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Determination of free radical scavenging activity of anthocyanins using diphenylpicrylhydrazyl radical

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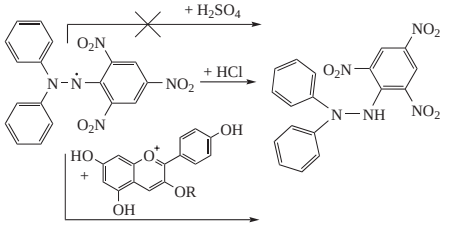
It has been demonstrated that hydrochloric acid reacts with the diphenylpicrylhydrazyl radical, which leads to a likely overestimation of the antioxidant capacity of anthocyanin extracts obtained by acidifying the extractants with hydrochloric acid. Therefore, during the extraction process it is recommended to replace hydrochloric acid with sulfuric acid.



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Anthocyanins are a unique subclass of the broad class of flavonoids, characterized by the existence of several pH-dependent forms.[1] Interest in anthocyanins is related to the presence of red color in flavylium forms with shades depending on the structure of anthocyanin aglycones, anthocyanidins,[2] as well as on acylation with cinnamic acid derivatives. This property allows the use of anthocyanins as natural dyes.[3] In addition, anthocyanins are the most important antioxidants, soluble in water, which determines their biological role for human health.[4] One of the most popular methods for determining antioxidant properties is to measure the free radical scavenging activity of substances using the diphenylpicrylhydrazyl (DPPH) radical. The method is based on the decolorization of a DPPH solution after DPPH removes a hydrogen atom from the antioxidant molecule.[5]

In this method, the test solution and DPPH solution are mixed and the drop in optical density at 517 nm is monitored. But for anthocyanins, strictly speaking, this method of determination using a simple solution is not entirely acceptable due to the coexistence of several pH-dependent forms that do not necessarily have the same antioxidant properties. Even when using anhydrous solvents in which the transition of the flavylium form to the pseudo-base form is excluded, there is no simple issue of controlling the completeness of the transition of all forms to the flavylium form. But if aqueous solutions of anthocyanins with pH < 1 are used, then the existence of anthocyanins predominantly in the flavylium form is considered guaranteed.[6] Therefore, acidified solvents are used for the extraction of anthocyanins, for which various acids can be used, among which hydrochloric acid is the most popular.[7] However, it is well known that hydrochloric acid is oxidized by strong oxidizing agents, such as, for example, potassium permanganate.[8] Therefore, the aim of this study was to determine the correctness of using hydrochloric acid to acidify anthocyanin extracts for the subsequent determination of the free radical scavenging activity of anthocyanins using DPPH as an example, as it was used in a number of works.[9–13]

In the first series of experiments, concentrated extracts of anthocyanins from blackcurrant fruits were used, obtained using hydrochloric acid and purified by solid-phase extraction. Equal aliquot volumes of these concentrates were diluted equally with a 0.05 M solution of sulfuric acid (sample 1) or a 0.1 M aqueous solution of hydrochloric acid (sample 2), obtaining solutions with the same optical density, *i.e.*, with the same anthocyanin content (Table 1).

A solution of DPPH in ethanol was mixed with sample 1 or sample 2 so that concentrations of anthocyanins, DPPH and H^+ ions in the sample were 2.0×10^{-6}, 6.2×10^{-4} and 8.0×10^{-3} mol dm^{-3}, respectively. Immediately after mixing, the optical density of the mixture was monitored for 45 min. The results obtained† are shown in Figure 1.

Note that changes in optical density in parallel observations were very close: the discrepancy did not exceed 1% (Figure 1). On the contrary, in a mixture of an ethanol solution of DPPH with an aqueous solution of hydrochloric acid (Figure 1, curve *4*), the optical density of the mixture quickly and significantly decreases, which indicates the oxidation of HCl by DPPH. At the same time, when replacing a solution of hydrochloric acid with a solution of sulfuric acid (Figure 1, curve *3*), the optical density decreased by only 1–2% in 45 min. From the results obtained, it follows that

Table 1 Solvents used to prepare anthocyanin extract samples.

Sample no.	Initial extractant	Solvent for dilution	Anthocyanin concentration[a]/M
1	0.10 M HCl	0.05 M H_2SO_4	$(1.0 \pm 0.1) \times 10^{-5}$
2	0.10 M HCl	0.10 M HCl	$(1.0 \pm 0.1) \times 10^{-5}$
3	0.05 M H_2SO_4	0.05 M H_2SO_4	$(1.0 \pm 0.1) \times 10^{-5}$
4	0.10 M HCl	0.10 M HCl	$(1.0 \pm 0.1) \times 10^{-5}$

[a] As cyanidin-3-glucoside equivalent.

† *Determination of DPPH radical scavenging activity of anthocyanins*. To a solution of DPPH (4 ml) in ethanol (with an optical density of about 1.0 at 517 nm), sample 1 or sample 2 (0.10 ml) was added and the volume was adjusted to 5 ml with ethanol. Immediately after mixing, the optical density of the mixture was monitored in a quartz cuvette with an optical path length of 1 cm on a Shimadzu UV-1550 spectrophotometer. In blank experiments, the extracts were replaced by the initial solutions of sulfuric and hydrochloric acids. The observation was continued for 45 min, repeating the measurements in the second series of experiments (samples 3 and 4) with the same loads.

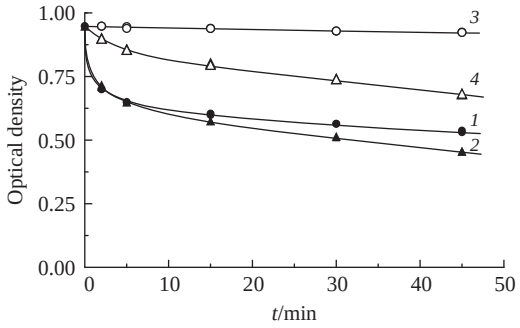

Figure 1 Changes in the optical density of a mixture of DPPH solution with samples depending on time: (*1*) sample 1, (*2*) sample 2, (*3*) blank sample with sulfuric acid and (*4*) blank sample with hydrochloric acid.

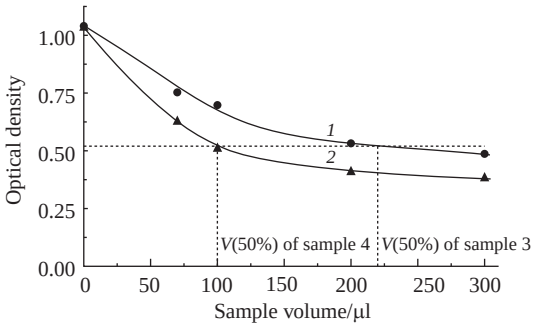

Figure 2 Determination of DPPH radical scavenging activity of anthocyanin extracts prepared (*1*) with sulfuric acid (sample 3) or (*2*) with hydrochloric acid (sample 4).

sulfuric acid is not oxidized by DPPH, and the observed slight decrease in optical density may be due to the presence of a small amount of HCl in the solution from the concentrated anthocyanin extract used for dilution.

When samples 1 and 2 were added to the DPPH solution, different results were also obtained: in the initial period, a rapid decrease in optical density was observed for both samples, which can be interpreted as the occurrence of the most rapid redox reactions between DPPH and anthocyanins. But already after 5 min, the decrease in the optical density of the solutions slows down, although in the case of the extract with HCl it occurs slightly faster than in the case of the extract with sulfuric acid.

Consequently, anthocyanin extracts in hydrochloric acid-acidified solutions should not be used to determine the free radical scavenging activity of anthocyanins, at least when DPPH is used.

In the second series of experiments, strawberry fruit extracts were initially obtained in a 0.05 M solution of sulfuric acid (sample 3) or in a 0.1 M aqueous solution of hydrochloric acid (sample 4). Then the optical density (*i.e.*, anthocyanin concentration) of the samples was equalized by diluting with solutions of the same acids. Subsequently, a traditional approach was used, in which the volume of the injected sample was varied for different samples, the reaction mixture was incubated for 45 min, and eventually the optical density of the final solutions was measured. Then the volume *V*(50%) of anthocyanin solution was determined for samples 3 and 4, at which the optical density decreases by 50% (Figure 2).[5]

From the presented data it follows that for the extract obtained using hydrochloric acid, the required sample volume (100 μl) is two times less than for the extract obtained using sulfuric acid (220 μl). Thus, the free radical scavenging activity of anthocyanins in extracts acidified with hydrochloric acid appeared to be significantly overestimated.

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