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HPTLC quantification of cocoa ingredients and their changes during different chocolate manufacturing steps

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The Institute of Food and Beverage Innovation of the Zurich University of Applied Sciences in Wädenswil deals with delicious, healthy, safe and sustainable food. Besides the education of food technology engineers, practical work with the food industry is fundamental to its strength in innovation. The research group, Food Chemistry analyzes food ingredients using different analytical techniques like HPLC-MS, FT-IR and HPTLC-MS. One of the research objectives is the determination of secondary plant metabolites like alkaloids and polyphenols in food. Monitoring the changes through the entire value chain is of primary importance. In this study, determination of secondary metabolites was performed on fresh cocoa beans and diverse intermediate products of the chocolate production process.

Introduction

Polyphenols are not only known for their astringency but also for their great antioxidant potential. They are found in large amounts in cocoa and its derived product chocolate. The characterization and determination of high molecular oligomeric proanthocyanidins (PA) are of great interest because of their higher antioxidant activity compared to low molecular monomeric flavan-3-ols [1]. The changes of the PA profile are also essential for the organoleptic evaluation, which shows that monomeric PAs are more responsible for the bitter taste in contrast to oligomeric PAs, which are responsible for the astringency. Also of great interest are the two alkaloids caffeine and theobromine, not only because of their bitter taste but also for their stimulating and

"prime pumping" effect. Anthocyanins can be used as general indicator for determining the degree of cocoa fermentation.

In the following application, HPTLC was used to determine oligomeric PAs, alkaloids and anthocyanins through the entire value chain – from fresh cocoa beans, through roasted cocoa, cocoa mass and up to molded chocolate bars. The established method has been proven as a suitable tool for comprehensive compound analysis in laboratories with high sample throughput. Besides the acquisition of the characteristic HPTLC fingerprint for the specific manufacturing step, the visual information can be used to build up an imaging database.

Chromatogram layer

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

Samples

One single batch of 10 kg fresh cocoa beans was studied across different processing steps [2]. Changes in the PA profile were recorded on a lab-scale model and may not be fully in line with real-life crop or industrial scale processing. Nevertheless, one batch was processed and aliquots of about 50 g were sampled from each processing step. Samples were collected from (1) raw fresh cocoa beans, (2) fermented, dried cocoa beans, (3) roasted cocoa mass, (4) 1 h conched cocoa mass, and (5) molded chocolate bars.

Sample preparation

1 g of the fine grinded and defatted cocoa powder was extracted three times with 3 mL acetone – water 1:1. The combined supernatant was diluted 1:10 with acetone – water 1:1.

Standard solutions

Anthocyanin standard solution (0.01 mg/mL in methanol) with cyanidin-3-*O*-arabinoside (cn-ara) und cyanidin-3-*O*-glucoside (cn-glc); alkaloid standard solution (0.2 mg/mL in acetone – water 1:1) with

caffeine and theobromine; PA standard solution (0.1 mg/mL in methanol) with (-)-epicatechin (EC), proanthocyanidins B2 (PA B2) and C1 (PA C1) as well as cinnamtannin A2 (Cinn A2)

Sample application

Bandwise with Automatic TLC Sampler (ATS 4), 15 tracks, band length 8 mm, distance from left edge 20 mm, distance from the lower edge 8 mm, application volume between 5 and 10 µL for standard solutions and 2 and 10 µL for the sample solutions

Chromatography

In the Automatic Developing Chamber (ADC 2) with chamber saturation (with filter paper) for 20 min and after conditioning at 33% relative humidity for 10 min using a saturated solution of magnesium chloride, development with ethyl formate – formic acid – water – toluene 30:4:3:1.5 to the migration distance of 70 mm (from the lower edge), drying for 5 min

Postchromatographic derivatization

The plate was heated at 100 °C and immersed with Chromatogram Immersion Device (immersion speed 5 cm/s, immersion time 0 s) in Fast Blue Salt B reagent (140 mg Fast Blue Salt B in 140 mL methanol, 10 mL water, and 50 mL dichloromethane), followed by 30 s drying in a cold air stream. Alternatively, the Derivatizer can be used.

Documentation

TLC Visualizer under UV 254 nm and white light, also after derivatization

Densitometry

TLC Scanner 4 and *visionCATS*, absorption measurement at 280 nm for alkaloids and 510 nm for anthocyanins and derivatized PA, slit dimension 5.00 x 0.20 mm, scanning speed 50 mm/s, evaluation via peak area, polynomial regression, spectrum recording from 190 to 600 nm

Mass spectrometry

Elution of target zones was done after derivatization with Fast Blue Salt B with the oval elution head (4 x 2 mm) of the TLC-MS Interface. Hereby, acetone – water 1:1 was used as elution solvent at a flow rate of 0.1 mL/min using a HPLC pump. The recording of mass spectra was performed in the positive ionization mode.

Results and discussion

The developed method showed a good separation and a quick quantification of the four oligomeric PAs, the two alkaloids and the two anthocyanins. The present study showed diminishing levels of the alkaloids and especially oligomeric PAs, especially during the fermentation and roasting process. Higher oligomeric PAs are presumed to decrease faster than monomeric PAs. In addition, the alkaloid content decreased throughout the manufacturing, whereas during fermentation, alkaloids may diffuse through the porous surface of the testa cotyledon [3]. During the roasting process, alkaloid content decreased due to diffusion into fat. Anthocyanins are responsible for the reddish coloring of the cotyledon. In general, the content of anthocyanins decreased constantly during all stages of fermentation and drops further during drying. Here, the initial
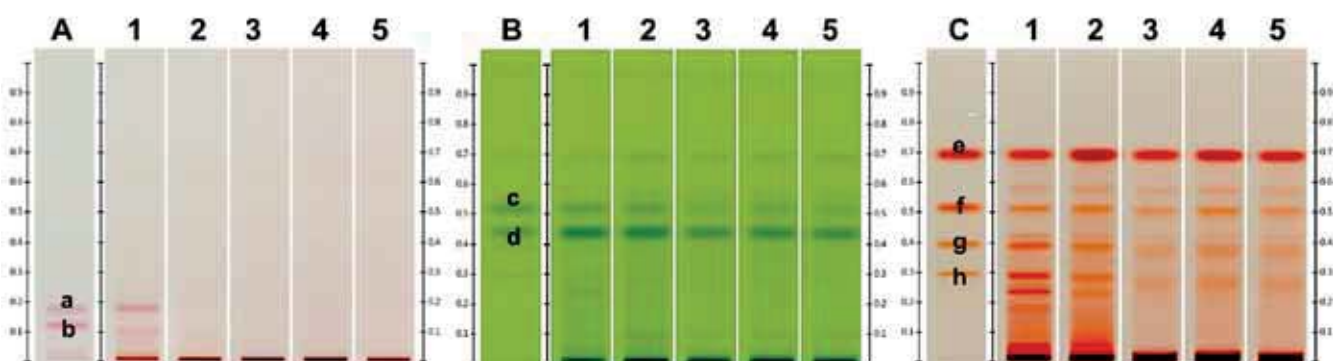

HPTLC chromatogram of different standard mixtures for (A) anthocyanins (a: cn-ara, b: cn-glc) illuminated under white light; (B) alkaloids (c: caffeine and d: theobromine) under UV 254 nm and (C) PAs (e: EC, f: PA B2, g: PA C1 and h: Cinn A2) under white light after derivatization as well as products from different steps of the chocolate manufacturing with 1: raw fresh cocoa beans, 2: fermented, dried cocoa beans, 3: roasted cocoa mass, 4: 1 h conched cocoa mass and 5: molded chocolate bars

content in fresh cocoa beans for cn-ara with 0.81 ± 0.01 mg/g and 0.40 ± 0.01 mg/g for cn-glc dropped drastically below the detection limit after six days of fermentation.

Polyphenol, alkaloid and anthocyanin mean content (mg/g non-fat dry matter, reproducibility inclusive sample preparation, n = 3) for products of five different chocolate manufacturing steps

Content [mg/g]	Caffeine	Theobromine	EC	PA B2	PA C1	Cinn A2
1	7.79 ± 0.70	16.38 ± 4.20	7.09 ± 1.09	2.98 ± 1.21	4.40 ± 0.08	5.06 ± 0.82
2	4.48 ± 0.64	11.12 ± 0.86	5.63 ± 0.45	2.04 ± 0.22	2.37 ± 0.22	2.01 ± 0.22
3	2.89 ± 0.39	7.42 ± 1.37	4.33 ± 0.36	1.72 ± 0.19	1.66 ± 0.19	1.10 ± 0.13
4	3.36 ± 0.19	9.00 ± 1.02	5.00 ± 0.15	2.23 ± 0.12	2.44 ± 0.26	1.35 ± 0.16
5	2.75 ± 0.17	7.41 ± 0.76	4.37 ± 0.04	1.81 ± 0.03	1.50 ± 0.20	0.92 ± 0.12
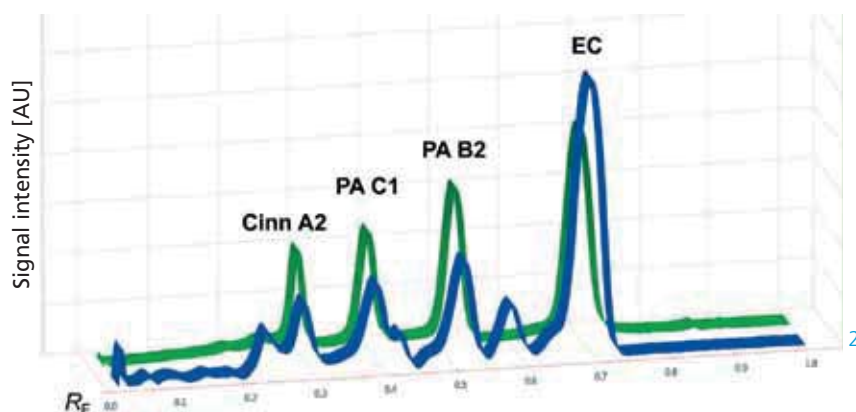
Densitogram of the raw fresh cocoa beans extract (blue) and PA standard solution (green), absorption measurement at 510 nm after derivatization

The quantification allows the determination of valuable ingredients through each specific processing step and can lead to optimization of the chocolate process.

[1] Pedan, V. *et al.* S. Food Res. Int. 89 (2016) 890–900

[2] Pedan, V. *et al.* Food Chem. 214 (2017) 523–532

[3] Timbe, D. *et al.* Food Sci. 43 (1978) 560–562

Further information is available on request from the authors.

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