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Fermentation monitoring based on HPTLC-OPLC technique: the effect of a complex biological matrix on quantification performances

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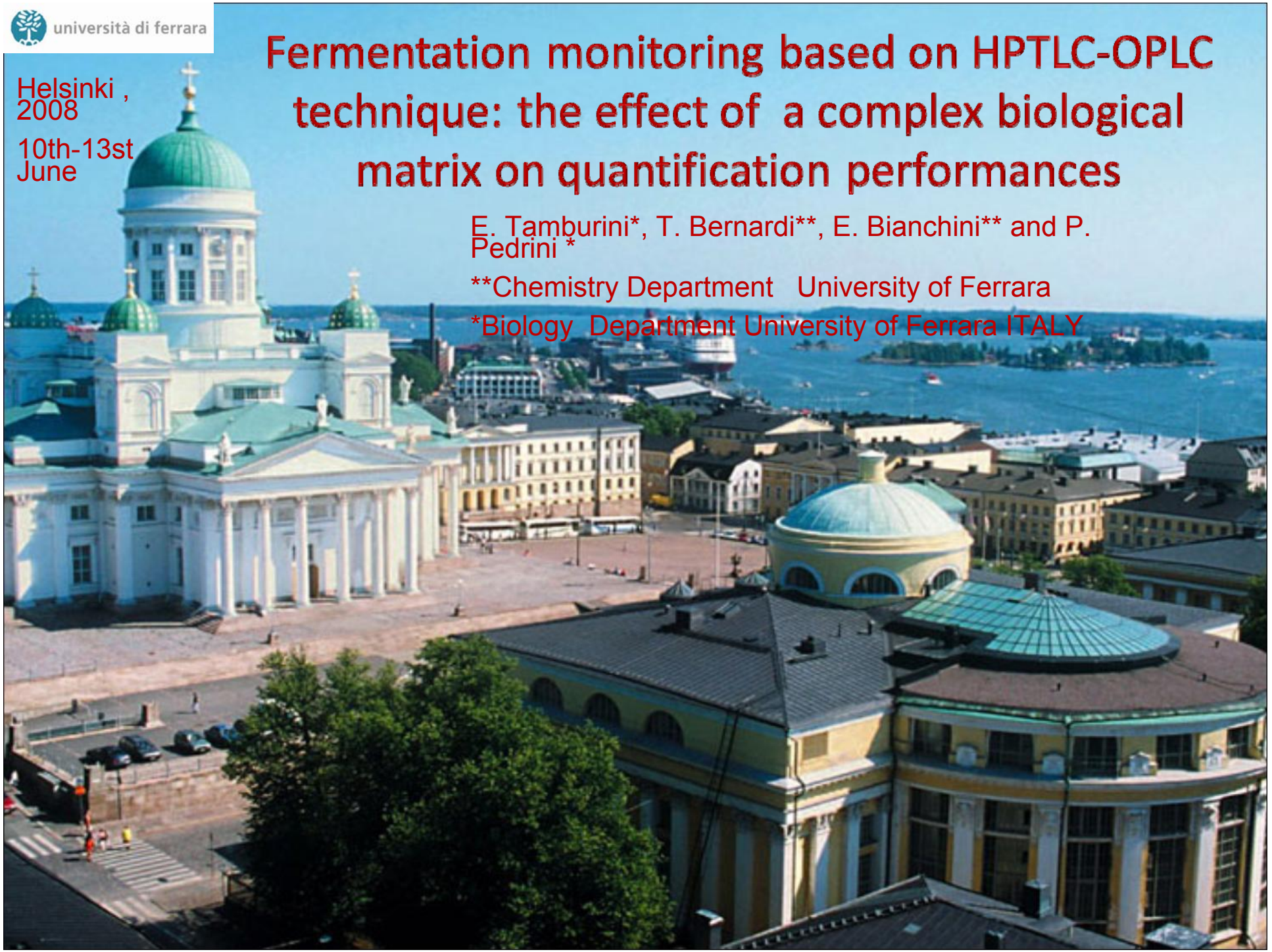

Which application for?

We applied HPTLC – OPLC technique for fermentation processes.

Fermentation broth is a complex mixture of microbial cells, nutrients and products/by-products.

To establish a hierarchy of sugars utilization in microbial metabolism we can monitor the concentration and depletion of single carbohydrates during the fermentation

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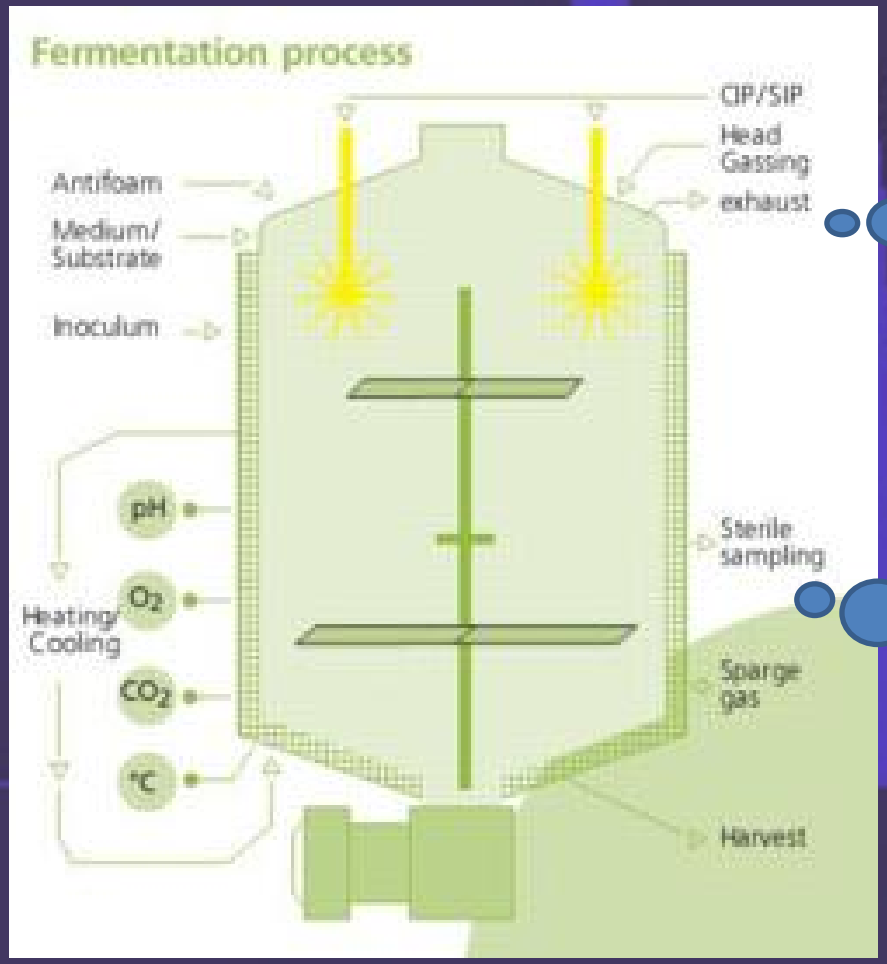
Knowledge of consumer (microbes)-resource (sugars) relationship

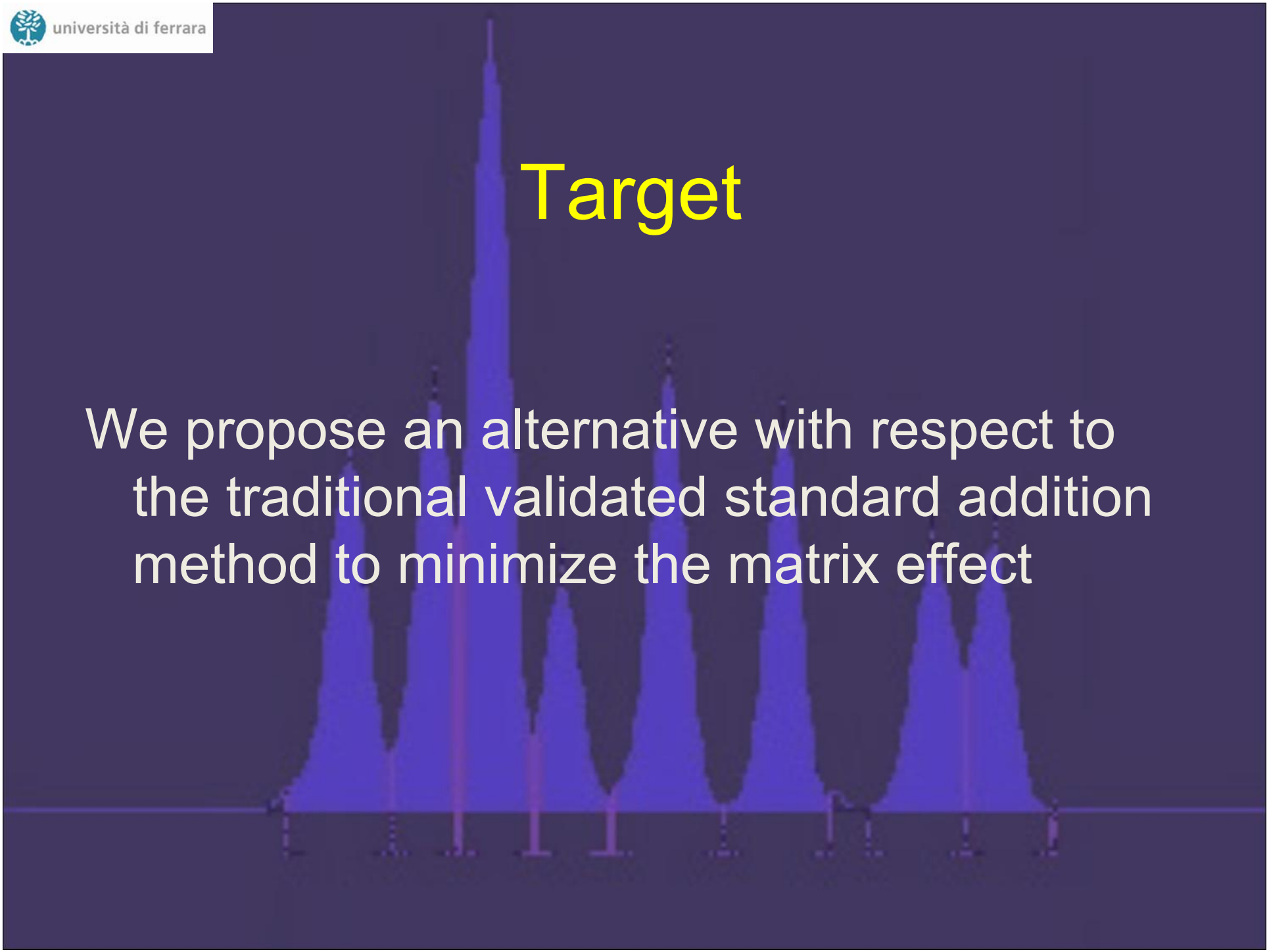


Bifidobacterium adolescentis MB239 as probiotics

Glucose, Fructose, Galactose, Lactose, Raffinose, 1-Kestose, F.Nystose, Sucrose as prebiotics

Target

We propose an alternative with respect to the traditional validated standard addition method to minimize the matrix effect



Chromatography conditions and derivatization

Separation optimized was performed on 20 × 20 cm aluminium foil-backed silica gel .

Samples were applied as 3 mm bands 15 mm from the bottom of the plate and 20 mm apart.

Plates were developed with acetonitrile-water 85:15 (v/v), external pressure 50 bar, mobile phase volume 10000 µl, flow rate 300 µl/min and rapid volume 300 µl

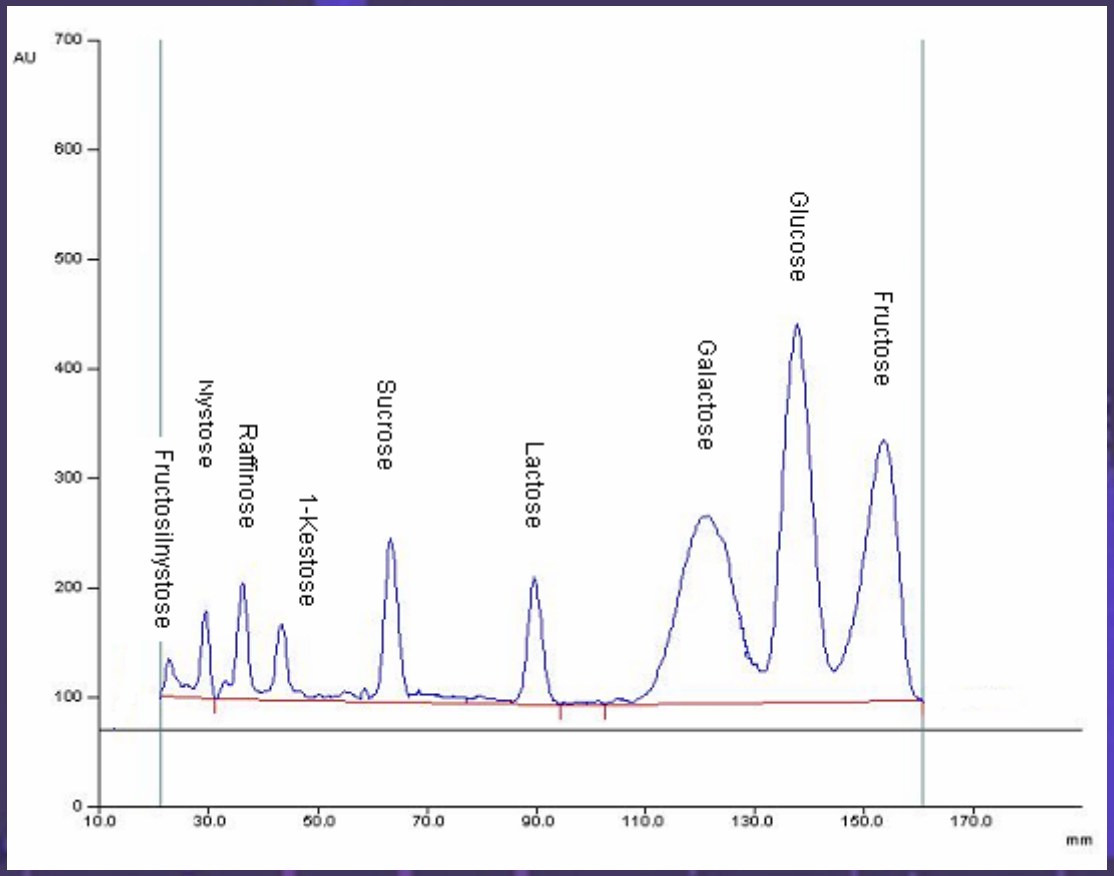
Elution time under these conditions was 2010 sec.

The plates were scanned in fluorescence mode with Hg lamp 313 nm after bands derivatization with diclorofluorescein

Samples

The samples were collected from *B.adolescentis* MB 239 fermentations every 2-3 hours, centrifuged, filtrated (syringe filter 0,22 µm) to avoid time-dependent changes in analytes concentration resulting from continued metabolism, and immediately chilled at 4°C.
Carbohydrates were analyzed in the supernatants, *AFTER ONLY SIMPLE DILUTION*

Densitogram



Matrix effect

The matrix effect may originate from the competition between the analyte and the co-eluting; the undetected matrix components could affect both the retention behaviour , the baseline and the detection response of the analyte.

This effect could compromise the PRECISION, SELECTIVITY and SENSITIVITY of the analysis

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Evaluation of matrix influence on calibration performance

Proteins

Salts

Free aminoacids

Metabolites from cells life

Peptides

Assessment of matrix effect

Calibration curves were validated by a working range and a linearity for each analytes on the basis of the calibration plot (peak area vs concentration)

Calibration curves were reported with correlation coefficient, slope, y-intercept and their confidence limits.

Success of the calibration curve critically depends upon *HOW* CLOSELY the matrix of the standard *RESEMBLES THAT* of the sample that is to be analyzed.

So we have decided to compare three regression models:

AQ = std solutions diluted in water-acetone 2:1

MX_D = std solutions in broth, diluted as AQ solutions in water-acetone 2:1

MX_C = std solutions in broth, diluted as AQ solutions but in broth

Subs c	Substan	y-intercept					
		AQ	±tsa	MXD	±tsa	MXC	±tsa
Fruc	Fructose	228,80	13,14	189,50	18,84	8,77	88,31
Gluc	Glucose	379,00	35,06	565,00	15,141	820,00	89,11
Gala	Galactos	538,00	11,31	392,00	24,94	375,00	84,51
Sucr	Sucrose	45,87	17,76	102,00	88,58	489,10	72,03
Lact	Lactose	742,00	72,60	559,00	27,51	386,00	65,21
1	1-	577,60	86,53	525,60	68,70	826,30	105,95
Raffi	Raffinose	410,20	78,56	394,40	11,30	344,30	22,36
Nyst	Nystose	89,18	17,17	83,66	27,86	174,30	7,19
F.nystose	F.nystose	246,50	42,79	237,70	38,90	65,17	10,11
	F.nystose	10-50		46,48		42,25	10,99
							0,34

AQ = std solutions in water-acetone 2:1
MXD = std solutions in broth diluted, as AQ solutions, in water-acetone 2:1
MXC = std solutions in broth diluted, as AQ solutions, but in broth

Comparisons between regression lines were performed by means of a t-test on lines slopes (α= 0,0025, degrees of freedom n1 + n2 – 4).

Substance	t-values (t = 2,306)	
	AQ/MX$_D$	AQ/MX$_C$
Fructose	1,410	19,068
Glucose	0,366	52,665
Galactose	0,095	27,158
Sucrose	1,490	30,990
Lactose	0,216	15,464
1-Kestose	0,708	12,381
Raffinose	0,281	25,346
Nystose	1,220	17,902
F.nystose	1,859	22,498

AQ = std solutions in water-acetone 2:1
MX$_D$ = std solutions in broth diluted, as AQ solutions, in water-acetone 2:1
MX$_C$ = std solutions in broth diluted, as AQ solutions, but in broth

What considerations can we make?

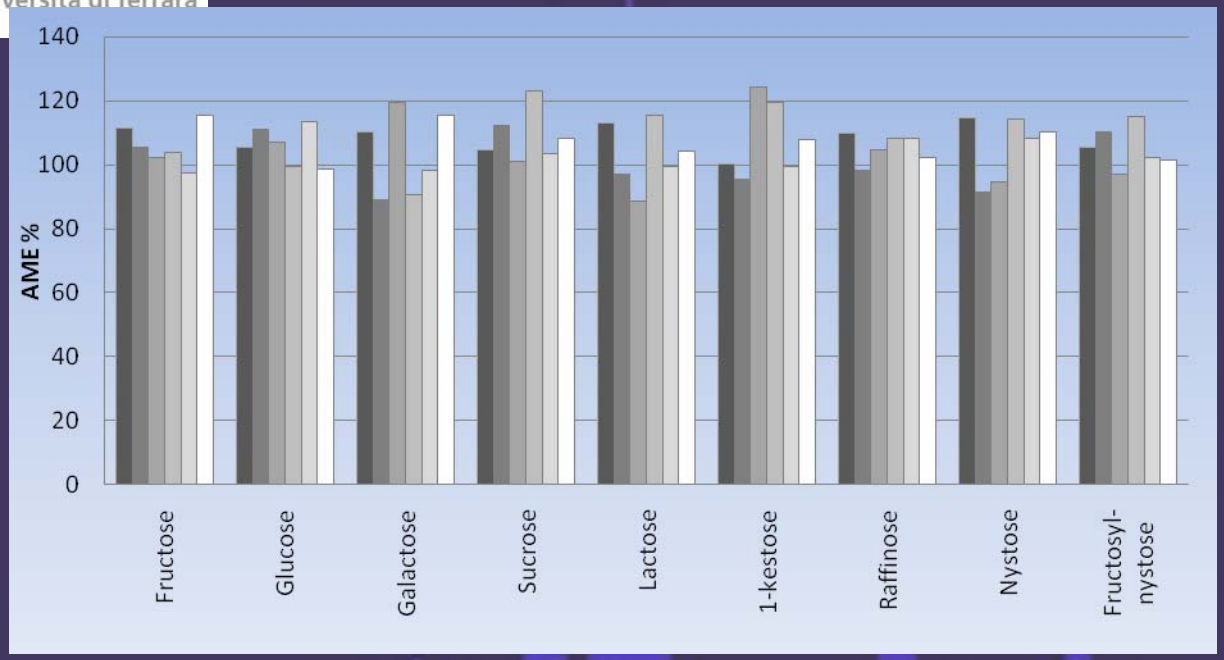
- The t-test points out a significant difference on the lines slopes in the case of the relevant matrix effect (MXc), while confirms the absence of interferences in the case of MX_D solutions.

Absolute matrix effect

AME = standard peak area in the presence of the matrix

$$\text{AME \%} = \frac{\text{std peak area in the presence of the matrix}}{\text{std peak area in the presence of water/acetone}}$$

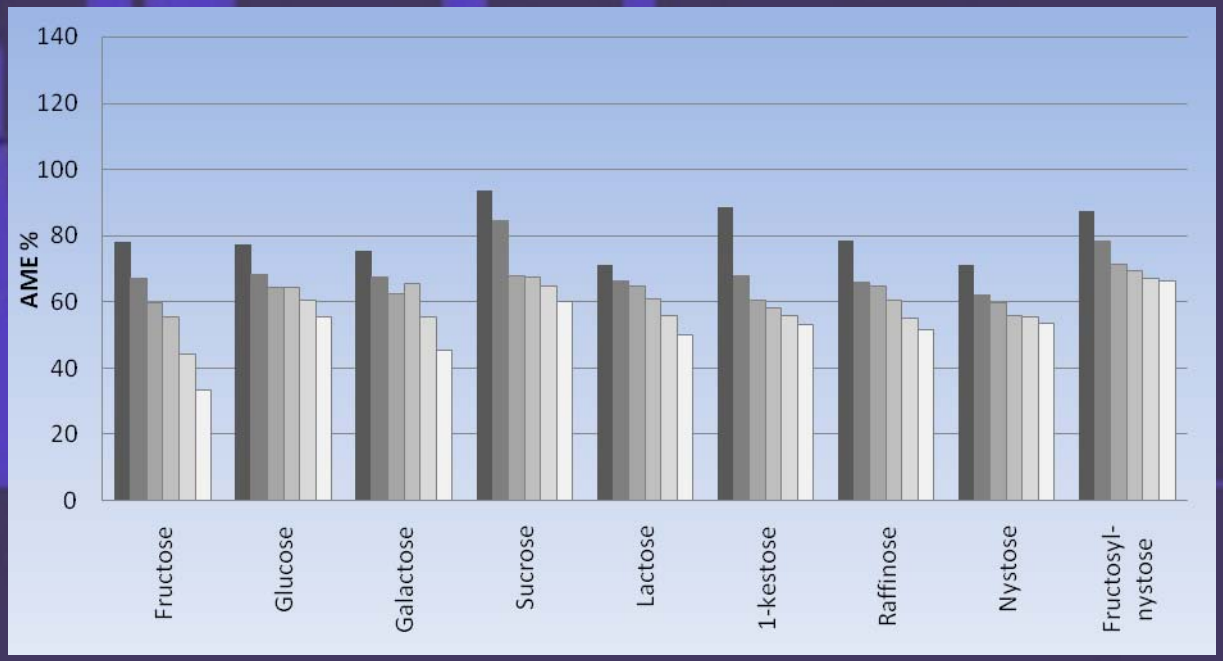
B.K. Matuszewski, M.L. Constanzer, and C.M. Chavez-Eng "*Strategies for the assesment of matrix effect in quantitative bioanalytical methods based on HPLC-MS-/MS"* Anal. Chem. 2003, 75, 3010-3030



The analytes were at a concentration from 20 to 200 ppm

AQ vs MXD

AQ vs MXC



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What considerations can we make?

- The t-test point out a significant differences on the lines slopes in the case of the rilevant matrix effect (MXc), while confirming the absence of interferences in the first two cases (AQ and MXD).

- The AME% values for MXD are all distributed around 100% and there aren't the same results for MXC, where all the responses are suppressed in a progressive manner.

Quantitative assesment of the method

For this reason calibration models as AQ and MX$_D$ for each analytes were constructed.

The working range, correlation coefficient, the estimated limit of detection LOD, and the limit of quantification LOQ were calculated.

The precision as coefficient of variation CV% was determined for replicated (n=6) applications of samples in one plate.

Substance	Correlation coefficient		Upper limit of linearity (ng)		LOD (ng)		LOQ (ng)		CV %	
	AQ	MXD	AQ	MXD	AQ	MXD	AQ	MXD	AQ	MXD
Fructose	0,99	0,99	200	200	9,61	11,0	19,03	18,69	4,87	5,01
Glucose	0,99	0,99	200	200	15,20	6,48	20,65	21,60	4,02	4,55
Galactose	0,99	0,99	200	200	4,91	10,8	16,37	15,99	4,24	3,99
Sucrose	0,99	0,99	200	200	18,55	7,33	30,83	24,43	6,22	5,78
Lactose	0,99	0,99	200	200	5,88	22,0	25,60	28,38	6,35	6,55
1-Kestose	0,99	0,99	70	70	5,01	4,09	16,71	13,63	3,54	2,98
Raffinose	0,99	0,99	200	200	7,98	11,6	26,60	18,69	5,98	5,55
Nystose	0,99	0,99	70	70	2,90	5,25	9,65	17,49	4,00	4,56
F.nystose	0,990	0,989	50	50	3,54	3,53	11,78	11,78	5,77	6,00

AQ = std solutions in water-acetone 2:1
MXD = std solutions in broth diluted, as AQ solutions, in water-acetone 2:1

Conclusions

The strategies proposed would like to be an alternative procedure with respect to the standard addition method when samples from fermentation processes have to be analyzed.

This IMPLIES that calibration curves can be built in water/acetone for a valid quantitative prediction of carbohydrates in a conveniently diluted matrix.

Our perspectives

OPLC technique represents a valid contribution towards the understanding of the metabolic behaviour of Bifidobacteria and their interactions with important prebiotics such as Fructo-oligosaccharides.

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