APPLICATION NOTE

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Methodology for the identification and quantification of human milk oligosaccharides (HMO) by HPTLC

A-139.1

Keywords

HMO human milk oligosaccharides, carbohydrates, polysaccharides, lacto-N-fucopentaose I (LnFP-I), difucosyllactose (DFL), 2'-fucosylactose (2FL), L-fucose, lacto-N-neotetraose (LNnT), lacto-N-tetraose (LNT), lacto-N-triose II, lactulose, para-lacto-N-neohexaose (para-LNnH), D-panose, lactose, glycan

Introduction

HMOs (Human Milk Oligosaccharides) are the general term for complex sugar molecules (oligosaccharides) occurring in human breast milk. They are important components of human milk that promote infant health [1]. HMOs comprise a group of structurally complex, unconjugated glycans that are highly abundant in human milk. HMOs are minimally digested in the gastrointestinal tract and reach the colon intact, where they shape the microbiota [2].

Scope

The proposed methodology describes how HMOs can be analyzed by HPTLC during different production steps (fermentation and finished products). Herein, we demonstrate the quantification of HMOs in different real case samples. Samples are directly applied after a simple dilution step. HMOs, which are lacking a chromophore/fluorophore, are sensitively detected in white light and longwave UV light after derivatization with aniline diphenylamine phosphoric acid reagent.

Required or recommended devices

Automatic TLC Sampler 4 or HPTLC PRO Module APPLICATION, Automatic Developing Chamber ADC 2 or HPTLC PRO Module DEVELOPMENT, optional: HPTLC PRO Module PLATE STORAGE (to run 5 plates autonomously), Derivatizer, TLC Plate Heater 3, TLC Visualizer 2, TLC Scanner 4, *visionCATS* (3.1 or latest version)

NOTE: The presented results are to be regarded as examples only!

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Sample

Liquid samples* (fermentation broth) are prepared at 10% (100 mg/mL) and 1 % (10 mg/mL), and powdered samples* (finished products) are prepared at 1% (10 mg/mL) and 0.05% (0.5 mg/mL) in acetonitrile-water 1:1 (*V/V*).

Standards

Standards* are prepared at different concentration levels, depending on the analytical task, *e.g.* for identity testing at a final concentration of 0.20 mg/mL in acetonitrile-water 1:1 (*V/V*); for quantification in the range of 0.05-0.50 mg/mL. A mixture of para-lacto-N-neohexaose (para-LNnH), lacto-N-triose (LNT), and lactose (0.2 mg/mL, each) in acetonitrile-water 1:1 (*V/V*) can be applied as System Suitability Test (SST) on each plate.

Chromatography

Stationary phase	HPTLC Si 60 F_{254}, 20 x 10 cm (Merck)
Sample application	Application as 8.0 mm bands with ATS 4 or HPTLC PRO Module APPLICATION (settings for ethanolic solutions), 15 tracks, track distance 11.4 mm, distance from left edge 20.0 mm, distance from lower edge 8.0 mm, application volume of 2.0 µL for sample and standard solutions (for quantitation individual standards at different concentration levels)
Developing solvent	Ethyl acetate, methanol, water, acetic acid 50:40:10:2 (*V/V*)
Development	In the ADC 2 **without chamber saturation** or in the HPTLC PRO Module DEVELOPMENT, both after activation at 33% relative humidity (saturated solution of magnesium chloride) for 10 min.
Developing distance	70 mm (from the lower edge)
Plate drying	Drying 5 min in the ADC 2 or in the HPTLC PRO Module DEVELOPMENT
Documentation	With the TLC Visualizer 2 in UV 366 nm and white light after derivatization with ADPA reagent.
Densitometry	Densitometric analyses are performed in a) absorbance mode at 512 nm (tungsten lamp), slit dimension 5.0 x 0.2 mm, scanning speed 20 mm/s, data resolution: 25 µm/step b) (requires adjustment of the applied concentrations) fluorescence mode with the TLC Scanner 4 at 366/>400 nm (mercury lamp), slit dimension 5.0 x 0.3 mm, scanning speed 20 mm/s, data resolution: 25 µm/step

Derivatization	Aniline diphenylamine phosphoric acid (ADPA) reagent Preparation: 0.2 g of diphenylamine and 0.2 mL of aniline are dissolved in 8.0 mL of methanol, 1.0 mL of o-phosphoric acid (85%) are added. The mixture is shaken until any precipitate is dissolved, and then another 1.0 mL of methanol is added. Use: Derivatize (Derivatizer: 3 mL, yellow nozzle, spraying level 6), heat the plate at 110°C for 10 min.

Results

SST in the ADC 2:

A zone at R_F ~ 0.10 (para-LNnH), R_F ~ 0.26 (LNT), and R_F ~ 0.53 (lactose) (in UV 366 nm or white light after derivatization)

SST in the Module DEVELOPMENT:

A zone at R_F ~ 0.09 (para-LNnH), R_F ~ 0.22 (LNT), and R_F ~ 0.44 (lactose) (in UV 366 nm or white light after derivatization)

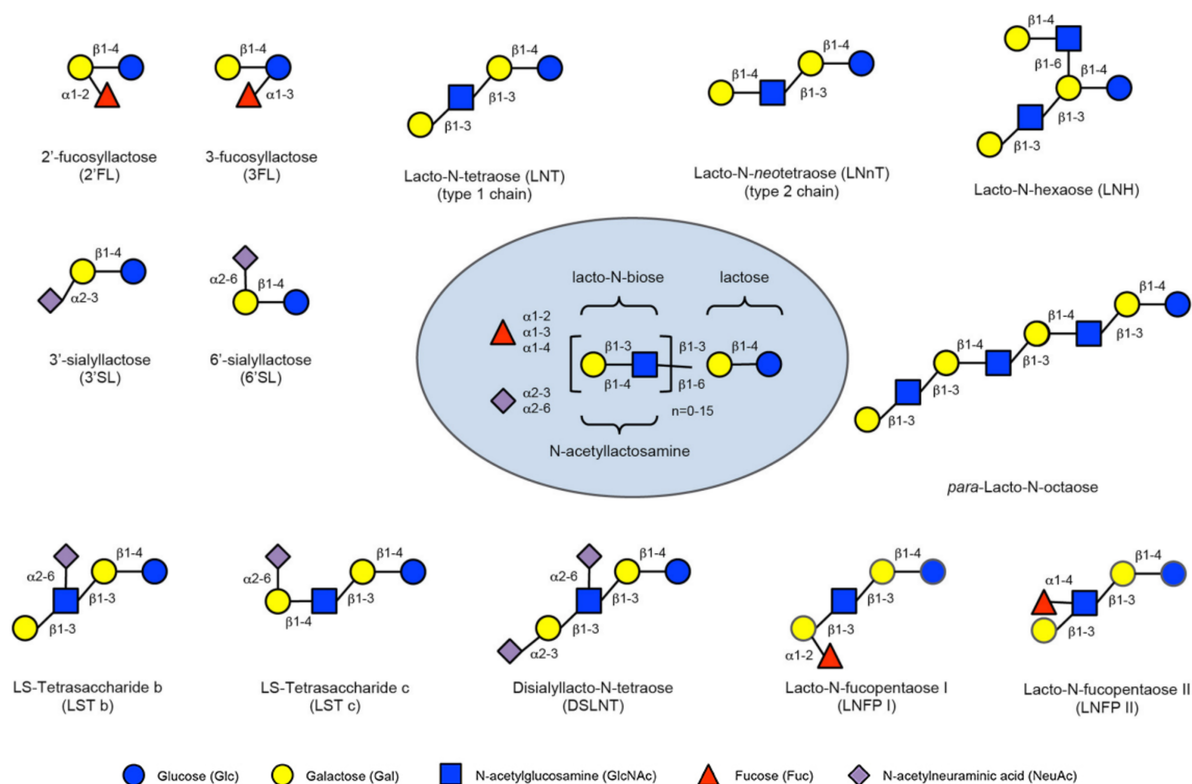

Figure 1: Composition of HMOs (composition blue print) [2]

In the following chromatograms real case samples are shown. The samples (fermentation broth and finished products) have been analyzed. For quantification of the major (2FL) and minor (lactose) HMO in one of the finished product, the samples were applied at different concentration levels (powdered sample at 1.00 and 0.05% in acetonitrile-water 1:1 (*V/V*)

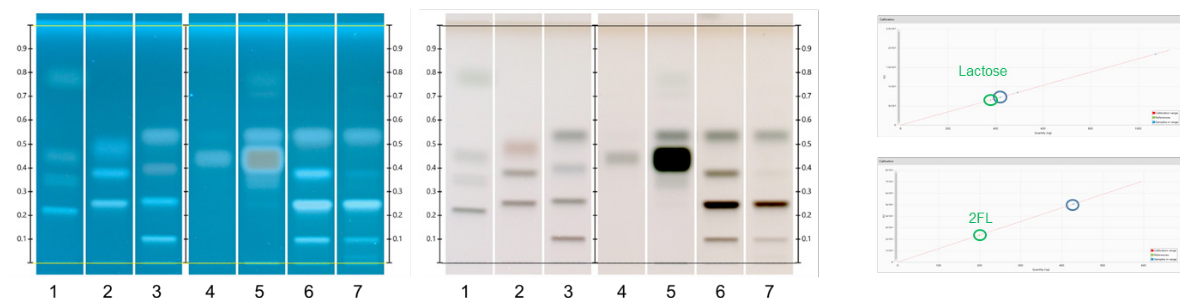

Figure 2: HPTLC chromatograms at UV 366 nm (left) and in white light (middle) after derivatization with ADPA reagent; HPTLC chromatograms of standards and three selected samples after derivatization with ADPA reagent under UV 366 nm (left) and under white light (right); Track 1: LNFP-I, DFL, 2FL, track 2: LNnT, lacto-N-triose II, lactulose, track 3: para-LNnH, LNT, D-panose, lactose with increasing R_F values, track 4: HMO sample 1 (finished product) at 0.05% (application volume 1 µL, absolute amount on plate: 0.5 µg), track 5: HMO sample 1 at 1% (application volume 1 µL, absolute amount on plate: 10.0 µg), track 6: HMO sample 5 (finished product) at 0.2% (application volume 2 µL, absolute amount on plate: 4 µg), track 7: HMO sample 8 (fermentation) at 1% (application volume 4 µL, absolute amount on plate: 40.0 µg); contrast 2.0 for both detection modes; and calibration curves (right) for lactose and 2FL (scan at 512 nm, single-level calibration, evaluation by peak area, linear-1, shown results have been obtained after chromatography in the ADC 2)

Area calibration for substance Lactose @ 512 nm:

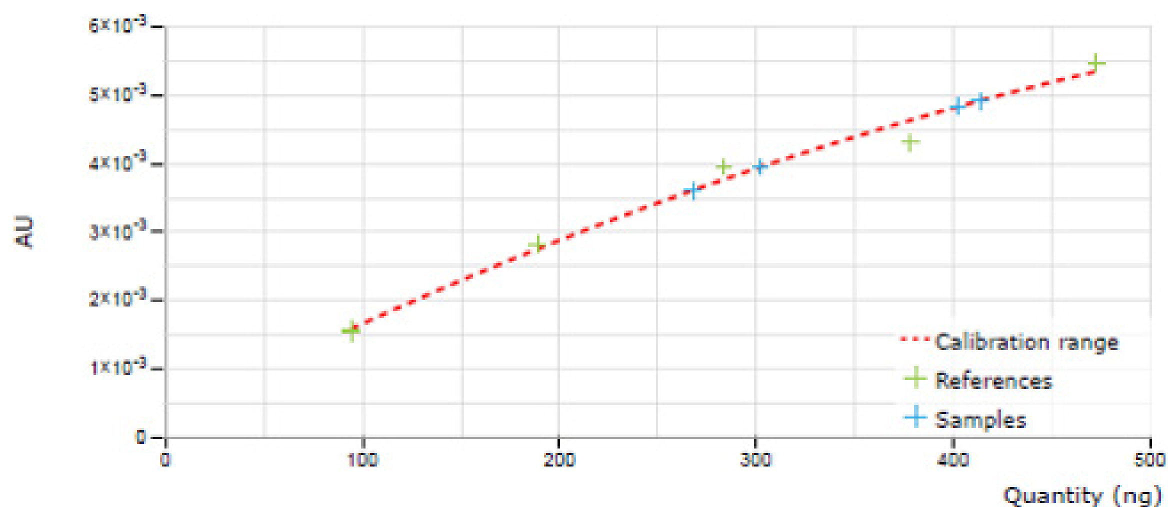

Figure 3: Calibration curve for lactose standards from 0.05-0.50 mg/mL and selected samples (scan at 512 nm, multi-level calibration, evaluation by peak area, polynomial regression)

HPTLC is well suited for in-process control during fermentation, monitoring of purification steps, and QC of finished products like HMOs. All production cycles can be followed by using the same methodology. Quantitation can be done by single-level or multi-level calibration.

For further information send an email to request@camag.com.

Literature

[1] Lars Bode (2009) Nutrition Reviews, Volume 67, Issue suppl_2, 1 pp. S183–S191. doi: 10.1111/j.1753-4887.2009.00239.x

[2] Vassilis Triantis *et al.* (2018). Front. Pediatr. 6:190. doi: 10.3389/fped.2018.00190

Acknowledgment

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