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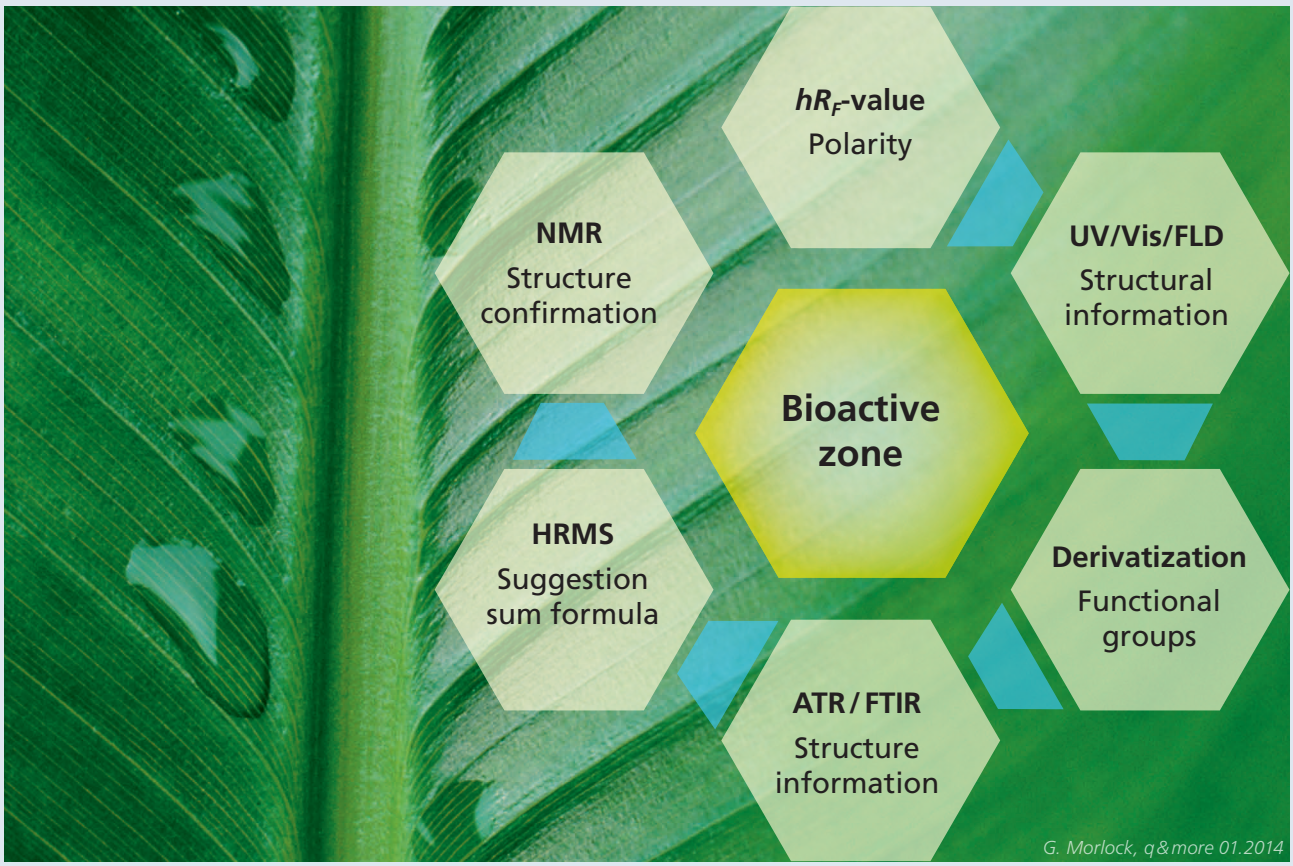
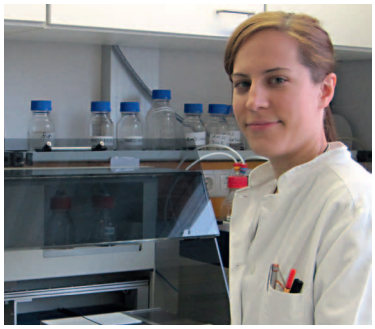


From the bioactive zone to the structure – hyphenations for effect-directed analysis at an analytical level

CAMAG BIBLIOGRAPHY SERVICE PLANAR CHROMATOGRAPHY

Anthocyanes in food and animal feed by HPTLC-Vis-(EDA-)MS



Stephanie Krüger

Ms Stephanie Krüger has developed this rapid HPTLC method to investigate anthocyanes as bioactive compounds in food and feed during her diploma thesis under supervision of Professor Gertrud Morlock.

Introduction

Anthocyanins are naturally occurring water soluble flavonoid dyes whose colors range from yellow through orange and red to bluish hues. Several health-promoting properties like anti-inflammatory, anti-microbial, antioxidant and cancer preventive activities are attributed to them, as well as a positive influence on stress triggered chronic illnesses like diabetes. [1] As food color E 163, anthocyanin extracts are added to various food stuffs. Also animal feed is supplemented with pomace of anthocyanin rich fruits to enrich the phenolic and therefore antioxidant content.

Food and animal feed are heavily loaded with matrices. Planar chromatography as a matrix-robust method allows to analyze anthocyanes in such samples without any purification steps. It is an additional asset that several anthocyanes of the same group (*e.g.* aglycones, monoglucosides, diglucosides) can be separated at the same time and, if required, both polar and apolar groups on the same plate. The determination of the 11 individual anthocyanes was performed using three complementary detection methods i.e. direct detection in the visible and UV range, detection of radical scavenging properties by derivatization with DPPH•, and detection of bioactivity after immersion in a suspension of *Aliivibrio fischeri* bacteria. By recording mass spectra after elution with the TLC-MS Interface, it was possible to characterize most of the unknown anthocyanin zones. The method allows a high sample throughput *i.e.* parallel analysis of up to 9 samples in duplicate [2].

Chromatogram layer

HPTLC plates silica gel 60 F_{254} (Merck), 20 × 10 cm

Standard solutions

Anthocyanes were dissolved in 0.5 % hydrochloric acid in methanol with stock solution concentrations of 1 mg/mL for the 5 anthocyanidins (aglycons) and 0.1 mg/mL for the 6 anthocyanins (glycosides). The stock solutions were pipetted together into one standard mixture in quantities between 6 and 32 ng/µL [2]. All solutions were stored in the dark at –20 °C.

Sample preparation

The solid samples (grape pomace, animal feed, pomace formulation AntaOx E (used as reference) and grape seed meal) were extracted with acidified methanol and the supernatant centrifuged for 5 min at 1000 g. The belladonna extract was purified using XAD-7 (1 mL equals about 25 g fresh great cherry fruits). Wine and fruit juice samples purchased at local shops in 2012 were centrifuged for 10 min at 10000 g and the supernatant was acidified with 1 % hydrochloric acid. All samples were stored in the dark at –20 °C.

Sample application

Bandwise (8.0 or 7.5 mm bands) using Automatic TLC Sampler 4 leading to 19 or 22 tracks, respectively, distance from the left side 15 mm, distance from the lower edge 8 mm. For quantitation, standard mix volumes ranged between 2.5 and 12 µL and samples volumes between 0.5 and 15 µL.

Chromatography

In the Automatic Development Chamber ADC 2 for anthocyanins with ethyl acetate – 2-butanone – formic acid – water 7:3:1.2:0.8. Before development the relative humidity was adjusted to

33 % by a saturated magnesium chloride solution for 2 min. The migration distance was set to 65 mm (from lower plate edge). When anthocyanidins (hR_F > 90) were detected, the lower part of the plate was cut off with the smartCUT Plate Cutter and the upper part was developed with ethyl acetate – toluene – formic acid – water (10:3:1.2:0.8, v/v/v/v). Plates used in the investigation with *A. fischeri* bioassay or DPPH• reagent were dried for 15 min to remove all acidic solvent residues.

Documentation

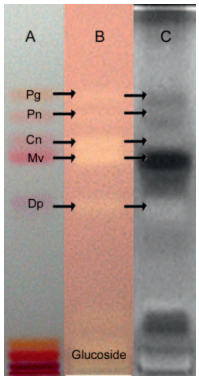
With DigiStore 2 System under UV 254 nm, UV 366 nm and under white light

Densitometry

With TLC Scanner 3 under winCATS, absorbance measurement using multi-wavelength scan at 505, (510), 520, 530 and 555 nm

Bioassay detection with *Aliivibrio fischeri*

A. fischeri culture medium was prepared according to DIN EN ISO 11348-1, section 5. Immersion with the TLC Chromatogram Immersion Device for 2 s at a speed of 2 cm/s. Immediate documentation with the BioLuminizer (10 images at 3 min time intervals, exposure time 50 s).



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Comparison of alternative detection methods: Vis (A), DPPH• reagent (B) and A. fischeri suspension (B), exemplarily shown for the apolar anthocyanidins pelargonidin (pg), peonidin (pn), cyanidin (cn), malvidin (mv) and delphinidin (dp)

Postchromatographic derivatization

Plates were dipped into the DPPH• reagent (0.5 mM 2,2-diphenyl-1-picrylhydrazyl radical in methanol) at a speed of 2 cm/s and an immersion time of 5 s using the TLC Chromatogram Immersion Device. Then they were first dried for 90 s in the dark at ambient temperature, followed by heating for 30 s on the TLC Plate Heater at 60 °C. Documentation under white light; the reverse DPPH• chromatogram (bright zones on

2

CAMAG BioLuminizer

Hyphenating TLC/HPTLC and bioassay is an excellent tool for identification of single toxic compounds in complex sample matrices.

The BioLuminizer is a compact, user-friendly detection system for capturing bioluminescence images. It shows an exceptional image quality and a high resolution for a short exposure time. The system is comprised of a compartment excluding any extraneous light, climate controlled for extended stability of the plate, and a 16 bit CCD digital camera of high resolution and high quantum efficiency.

In this CBS issue, the BioLuminizer was used to detect the impact of compound zones on the luminescence and bioactivity of selected bacteria. Such a luminescent bioassay detection was used in the contributions on pp. 2–5 and 12–15 for detection of the luminescence inhibition or intensification of *Pseudomonas savastonoi* and *Aliivibrio fischeri* bacteria. As the bioassay indicates single bioactive compound zones, it is an effective strategy for finding bioactive compounds in complex mixtures. It directly links to a bioactive compound.

Further information can be found under **www.camag.com/bioluminizer** or in the special brochure CAMAG BioLuminizer.

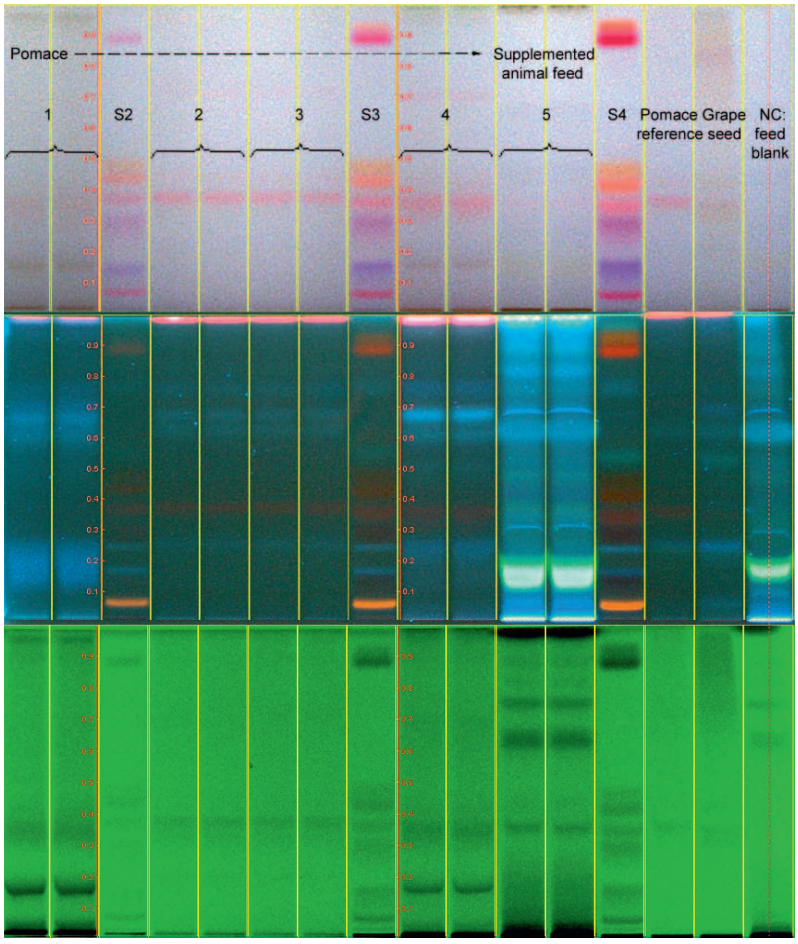
rose-violet background) was scanned at 517 nm by selecting "fluorescence mode" in order to invert the otherwise negative peaks.

Mass spectrometry

Zones were eluted with the TLC-MS Interface with oval elution head (2 x 4 mm) using methanol at a flow rate of 0.1 mL/min (pump of Agilent HP 1100 Chem-Station) All spectra were recorded with a single-quadrupol MS with electrospray ionization (ESI) in the positive mode (capillary voltage +4 kV, fragmentation voltage 160 eV or 300 eV for anthocyanins and anthocyanidins, respectively). A plate background spectrum recorded aside the analyte zone was subtracted from the analyte spectrum.

Results and discussion

After minimal sample preparation, the HPTLC separation of the samples took merely 20 min, and in case of presence of anthocyanidins further 13 min. Grape pomace, animal feed, grape juice, wine samples and various other fruit juice samples were analyzed. Anthocyanin patterns corresponded when juices originated from the same fruit.



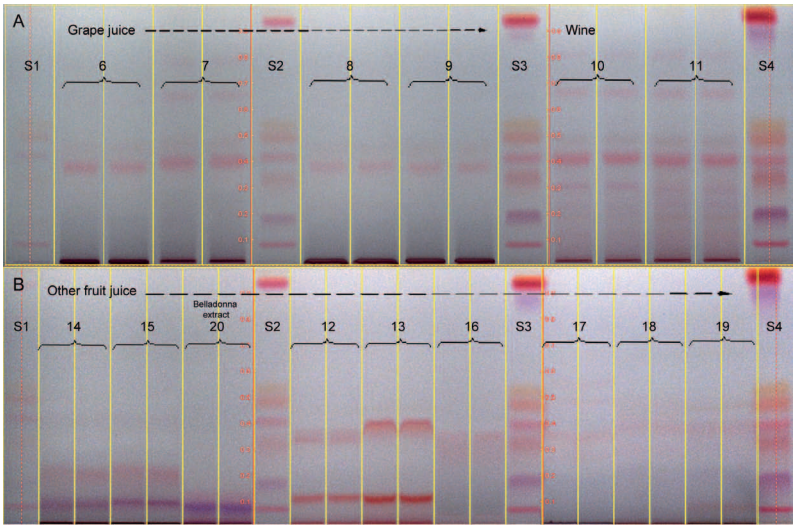
HPTLC chromatograms of pomace and animal feed samples and standard mixture (S2–S4) under white light, 366 nm and 254 nm (reprinted from [2] with permission)

The method showed good validation data. The correlations coefficients for all 11 anthocyanes were between 0.9993 and 0.9999. For the analysis of mv-3-glc in grape pomace and animal feed, the mean repeatabilities (*%RDS*, n = 2) were 1.4 %. Intermediate precision (*%RDS*, n = 3) was ≤6.7 % and the method's ruggedness was ≤ 5.5 %. In the visible range LOD and LOQ (signal-to-noise ratio of 3 and 10) were ≤90 ng/zone for all anthocyanes. Interestingly anthocyanins had generally better LODs/LOQs than their aglycones. For example, pn-3-glc and pg-3-glc had LOQ values ≤7 ng/zone, which were better by a factor 3 and 5, respectively, compared to their sugar-free counterparts.

To ascertain anthocyanes' bioactive properties two effect-directed detections were used. For DPPH$^{\bullet}$ the values for LOD and LOQ were slightly increased, but generally still ≤100 ng/zone. The evaluation for *A. fischeri* was done visually, and the LODs for anthocyanidins were in the same range as the other two detection methods. However, the values for anthocyanins were increased up to 20 times (<1550 ng/zone).

However, it was not always possible to assign sample zones to an anthocyanin of the standard mix. To characterize these zones HPTLC-ESI-MS spectra were recorded. Since little is known about the anthocyanin pattern of the great cherry fruit (belladonna), the identification of the two anthocyanins was of special interest. The mass spectra of the higher, pink colored zone showed an ion at *m/z* 287 corresponding with cyanidin [A$^+$]. With regard to hR_F-value one can conclude a multi-glycosylated derivative. The lower, lila colored zone showed two ions, one at *m/z* 317 corresponding with petunidin [A$^+$] and another at *m/z* 657 corresponding with the sodium adduct of the dimer [2A+Na]$^+$. Further sodium adducts

of the monoglycoside $[Hex+Na]^+$ and diglycoside moiety $[Hex_2+Na]^+$ as well as their dimer $[2Hex_2+Na]^+$ also indicated a multiple glycosylated anthocyanin.



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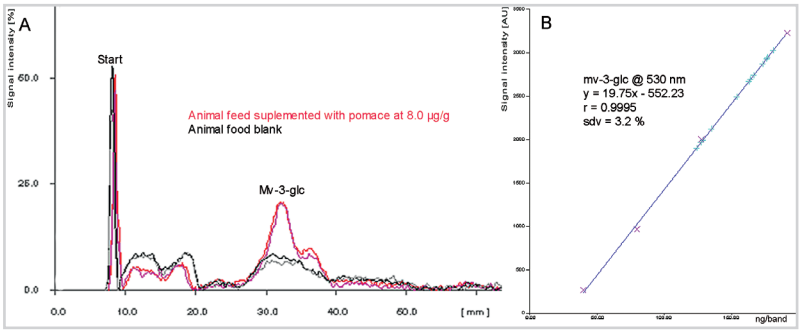
HPTLC Chromatograms of grape juices and wine (A) as well as other fruit juices (B) (reprinted from [2] with permission)



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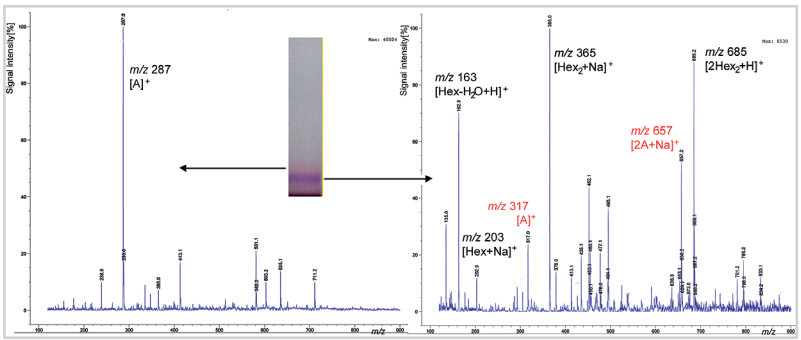
Overlaid densitograms obtained by absorbance measurement at 530 nm of animal feed supplemented with mv-3-glc pomace at 8.0 µg/g (twofold determination) (A); calibration curve of mv-3-glc (B) (reprinted from [2] with permission)



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HPTLC-ESI+-MS of two unknown anthocyanine zones in a belladonna extract (reprinted from [2] with permission)

[1] J. Shipp, E.-S. Abdel-Aal, The Open Food Science Journal 4 (2010) 7

[2] S. Krüger, O. Urmann, G.E. Morlock, J. Chromatogr. A 1289 (2013) 105

Further information is available on request from the authors.

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