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Research Article

Tissue distribution of anthocyanins in rats fed a blackberry anthocyanin-enriched diet

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Anthocyanins are natural dietary pigments that could be involved in various health effects. The aim of this study was to investigate the distribution of anthocyanins to various organs (bladder, prostate, testes, heart and adipose tissue) in rats fed with a blackberry anthocyanin-enriched diet for 12 days. Identification and quantification of anthocyanins were carried out by HPLC-DAD. The urinary excretion of total anthocyanins (native anthocyanins and their metabolites) was low (0.20 ± 0.03%, $n = 8$). Proportions of anthocyanin derivatives (methylated anthocyanins and glucurono-conjugated derivatives) differed according to the organ considered. The bladder contained the highest levels of anthocyanins followed by the prostate. Prostate, testes and heart contained native cyanidin 3-glucoside and a small proportion of cyanidin monoglucuronide. Cyanidin 3-glucoside and methylated derivatives were present in adipose tissue. Thus, anthocyanin feeding in rats resulted in a wide distribution of anthocyanin derivatives to several organs. Identification of target tissues of anthocyanins may then help to understand the mechanisms of action of anthocyanins *in vivo*.

Keywords: Anthocyanins / Blackberry / Cyanidin 3-glucoside / Rats / Tissue distribution

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1 Introduction

Anthocyanins, which belong to the flavonoid family, are reddish natural pigments widely distributed in fruits and especially in berries [1]. Their average daily intake was estimated for a long time at around 200 mg/day in the United States [2], but lower intake values were determined by others (6.6 mg/day in Germany, 12.5 mg/day in the United States and 47 mg/day in Finland) [3–5]. However, high intakes (>100 mg/day) could be easily achieved with regular consumption of red fruits or berries. Natural food colorants rich in anthocyanins may also significantly contribute to the total anthocyanin dietary intake.

Anthocyanins have been reported to promote health and prevent various chronic diseases [6, 7]. They may reduce the risk of cardiovascular diseases, exert anticarcinogenic and neuroprotective activities, reduce inflammatory insult and modulate the immune response [8–11]. They are also reported to exert antidiabetes and antiobesity effects [12, 13].

Health-enhancing properties of anthocyanins would depend on their absorption, metabolism, tissue distribution and excretion. These last years, there have been a number of studies on the absorption of anthocyanins in humans or experimental animals (for review, see [14]). Most of them reported that the proportion of total anthocyanins absorbed and excreted in the urine (in the native form and as metabolites) was below 1% of the ingested amount, which is lower than that found for most flavonoids [6, 14]. We have previously shown that anthocyanins are rapidly absorbed from both stomach and small intestine [15, 16]. They then appear in blood circulation and urine in intact, methylated, glucurono- and/or sulphoconjugated form [17–20].

Knowledge on anthocyanin tissue distribution is also important in understanding the mechanisms by which health effects could occur. However, to date little is known about their distribution in tissues. A few studies have shown the presence of anthocyanins in some organs such as liver, kidney, brain or eyes [18, 21–24]. However, no information was available for other tissues where anthocyanin effects have been observed.

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The aim of the present work was to examine the tissue distribution and metabolism of anthocyanins in rats fed a diet supplemented with a blackberry extract. We analysed anthocyanins in tissues such as heart, adipose tissue, testes, prostate gland and bladder. The blackberry extract was selected as it is characterised by the presence of one major anthocyanin, cyanidin 3-glucoside.

2 Materials and methods

2.1 Chemicals

Cyanidin 3-glucoside and cyanidin 3,5-diglucoside were purchased from Extrasynthèse (Genay, France). Blackberry anthocyanin extract was supplied by Ferlux Mediolanum (Cournon d'Auvergne, France).

2.2 Animals and diets

Sixteen male Wistar rats born at the Institut National de la Recherche Agronomique and weighing ~220 g were housed two *per* cage in temperature-controlled rooms (22°C) with a dark period from 8 to 20 h and access to food from 8 to 16 h. They were fed a semi-purified control diet (755 g wheat starch, 150 g casein, 50 g peanut oil, 35 g AIN-93M mineral mixture, 10 g AIN-93A vitamin mixture *per* kg) for 1 wk [25]. They were then randomly divided into two groups (each group comprising eight rats) and individually housed in metabolic cages fitted with urine and faeces separators. They received for 12 days (25 g diet/rat/day) either the control diet (control rats) or the control diet supplemented with 20 g blackberry extract *per* kg diet (*i.e.* 14.4 mmol anthocyanins *per* kg diet) (anthocyanin fed rats).

All animals were maintained and handled according to the recommendations of the Institutional Ethics Committee (INRA), in accordance with French decree no. 87-848.

2.3 Sampling procedure

The day before killing, urine was collected over 24 h in tubes containing 1 mL of 3 M HCl and exact food consumption was checked. Rats were killed at 11 h (*i.e.* during the dark period and 3 h after they have begun to eat) after being anaesthetised with sodium pentobarbital (40 mg/kg body weight). At this time, rats had ingested about 40% of the amount of anthocyanins consumed *per* day. Anthocyanin absorption and disappearance from plasma are fast (for review, see [6]). This time point was thus chosen to make a compromise between absorption, distribution to tissues and excretion. All the blood (8 – 10 mL) was withdrawn from the abdominal aorta into heparinised tubes and urine was collected in the bladder. Blood samples were centrifuged at 12 000 × *g* for 5 min. Plasma and urine samples were rapidly acidified with 240 mM HCl after collection and stored at −20°C until analysis. Next, 25 mL of phosphate buffer saline was perfused into the portal vein to clean up the tissues. Tissues (heart, prostate, testes, epididymal white adipose tissue and bladder) were removed from bloodless rats. All tissue samples were weighed, rapidly frozen in liquid nitrogen and stored at −80°C until analysis.

2.4 Sample preparation

Urine samples were centrifuged at 12 000 × *g* for 5 min and the supernatant (20 μL) was injected and analysed by HPLC as described below.

Anthocyanins present in plasma samples were extracted with a Sep-Pak C_{18} Plus SPE cartridge (Waters, Milford, MA, USA), using cyanidin 3,5-diglucoside as the internal standard as previously described [15], and then analysed by HPLC (60 μL).

Extraction of anthocyanins was carried out on frozen bladder, prostate, heart, one of the testes and adipose tissue (0.6 g). Bladder was first opened and washed with 1 mL of distilled water to clean up this organ from residual urine. Tissues were crushed using a homogeniser (Ultra-Turrax) in methanol containing 1% HCl (9 mL/g of tissue), spiked with 1.60 nmol cyanidin 3,5-diglucoside as internal standard and centrifuged (3250 × *g*, 10 min). Use of the internal standard allowed to correct for the loss of anthocyanins during sample preparation. Supernatants were collected and pellets were reextracted with 1% HCl in methanol (4.5 mL/g of tissue). The two methanolic supernatants were mixed and evaporated to dryness using a rotary evaporator at room temperature under reduced pressure. Dried tissue extracts were dissolved in 250 – 400 μL of 1% HCl aqueous solution. After centrifugation (12 000 × *g*, 5 min), an aliquot (100 μL) was immediately analysed by HPLC. The internal standard recovery was 22.6 ± 3.0%, 46.1 ± 3.6%, 34.0 ± 3.4%, 28.2 ± 1.8% and 66.1 ± 3.6% in the heart, prostate, bladder, testes and adipose tissue, respectively. This low recovery of internal standard was partly related to the type of tissue and duration of the extraction process and was in the same order of magnitude than that previously published by us and others [18, 21, 26].

We have verified in various control samples (tissues, plasma, urine) spiked with cyanidin 3,5-diglucoside or cyanidin 3-glucoside that neither HCl nor evaporating to dryness under reduced pressure at room temperature broke down glycosides to aglycones during HPLC sample preparation. Moreover, cyanidin 3,5-diglucoside used as internal standard was not degraded into cyanidin 3-glucoside. The recovery of cyanidin 3-glucoside was similar to that of cyanidin 3,5-diglucoside in control tissue samples.

2.5 HPLC analysis

Identification and quantification of anthocyanins were performed by HPLC using a photodiode array detector (DAD

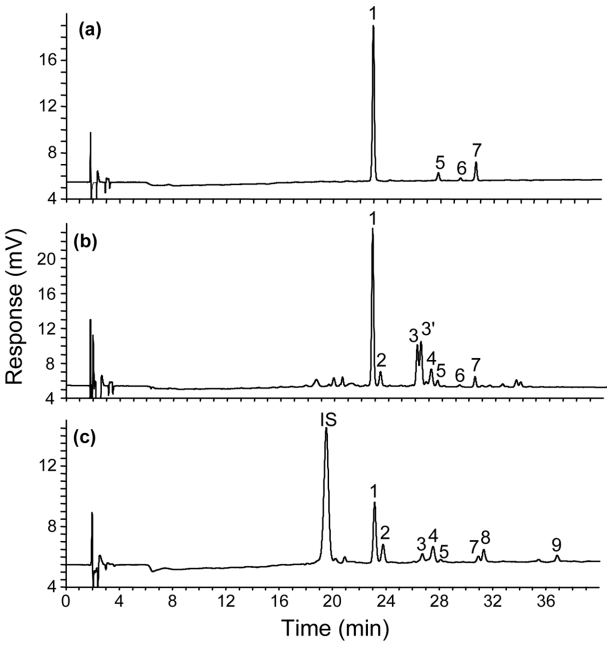

Figure 1. HPLC chromatograms of blackberry anthocyanin extract (a) and urine (b) and plasma (c) collected from rats fed a diet supplemented with the same extract. Detection was performed at 524 nm. (1) cyanidin 3-glucoside, (2) cyanidin monoglucuronide, (3) and (3′) methylated cyanidin 3-glucosides, (4) methylated cyanidin monoglucuronide, (5) cyanidin 3-pentose, (6) cyanidin 3-malonylglucoside, (7) cyanidin 3-dioxalylglucoside, (8) cyanidin, (9) methylated cyanidin. IS, internal standard (cyanidin 3,5-diglucoside).

200; Perkin Elmer, Courtabœuf, France) and a UV–Vis detector (785A; Perkin Elmer) at 524 nm. Samples were loaded onto a Uptisphere 3 ODB C_{18} 3 µm column (150 × 4.6 mm id) protected by a Uptisphere 3 ODB C_{18} 3 µm guard column (10 mm × 4 mm id) (Interchim, Montluçon, France). Elution was performed using water/H_3PO_4 (99:1) as a solvent A and ACN as a solvent B at a flow rate of 1.0 mL/min. Analyses were carried out with linear gradient conditions from 100 to 90% A for 10 min and then to 75% A for 30 min. Peak identification and assignment were based on the comparison of their retention time and spectral data with that of the blackberry anthocyanin extract, standards or our previous data obtained by HPLC-MS/MS analysis [18, 27]. Anthocyanin quantification was expressed as cyanidin 3-glucoside equivalents.

3 Results

The blackberry extract contained ~35% anthocyanins. Their identification was previously performed by HPLC-ESI-MS-MS [18]. This extract contained only derivatives of cyanidin (Fig. 1a): cyanidin 3-glucoside (peak 1, 85.1% of total anthocyanins), cyanidin 3-pentose (peak 5, 4.7% of total anthocyanins), acylated derivatives of cyanidin 3-glucoside (cyanidin malonylglucoside, peak 6; cyanidin dioxalylglucoside, peak 7; 10.2% of total anthocyanins).

Urine of the anthocyanin-fed rats (Fig. 1b) contained blackberry anthocyanins (46.1% of total anthocyanins) as well as glucurono-conjugated and methylated derivatives: a cyanidin monoglucuronide (peak 2), methylated cyanidin 3-glucosides (peaks 3 and 3′), and a monoglucuronide of methylated cyanidin (peak 4). Peaks 3 and 3′ correspond to methylated cyanidin 3-glucosides: peonidin 3-glucoside and an isomer of peonidin 3-glucoside as described by Ichiyanagi *et al*. [28, 29]. Urinary excretion of native anthocyanins plus metabolites detected at 524 nm was 0.47 ± 0.10 µmol/24 h (n = 8). Since the amount of anthocyanins ingested on the day before sacrifice was 250 ± 28 µmol, the total urinary excretion of anthocyanins accounted for 0.20 ± 0.03% of anthocyanin intake. Plasma from anthocyanin-fed rats (Fig. 1c) contained native anthocyanins as well as several anthocyanin metabolites: cyanidin monoglucuronide, methylated forms of cyanidin 3-glucoside and cyanidin monoglucuronide, and aglycones (cyanidin and methylated cyanidin).

Total anthocyanin concentrations in various tissues and in plasma are given in Table 1. Highest levels of anthocyanins were observed in the bladder; native anthocyanins were accompanied by a high proportion of methylated

Table 1. Anthocyanin content in tissues from rats fed during 12 days a diet supplemented with a blackberry anthocyanin extract[a, b]

Tissue	Cy 3-glc concentration[c] (% total anthocyanins)	Total anthocyanin concentration
	(nmol Cy 3-glc/g of tissue)	(nmol Cy 3-glc equivalents/g of tissue)
Bladder	1.37 ± 0.55 (58.1%)	2.37 ± 0.91
Prostate	0.257 ± 0.081 (84.5%)	0.304 ± 0.095
Testes	0.051 ± 0.011 (82.3%)	0.062 ± 0.011
Adipose tissue	0.079 ± 0.014 (79.8%)	0.099 ± 0.020
Heart	0.051 ± 0.030 (85.0%)	0.060 ± 0.029
	(nmol Cy 3-glc/mL of plasma)	(nmol Cy 3-glc equivalents/mL of plasma)
Plasma	0.114 ± 0.024 (57.0%)	0.200 ± 0.043

a) Mean values with their standard errors (n = 6–8).
b) The diet contained 14.4 mmol of anthocyanins, *i.e.* 12.3 mmol (85.1%) of cyanidin 3-glucoside *per* kg diet.
c) Cy 3-glc, cyanidin 3-glucoside.

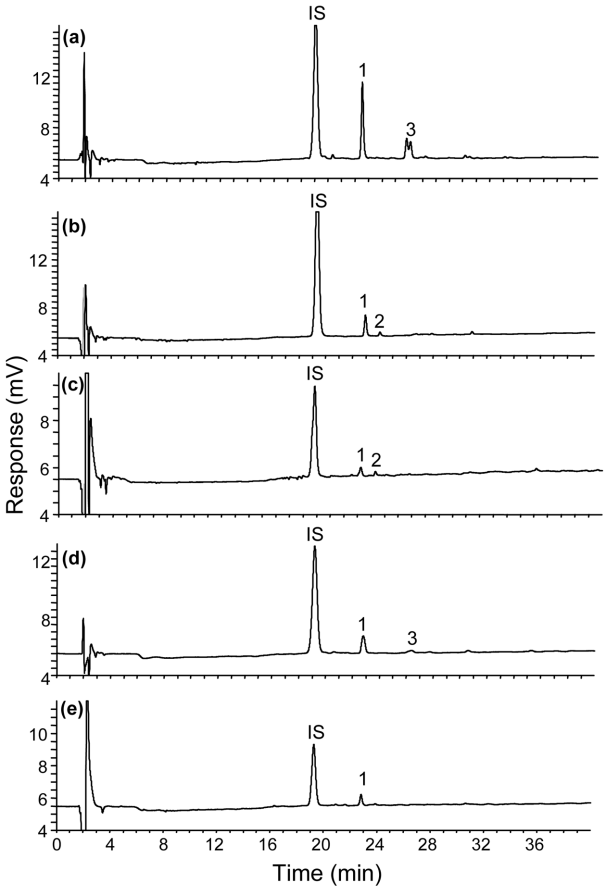

Figure 2. HPLC chromatograms of the extracts of tissues collected after feeding rats with a diet supplemented with blackberry anthocyanins. (a) Bladder, (b) prostate, (c) testes, (d) adipose tissue, (e) heart. Detection was performed at 524 nm. (1) cyanidin 3-glucoside, (2) cyanidin monoglucuronide, (3) methylated cyanidin 3-glucosides. IS, internal standard (cyanidin 3,5-diglucoside).

derivatives (~40% of total anthocyanins) (Fig. 2a). The concentration of anthocyanins in prostate gland was one of the highest among the studied tissues. A monoglucuronide of cyanidin was detected in most prostate samples (7/8) and methylated derivatives were observed in only one sample (Fig. 2b). Similarly, the HPLC profile of anthocyanins in testes showed native cyanidin 3-glucoside as well as a small amount of cyanidin monoglucuronide (Fig. 2c). Native cyanidin 3-glucoside as well as its methylated derivative were found in adipose tissue (Fig. 2d). Cyanidin 3-glucoside was detected in four heart samples out of six and only two heart samples contained a glucurono-conjugate of cyanidin. No anthocyanins were detected in the tissues and plasma of the control rats.

4 Discussion

We previously reported the distribution of anthocyanins in the digestive area organs (stomach, jejunum, liver), kidney and brain of rats fed a similar blackberry extract [18]. In the present work, we examined the capacity of anthocyanins to reach other inner tissues such as heart, adipose tissue, testes, prostate gland and bladder. We used a model that mimics a regular intake of anthocyanins: rats were fed for 12 days with a diet supplemented with a blackberry extract mainly containing cyanidin 3-glucoside.

As we have previously reported [18], native blackberry anthocyanins as well as methylated and glucuronidated metabolites were excreted in urine in a low proportion as regard to the ingested amount (~0.2%). Circulating forms detected in plasma provided evidence of anthocyanin metabolism in various tissues such as mainly intestine and liver [18].

Excision of tissues was carried out in bloodless rats after perfusion of phosphate buffer saline to remove residual blood in organs. Anthocyanins detected in tissue homogenates were thus a reflection of their presence in the tissues and not in contaminating blood. The bladder contained the highest amount of anthocyanins as compared to the other tissues analysed. Anthocyanin concentration was close to that we previously found in kidney and the proportion of methylated derivatives was also similar to that observed in both urine and kidney [18]. It has recently been shown that quercetin inhibited growth of various bladder cancer cells and induced apoptosis in such cells [30]. By their presence in this tissue, other flavonoids such as anthocyanins could also exert beneficial effects at this level.

Anthocyanins were shown to be cytotoxic and to induce apoptosis in prostate cancer cells [31 – 33]. However, the presence of anthocyanins has never been described in prostate gland. We show here for the first time that cyanidin 3-glucoside and a glucurono-conjugate of cyanidin reach this organ. Various other polyphenols such as tea polyphenols and isoflavones were previously detected in the prostate [34 – 36]. Anthocyanins could thus play a role in the prevention of prostate cancer, as has been suggested before for tea polyphenols [34, 37].

Flavonoids such as isoflavones and quercetin have been identified in testes from rats fed flavonoid-enriched diets [35, 36, 38]. They were largely present as glucurono and/or sulphoconjugate forms [35, 36]. A methylated derivative of quercetin (isorhamnetin) was also reported as the main metabolite in testes of quercetin-fed rats [38]. As previously described for genistein and daidzein [35], anthocyanin concentration was lower in the testes than in the prostate. In both organs, native cyanidin 3-glucoside was accompanied by a small proportion (~15%) of a cyanidin monoglucuronide.

Anthocyanins were shown to prevent obesity when administered to high-fat fed mice [12, 13, 39]. Anthocyanins added to the culture medium of adipocytes also enhanced adiponectine and leptin secretion and upregulated the expression of several adipocyte specific genes [40]. They were also shown to improve insulin sensitivity in

mouse adipocytes treated with H_2O_2 and TNFα [41]. The present study shows that anthocyanins enter adipose tissue. They could thus contribute to the antiobesity effects of anthocyanins by directly influencing the metabolism of adipocytes. Contrary to what was observed in prostate and testes, cyanidin 3-glucoside was accompanied in fat by a small proportion of a less polar metabolite, methylated cyanidin 3-glucoside. On the other hand, only methylated derivatives of quercetin have been found in white fat from quercetin-fed rats [38]. The level of methylation and/or tissue uptake of methylated metabolites thus differed between tissues [38].

Various studies conducted both *in vivo* and *in vitro* have shown that anthocyanins could be beneficial to cardiovascular health [9, 42 – 44]. No anthocyanins were detected in the heart from rats receiving the aglycone pelargonidin [22]. However, after feeding rats with blackberry anthocyanin glycosides, we have found cyanidin derivatives (cyanidin 3-glucoside and a cyanidin monoglucuronide) in this organ. In the heart from quercetin-fed rats, the main compound was a methylated derivative, isorhamnetin, accounting for around 2/3 of the total flavonol content [38].

This study thus shows for the first time that anthocyanin feeding in rats resulted in the distribution of anthocyanin derivatives to several organs such as bladder, testes, prostate, adipose tissue and heart. In relation to the complex chemistry of anthocyanins, some other important metabolites may also be present in organs but are still not identified [6]. Information on tissue distribution and evaluation of the biological properties of anthocyanin metabolites may help to understand the mechanisms of action of anthocyanins *in vivo* and their role in health promotion.

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The authors have declared no conflict of interest.

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