extracted with the same solvents and disrupted mechanically versus disrupted mechanically plus sonication

	Mechanical disruption *vs.* mechanical disruption + sonication Student´s criterion t calculated; $t_{crit\ (n=4)} = 3.182$			**Mechanical disruption *vs.* mechanical disruption + sonication**		
	Acetone-hexane 2:3			**Acetone-water 4:1**		
	Strawberry	Apricot	Rapsberry	Strawberry	Apricot	Rapsberry
Chlorophyll a	4.22> t_{crit}	1.31	ND	17.45> t_{crit}	1.10	6,25> t_{crit}
Chlorophyll b	3.01	4.32> t_{crit}	ND	5.62> t_{crit}	0.88	1.81
Lycopene	5.82> t_{crit}	1.90	ND	----	----	----
β - carotene	ND	0.93	0,95	----	----	----
T Carotenoids	----	----	----	0.46	0.03	1.06

CONCLUSION

Based on the results presented in this contribution we may conclude that in almost all experimental variants discussed above, regardless of the type of solvent used, the mechanical processing combined with sonication increased the extraction yield of natural pigments. A simple spectrophotometric method appears to be suitable for determination of pigments in some fruits, however different solvent mixtures and conditions of homogenization must be experimentally tested for each type of fruit in order to increase the pigment yields. If the above mentioned conditions are specified for each fruit, the spectrophotometric determination of pigments can provide a fast and less expensive method for the obtaining of original data on pigment content, directly linked to the nutritional value of the fruits. However, there are many others of factors that must be taken into account, when comparing the content of natural pigments. For example, climatic conditions, soil properties, environmental stresses and different types of fruits carry great variability in the abundance of pigments. Another important aspect of the experimental design is to eliminate potential sources of experimental errors and to achieve a high degree of reproducibility.

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