Research article

Staining of RNA and DNA on electrophoretic gels and in cytology with juice of *Vaccinium myrtillus* berries

Hannu Ahokas*

MTT-Agrifood Research Finland, Biotechnology and Food Research, FI-31600, Jokioinen, Finland

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A B S T R A C T

Background: My early results of cytological chromosome staining with berry juice of blueberry or bilberry (Vaccinium myrtillus) was re-evaluated with staining of electrophoretic agarose and polyacrylamide gels, fractionating DNA, RNA, and proteins.

Results: Electrophoretic gels were stained with juice from berries of *V. myrtillus*, only filtered, or the diluted filtrate was mixed with acetic acid and 2-propanol. The staining starts in 2 min, with the highest intensity over the background usually appearing in about 30 min. The berry juice stains DNA, RNA, and an unidentified contaminant of molar mass 200–700 g. After differentiation of the gel background, the stained zones appear purple or black. The berry juice staining with or without acetic 2-propanol shows sharp RNA and DNA zones on gels in UV (visible) light, and the berry juice displaces a preceding staining with ethidium bromide. A secondary staining with ethidium bromide is not able to displace the berry juice in nucleic acids. Cytological staining of sectioned mature barley (Hordeum vulgare) grains with *Vaccinium myrtillus* juice followed by differentiation in dilute acetic acid shows nuclei and apparent RNA storing compartments in the aleurone cells and stains suberin-containing chalazal cells of the grain crease deeply red, which keeps long. The anthocyanins faintly stain some proteins on gels. The preparation of polymers from cytochrome c and myoglobin was described.

Conclusions: The staining molecules of *V. myrtillus* anthocyanins apparently intercalate in the nucleic acids competing for the sites, which could be intercalated by ethidium bromide. The juice is suggested to have use in the exchange of intercalating toxic molecules from DNA and RNA, in the inactivation of viruses, and phytotherapy. The juice may have a use as a nontoxic stain in cytology.

1. Introduction

The blueberry (*V. myrtillus*) is a common wild berry species native to coniferous forests in the Nordic countries with a wide Eurasian distribution. The blueberry is an important berry gathered in the forests of Finland, where the mean annual production of its berries was calculated to be $168.4 \times 10^{-6}$ kg [1]. Its berry production varies from year to year. Its matured, thoroughly blue berries and staining capacity are familiar to people in the area of blueberry distribution. A variation of the contents and types of anthocyanins occur within the species *V. myrtillus*, which has one of the highest contents among species with blue berries. *V. myrtillus* from Northern Europe were shown to genetically vary in their anthocyanin contents, the northern proveniences having high contents [2] also within the territory of Finland [3]. The total anthocyanin content in *V. myrtillus* berries is several times higher than that in the domesticated *V. corymbosum* on dry weight basis [4].

As a university student, I made my first cytological staining of chromosomes for light microscopy with acetic *V. myrtillus* juice in 1968. I could see plant chromosomes through the microscope, though the resolution against the stained cytoplasm was not good enough in these preparations and was perhaps due to the presence of RNA in the cytoplasm. The spread preparations of chromosomes with only little cytoplasm around might be better stained with *V. myrtillus* juice. The present study is to confirm the staining of nucleic acids, both DNA and RNA, and possibly of proteins with *V. myrtillus* juice using electrophoretic gels carrying these macromolecules. My early studies on the *Vaccinium* species also resulted in a few publications in the 1970s [5, 6, 7, 8].

In the past, blueberries have been used or tried to stain garn or cloth in black and blue colors in Finland, Estonia, and Livonia [9]. Furthermore, blueberry anthocyanins have many therapeutic effects to human beings [10, 11, 12, 13] and supposedly to animals of many berry-eating species as well. The *V. corymbosum* extract also showed antimicrobial

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* Corresponding author.
E-mail address: hannu.ahokas0@saunalahti.fi.

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activity [14]. It may be of interest, if any of the beneficial effects are regulated on the nucleic acids level. A direct interaction with DNA may also raise concern about the mutagenic or teratogenic effects of anthocyanins. On the other hand, the anthocyanins may compete with harmful molecules able to intercalate into DNA and may have a detoxifying effect as the competitor.

2. Materials and methods

2.1. Berries and staining with their juice

I collected mature, qualified berries of blueberry (V. myrtillus) free from berries of other species in natural forest stands in Southern Finland, froze them in zip-lock polyethene bags in 320 g (about half-liter) lots, and stored at -18 °C. Usually, 320 g of frozen berries were partly melted in a microwave oven at 360 W in a glass vial in 1 min lasting pulses, mixing the berries after each pulse. The melted but cold berry mass was filtered through Nylon gauze in glass funnels, gently pressing the berries with a glass test-tube as a pestle. The V. m. (Vaccinium myrtillus) berry juice filtrate (about 100 ml) was gathered in 500 ml glass bottles standing in an ice bath. Either used as such or the filtrate (about 100 ml) was made up to 195 ml with MQ (Milli-Q water), and 75 ml 2-PrOH (2-propanol) was vigorously mixed, and rapidly 30 ml AcA (acetic acid) was mixed successively; the freshly made mixture was poured on the gel to be stained. The gel was gently shaken first by hands and then horizontally in a shaker, usually 25 rpm for various lengths, from minutes to several hours.

2.2. Gel electrophoresis

Protein samples of barely grain and commercial purified proteins and part of the pssRNA (yeast soluble RNA) samples were extracted with Tris-buffered (tris(hydroxymethyl)aminomethane, 40 mM, pH 8) 2-PrOH (50 % v/v) and βME (β-mercaptoethanol) (5 % v/v) followed by a precipitation step with 2.5 vols of MetOH (methanol) [15, 16] or with the evaporation of the extraction supernatant with a vacuum centrifuge. The methanolic supernatant usually also evaporated with the vacuum centrifuge. The extraction steps were repeated once. The samples were usually loaded in Laemmli’s [17] sample buffer. If a reducing agent, βME or DTT (DL-dithiothreitol) was included, it was added after the heat treatment of the samples (95 °C, 2 min). In some trials, fresh V. m. juice was additionally loaded in the sample wells before the start of running. The vertical SDS-PA (sodium dodecyl sulphate polyacrylamide) gels, with 5 % stacking gel and 10.8 % fractionating gel, were prepared and run as described [15]. The gels were stained with the V. m. juice as above or sometimes post-stained with CBB (Coomassie Brilliant Blue R 250), as described [15]. The horizontal 11 mm thick agarose gels (0.9 %) were described [15]. The horizontal 11 mm thick agarose gels (0.9 %) were run as described [15]. The gels were transilluminated with UV light of wave length 312 μm. Primary staining with EtBr (ethidium bromide) 5 μg ml -1 was done in 1×TBE buffer, in later stages in MQ.

2.3. Gel photography

The stained gels were photographed with a Canon EOS 1000D digital camera. The gels were transilluminated with UV light of wave length 312 nm (B&Q-Vision, Spectrolite) or with visible light, usually fluorescent tubes as the source. Kodak UV barrier filters, though changed the image, did not improve the fluorescence resolution with this camera, and the UV illumination set-up and exposures were made without UV filters. The gel figures are unedited records with the camera system.

2.4. Double-diffusion test

For double-diffusion tests introduced for the purpose, 10.8 % SDS-PA
similar to that of electrophoresis separation gel was poured in Petri dishes covered with a home-made Teflon lid (thickness 1 mm) with tight holes, adopting two gel combs side-by-side. The Teflon lid lining the upper level of the gel in the dish was additionally covered with water to reduce oxygen in the polymerizing acrylamide to make a smooth surface. The sample solutions to be tested were pipetted in one of the well rows, V. m. juice in the other.

2.5. Cytology with berry juice staining

For microscopy, hand-cut sections of mature, dry barley (Hordeum vulgare) caryopses, cv. ‘Adora’ and landrace Line 269 were pretreated for 1 or 3 h with MetOH or mixture of 1 volume of Tris-buffered (40 mM, pH 8) 2-PrOH (50 % v/v) and βME (5 % v/v) plus 2.5 volumes of MetOH. Subsequently, the sections were soaked in a mixture of V. m. berry juice: AcA: 2-PrOH (65: 10: 25 % v/v, respectively) for about 8 h. The stain was differentiated with changes and mounted on slides in 10 % AcA, which was displaced with glycerol. The light microscopic views (Zeiss Axioskop) were photographed with Kodak Ektar 100 or Agfaphoto CT Precisa 100 film using a 40× Plan objective.

2.6. Sources of used laboratory chemicals

Acetic acid ca. 100% (Bang & Co.) or Emprove (Merck). Acrylamide (99.9%) and N,N'-methylene-bis-acrylamide (electrophoresis purity) (Bio-Rad). Agarose (GTG Agarose, Biometra). Ammonium persulfate (electrophoresis purity) (Bio-Rad). β-Mercaptoethanol puriss (Fluka). Boric acid p.a. (Merck). Bromophenol blue (Merck). Coomassie Brilliant Blue R 250 (Serva). Cytochrome-C, type III (Sigma C-2506). DL-dithiothreitol (Sigma D-0632). DNA from Calf Thymus Type I, Na salt, highly polymerized, (Sigma D-1501). Ethidium bromide (Sigma E8751). Ethylenediamine tetraacetic acid disodium salt p.a. (Merck). Gel Drying Films (Promega). Glycine p.a. (Riedel-deHaen 33226). Methanol p.a. or SeccoSolv (Merck). Myoglobin from Horse Heart (Sigma M-9267). Plasmid pCaMVN (Pharmacia) 4177 bp, linearized with Cla I (NEB). Precision Protein Standards (Bio-Rad 161-0362) with given molar masses of 250, 150, 100, 75, 50, 37, 25, 15, & 10 kDa. 2-propanol, p.a. Emsure or SeccoSolv (Merck). RNA, soluble from Yeast, Type III (Sigma R-7125). Sodium dodecyl sulphate (BDH 44215). TEMED (electrophoresis purity) (Bio-Rad). Tris, Trizma preset pH, base and HCl (Sigma).

3. Results

3.1. Staining gels with Vaccinium myrtillus berry juice

The melting and filtering of the V. m. juice from berries were done at a chilled temperature to keep the anthocyanins. The staining solution usually had low pH due to AcA, the berry juice also being naturally acidic. Genomic ctDNA (calf thymus DNA) was used as the test material for the staining with V. m. berry juice on agarose gels. After a 1 h rinse in MQ water, the DNA and gels stain in different ways depending on the components of the stain. The V. m. berry juice alone stains efficiently, but the gel itself retains more the stain than, if AcA and 2-PrOH were added to the stain solution (Fig. 1, tracks A and B). After soaking in water, if the gel was stained with V. m. berry juice, EtBr staining is inefficient, even after a prolonged EtBr soaking (Fig. 1, tracks C1 - C4, the single track treated sequentially). In Figure legends, an arrow (→) indicates the change to a next-step solution at gel staining or destaining, the duration of which is given in hours (h) and minutes (min). Even a prolonged (>48 h) soaking in EtBr is not capable of replacing V. m. juice color from DNA (Fig. 1, track C4), though this happens in the gel itself, which starts to show the typical fluorescence in UV after a prolonged incubation in EtBr (Fig. 2, tracks A1, A6). EtBr is not quenched due the presence of V. m. juice (Fig. 2, track C series), but V. m. juice competes with EtBr for the staining sites in DNA (Fig. 2, track A and B series).

V. m. juice stains RNA on gels with high specificity. Adding V. m. juice (26 µl) in the gel wells of the buffered sample (14 µl) before the start of running affects the relative mobility of the RNA bands (Fig. 3, tracks A and B vs. C series) and partly precipitates DNA in the sample well (Fig. 3, track C8). The V. m. juice reacts directly with nucleic acids. Some bands which stain blue-black fast and strongly with the berry juice solution alone for 11 h and washed with MQ, AcA, and 2-PrOH for 2 h. B1. Stained with V. m. juice solution alone for 11 h and washed with MQ, AcA, and 2-PrOH for 2 h. B1. B1 post-stained with CBB over night and washed with lowering proportions of 2-PrOH in 10 % AcA. Tracks C1–C12. Stained with EtBr for 2 h; UV without a filter, and C4–C6 using UV filters which did not improve the image. C7–C9. C1–C3 post-stained with V. m. juice solution for 17 h → MQ for 3 h 15 min: Vis. This staining gives sharper images of the bands than in the fluorescence with EtBr and UV illumination. V. m. juice in the sample well partly precipitates DNA in the entry into the gel (C8). C10–C12. C7–C9 photographed in UV transillumination, showing a total lack of fluorescence of the nucleic acids and the gel. The V. m. juice included in the sample well (C1 – C12) changes the mobility of the ystRNA zones from those without the juice in the sample wells (A, B, and B2).
running, the contaminant moved associated with the RNA (Fig. 4, tracks A–F). Tracks on the same gel. The wells were loaded with 600 μg of ystRNA. The gel was stained with V. m. juice alone. Samples without a reductant (A and B), with the contaminated MetOH treatment in B, causing the association of the contaminant with ystRNA. – C. Like B, but with 5 % v/v of βME, which dissociated the contaminant. – D. Like C, but with 0.03 % w/v of DTT instead of βME, which caused faster movement of the dissociated contaminant than βME. – E and F. A & B stained for 9 h 30 min with V. m. juice and post-staining with CBB, washed with 10 % AcA, and lowering proportion 2-PrOH in water. CBB also stains the contaminant in the ystRNA complex.

Contaminants to 2-PrOH from polyethene plastic containers were found to cause precipitation in the V. m. juice when staining gels. Solvents stored in glass bottles worked better. The amount of the contaminated MetOH stored in the 100 ml lab bottle was so small, that the suspected contaminant could not be chemically identified. The MetOH precipitation of solved proteins with Tris-buffered 2-PrOH [15, 16] was observed to promote the polymerization of cyt c (cytochrome c) up to heptamer and that of myoglobin to trimer (Fig. 5, tracks F and G). The precipitation with MetOH was not observed to increase protein zones, i.e., to cause polymerization in barley grain proteins (Fig. 5, tracks C1 and D1 vs. B1). The addition of MetOH at the precipitation was measured to lower the pH of the extraction solution by about 2 units. The fast-running stainable band was also observed where proteins were precipitated with the contaminated MetOH lot (Fig. 5, tracks C, C1, D, and D1). Such contaminated samples in the double-diffusion test in gel formed staining bands between the V. m. juice and the contaminant (Fig. 6), which behaved as could be expected from results with the SDS-PA electrophoresis of the same samples. The meeting of the

With the analytical quality of AcA and 2-PrOH as the components of the V. m. juice stain, the mixed stain keeps several hours at room temperature, and the stained gels could at times be taken in 10 % AcA on a glass plate wiped with cotton for the photography, after which, the staining in V. m. juice with AcA and 2-PrOH could be continued.

![Fig. 4. Vertical SDS-PA gel fractionating ystRNA with or without a sulphydryl-reductant affecting the mobility of the black-staining contaminant caused by the MetOH treatment. – A-F. Tracks on the same gel. The wells were loaded with 600 μg of ystRNA. The gel was stained with V. m. juice alone. Samples without a reductant (A and B), with the contaminated MetOH treatment in B, causing the association of the contaminant with ystRNA. – C. Like B, but with 5 % v/v of βME, which dissociated the contaminant. – D. Like C, but with 0.03 % w/v of DTT instead of βME, which caused faster movement of the dissociated contaminant than βME. – E and F. A & B stained for 9 h 30 min with V. m. juice and post-staining with CBB, washed with 10 % AcA, and lowering proportion 2-PrOH in water. CBB also stains the contaminant in the ystRNA complex.

![Fig. 5. Vertical SDS-PA gels fractionating proteins. Photography in Vis. – Tracks A-D. Proteins from embryo-less barley grains prepared as described [15] and stained with V. m. juice in AcA + 2-PrOH solution. – A. Residual proteins from extraction in B. – B. Proteins solved by the Tris, 2-PrOH, βME solution and evaporated to dryness. – C. Proteins solved by the Tris, 2-PrOH, βME solution and precipitated with 2.5 volumes of the contaminated MetOH. – D. Proteins in the evaporated, contaminated MetOH supernatant from C. The contaminant stains black in the lower edge of the gel. A faint staining of the major protein zones and the upper smear (white bars). – Tracks A1–D1. The gel with A – D was post-stained with CBB to show the proteins. The angles show the position of the nine Precision Protein Standards. – Track E. Myoglobin from a MetOH supernatant evaporated and electrophoresed on a gel pre-run for 2 h before the samples were loaded. The gel was directly stained with V. m. juice for 12 h 30 min → MQ for 30 min. Bract shows myoglobin band stained with V. m. juice. – Track F. Myoglobin polymers up to trimer (angles) from a MetOH precipitate, the gel stained with CBB. – Track G. Cyt c polymers at least up to heptamer (angles) from a MetOH precipitate, the gel stained with CBB.](image-url)
V. m. juice and the contaminant in the gel also stopped further diffusion. When the Petri dish was sealed with Parafilm against drying out, the double-diffusion could continue for days, intensifying the stained zones and, thus, showing good stability of the anthocyanins in such an environment with SDS (sodium dodecyl sulphate) and buffer (Fig. 6).

3.2. Staining with Vaccinium myrtillus berry juice in cytology

In mature barley grains, V. m. juice with acetic 2-PrOH principally stains the nuclei in the caryopses after differentiation of the background in 10 % acetic acid (Fig. 7a). In the aleurone cells, the cytoplasm also retains the stain, which is probably due to the stored RNA in the cytoplasm. The degraded nuclei in the mature, undividable starch endosperm also stain, while all the starch grains remain unstained (Fig. 7b). In barley caryopses, strong staining was observed in the crease (Fig. 7c), where the staining was strong in the chalazal cells and has remained so for over five years in dry-out preparation in the dark. The chalazal cells are known to contain suberin [18], which may thus be highly reactive with the V. m. juice.

4. Discussion

The earlier tried and observed cytological stainability with the V. m. juice of metaphase chromosomes and interphase nuclei appears to be explainable as the staining of DNA because the juice also stains genomic and plasmid DNA and additionally RNA on gels. The V. m. juice staining of proteins, e.g. those associated with chromatin in the cells, is much weaker than that of the nucleic acids and could be less responsible for the staining of chromatin in cells.

The V. m. juice staining is strongly competitive with EtBr and displaces EtBr from nucleic acids on electrophoretic gels (Fig. 1, tracks C1–C4, and Fig. 2). Molecular similarity of some anthocyanins and EtBr suggests that many of the anthocyanins are also intercalating dyes in nucleic acids, which was also supported by sequential staining of a single gel with EtBr and V. m. juice in various orders with or without washes in between. The observed competing interaction of blueberry anthocyanins with the binding of EtBr suggests an intercalative reaction of one or some of them into DNA. The intercalation of anthocyanins in DNA was also reviewed [19]. The interaction of the flavonoids hesperitin and naringenin with DNA was shown to be intercalative [20]. Naringenin occurs as an intermediate in the flavonoid pathway leading to the anthocyanins in V. myrtillus fruits [21].

It is apparent that V. m. juice competes with EtBr. Therefore, V. m. juice might be used to detoxify accidental exposure of mouth and upper alimentary tract to EtBr or perhaps to other compounds intercalating in nucleic acids. A commercialized preparation of V. myrtillus anthocyanins was not found to be mutagenic or teratogenic [10]. On the contrary, anthocyanins instead belong to the colorants causing curing apoptosis in cancer tissue as reviewed [22]. A related flavonol, quercetin occurs in V. myrtillus flowers and fruits [21, 23]. There has been concern about the mutagenic or teratogenic effects of quercetin. Evidence for such in vivo toxicity of quercetin is, however, lacking [24]. The inactivation of viruses with V. m. juice thought to occur in human cures is conceivable through the affinity of one or more components of the juice with viral nucleic acids. The upregulation of genes directly by an anthocyanin interference with DNA might occur; V. m. juice anthocyanins are known to upregulate the heme-oxygenase-1 gene in human retinal pigment epithelial cells [25]. In rats with diabetes-induced oxidative stress and inflammation in retinas, blueberry anthocyanin feeding increased the mRNA levels of the heme-oxygenase-1 and its regulator, the nuclear factor-erythroid 2 in retinas [12]. It is to be seen, if the blueberry anthocyanins directly interfere with DNA in the regulation of the heme-oxygenase-1 gene or other genes in vivo. Anthocyanin-DNA copigment protects from OH radical damages and was suggested to also operate in the nuclei of plant cells [26].

Diluted V. m. juice might be used as a vital staining as an ultrafiltrate
Fig. 7. a-c. Dry sections of mature barley grains pretreated with MetOH (a & b) or Tris-buffered 2-PrOH – MetOH (c) stained with V. m. juice in Aca and 2-PrOH, and destained in 10 % Aca. – a. Caryopses has an aleurone (Al) with three cell-layers, showing interphase nuclei stained purple and cytoplasmic staining, probably by RNA. The subaleurone (Subal) cell-layer has nuclei with less intact appearance. The starch endosperm (St_end) has remains of nuclei. – b. Degraded chromat in the starch endosperm tissue with plenty of unstained starch granules. – c. In the transversally cut crease, the chalazal (Cal) cells stain strongly, apparently due to their suberin. The aleurone (Al) layers border the crease of the grain on two sides. Scales 100 μm, in a common for b.

5. Conclusion

The anthocyanins from V. myrtillus berries efficiently and fast stain DNA and RNA fractionated on electrophoretic gels. The staining molecules apparently intercalate in the nucleic acids competing for the sites, which could be intercalated by EtBr. The nucleic acids can be seen in visible light without exposure to UV. The juice is suggested to have a use to inactivate viruses and to exchange intercalating toxic molecules from DNA and RNA in vitro and in vivo. The juice may have a use as a nontoxic stain in cytology and could show an otherwise unnoticed, unidentified contaminant in a laboratory chemical. The suitability of the V. m. juice-stained RNA and DNA to enzymatic modifications and the possibility to remove the stain from nucleic acids is to be studied.

Declarations

Author contribution statement

Hannu Ahokas: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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The authors declare no conflict of interest.

Additional information

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