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# À côté calibration – Making optimal use of time and space in quantitative high performance thin layer chromatography

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ABSTRACT

Quantitative High Performance Thin Layer Chromatography (HPTLC) requires the application of several standards to each plate, reducing the number of actual samples that can be analyzed in a single run. Using pure standard compounds and a selective detection method, the standards for quantitation can be applied besides – à côté – the chromatography area. This frees the sample application space to accommodate the maximal number of sample on each plate. Also, analysis time is spent exclusively on samples, drastically shortening the effective analysis time per sample and increasing sample throughput.

Using this new calibration approach, the sample capacity of regular HPTLC methods can be increased or their scope be extended by an additional quantitative analysis. As a limitation, changes to the distribution of samples and standards within the plate as well as interferences from matrix compounds must be observed.

We demonstrate the feasibility of this method by complementing an HPTLC method with a quantitative analysis of total anthocyanin content in colored wheat varieties. The quantitation was validated and compared to the conventional photometric analysis. As outcome, the additional photometric analysis could be replaced and rendered unnecessary, saving time, effort and equipment. This approach could also be employed to quantify highly retained substances, which are usually inaccessible for quantitative analysis.

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## 1. Introduction

High Performance Thin Layer Chromatography (HPTLC) is routinely applied for quantitative analyses. Unlike other chromatographic methods, it uses a new and untested stationary phase – the plate – for each analytical run. This necessitates the application of standards to every single plate to account for plate-to-plate variations [1]. These standards are applied near the bottom edge of the plate to be chromatographed together with the samples. Developing a plate according to a certain method always takes the same time; the analysis time per sample is therefore depending on the number of samples analyzed on each plate. The more samples are applied to a plate, the shorter the analysis time per sample becomes. The necessity to chromatograph standards on each plate reduces the number of samples that can be analyzed with one plate, effectively increasing the analysis time per sample. Inversely, reducing the number of standards on a plate frees space for samples and thus decreases the analysis time per sample. Following the guidelines of the European Pharmacopeia [2], a plate can hold a total of 15 samples and standards. For quantification, it is reasonable to apply four to five standard concentrations to each plate – a third of the total capacity! – to account for the non-linear response curve in densitometric detection. If these application positions were available for samples, analysis time per sample, resource consumption, and costs would be reduced by a third.

Our hypothesis was that it is not necessary to include the standard into the chromatographic system under certain conditions: if matrix interferences are negligible, if a pure standard is available, and if the distribution of the target analyte within the plate is the same for the standards and the samples. The final condition holds true in two cases: if standards and samples are applied under the same conditions and are not yet chromatographed; and if the target

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**Fig. 1.** Diversity in wheat grain color: blue, deep purple, red and purple (from left to right). Blue and purple hues are due to the accumulation of anthocyanins in the aleurone layer and pericarp, respectively. The red pigmentation is caused by phlobaphene and proanthocyanidins [7]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

analyte is not eluted during development. In these cases, the concentration standards for quantification could be applied anywhere on the plate, even outside the chromatography area, freeing up all precious sample positions, maximizing sample throughput, and minimizing analysis time per sample. The obvious position to apply standards that do not need to be chromatographed is in the unused upper part of the plate, beside – à côté – the chromatography area.

Anthocyanins are phenolic compounds responsible for most blue to blue-black, and red to purple colors of diverse plant organs [3]. They are alternatives to artificial food colorants and research suggests potential health benefits due to their antioxidant properties [4,5]. In wheat grains, anthocyanins can be expressed in either the pericarp or the aleurone layer (Fig. 1); breeding lines with anthocyanins in both layers have been identified by HPLC [6]. Today, anthocyanin pigmented wheat grains are processed into whole-grain products with specific color and taste, as well as into anthocyanin extracts for further processing into functional foods.

In this paper, we tested the applicability of the new à côté calibration by the determination of the total anthocyanin content in colored wheat samples.

## 2. Material and methods

### 2.1. Sample preparation and extraction of anthocyanins

Forty samples of anthocyanin pigmented winter wheat (*Triticum aestivum* L.) were tested, which had been grown in 2014 under conventional farming practice in the wheat breeding nursery at the BOKU Experimental Station Groß-Enzersdorf, Lower Austria.

Grain samples (25 g) were milled with an AQC806 lab mill (Agromatic AG, Laupen, Switzerland). The different fractions were separated by a Promylograph LS laboratory sieving machine (Max Egger Gerätebau, St. Blasen, Austria). Only the bran fraction >710 μm was collected and subsequently milled with a Cyclotec™ 1093 mill (Foss GmbH, Austrian subsidiary, Vienna) equipped with a 1 mm sieve. The milled samples were stored in a freezer at −20 °C. The moisture content of the bran samples was measured with a MA35 moisture analyzer (Sartorius, Göttingen, Germany) and was typically 10%.

All samples were extracted according to Abdel-Aal and Hucl [8]. In brief, 8 mL of a mixture of methanol and 1 M HCl in a proportion of 85:15 (v/v) were added to 1 ± 0.002 g milled bran in a 15 mL centrifuge tube. The tubes were shaken shortly by hand and then agitated in an overhead shaker (Heidolph Instruments, Schwabach, Germany) for 30 min at 150 rpm. Then the tubes were centrifuged for 5 min at 4000 rpm in a Z206A compact centrifuge (Hermle, Wehingen, Germany). The supernatant was decanted and made up to 6 mL with the extraction solvent. The extracts were stored in a freezer at −20 °C for further analysis.

### 2.2. Determination of total anthocyanin content by UV/Vis spectroscopy

Total Anthocyanin Content (TAC) was determined by photometry using a LAMBDA 45 UV/Vis Systems instrument (Perkin Elmer, Waltham, MA). The extracts were warmed up to room temperature, filtered through a 0.45 μm PTFE syringe filter (VWR International, Darmstadt, Germany) and transferred into plastic cuvettes. Absorbance was recorded from 450 to 580 nm in 1 nm steps. TAC was calculated according to the absorption at 535 nm as proposed by various authors [8–10].

For calibration, kuromanin (cyanidin-3-*O*-glucoside) chloride (Extrasynthese, Genay, France) was used at concentrations from 0 to 28 μg $mL^{-1}$. TAC was expressed as kuromanin equivalents (kur-eq) per bran g.

### 2.3. Determination of total anthocyanin content by à côté calibration

For all analyses, HPTLC plates (200 × 100 mm, 200 μm silica gel 60 F254, glass plates; Merck, Darmstadt, Germany) were used. Samples – thawed and filtrated through a 0.45 μm PTFE syringe filter (VWR International, Darmstadt, Germany) – and anthocyanin standards, i.e. kuromanin and myrtillin (delphinidin-3-*O*-glucoside), were applied with an Automatic TLC Sampler 4 (ATS 4, CAMAG, Muttenz, Switzerland), using the following settings for 18 sample tracks per plate: band length 8.0 mm, track distance 10.0 mm, dosage speed 150 nL $s^{-1}$, first application position: 15 mm from the left edge (x-axis), 8 mm from the bottom edge (y-axis); 14 μl of each sample and standard were applied. Scanning densitometry was performed with a TLC Scanner 3 (CAMAG, Muttenz, Switzerland) both directly after sample and standard application using the following settings: scanning speed: 20 mm $s^{-1}$; data resolution: 100 μm per step; slit: 5 × 0.2 mm, micro. All instruments were controlled with VisionCats 2.0 software (CAMAG, Muttenz, Switzerland).

For TAC determination with à côté calibration, kuromanin standards were applied to each plate outside the area required for chromatography (70 mm, y-axis), while the samples were applied at their usual position near the bottom edge (8 mm, y-axis) (Fig. 2). The plates were scanned at 535 nm directly after application of the standards and then again after sample application. Then, the plates

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