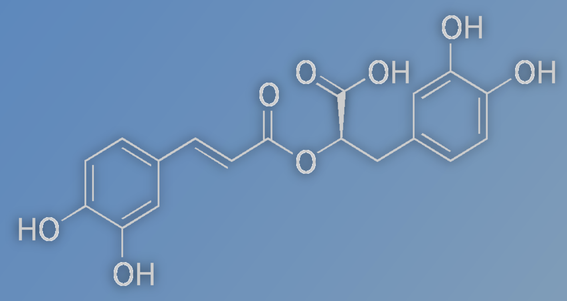
MULAS S.,
PICCINI L.,
MULINACCI N.,
CORAN S.A.

Dipartimento di Scienze Farmaceutiche,
Università di Firenze,
via Ugo Schiff 6,
50019 Sesto Fiorentino
(Florence) Italy

CRUCIAL ASPECTS OF QUANTITATIVE VALIDATION

DETERMINATION OF ROSMARINIC ACID



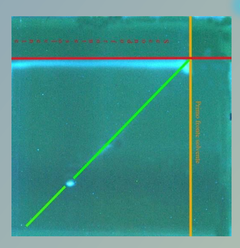
Rosmarinic acid (RA), a natural phenolic compound found in many Lamiaceae herbs, is known for having a number of interesting biological activities, e.g. antiviral, antibacterial, antiinflammatory , antioxidant and moreover for its effects on Alzheimer's Disease [1-2].
The main source of this compound is Rosmarinus officinalis L.. However reports have been published on the TLC determination of RA in a variety of herbal extracts [3-7], but none provided reliable quantitative results as the proposed methods are impaired by some methodological weakness.
Our work is focused on the analytical aspects of HPTLC quantitative validation. Here we present the pre-validation procedure, the linearity claiming and the calibration matrix effect as focal points in developing a validated HPTLC method.
The method was validated giving rise to a dependable and high throughput procedure well suited to routine application. RA was quantified in the range of 132 - 660 ng with RSD of repeatability and intermediate precision not exceeding 2.0% and accuracy inside the acceptance limits. The method was tested on several commercial preparations containing RA in different amount.

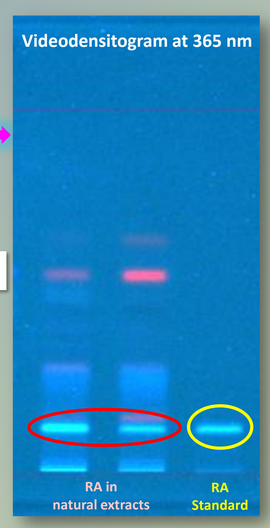
Stationary phase	
material	HPTLC Lichrospher Si 60 F 254s
manifacturer	Merck KGaA
batch	HX754450
pre-washing	Methanol dipping
drying device	120°C for 30 min.
Linomat 5 application parameters	
spray gas	N_2
dosage speed	60 nL/s
pre dosage vol.	0.5 µL
pressure of spraying	1 bar
syringe size	100 µL
number of tracks	15
application position Y	10.0 mm
first application position X	15.0 mm
band lenght	7.0 mm
Development-ADC2	
chamber type	ADC2
pre-drying	enable
humidity control	10 min ($MgCl_2$ saturated solution)
tank saturation	10 min with mobile phase
plate preconditioning time	10 min
mobile phase	toluene ethylformiate HCOOH 6:4:1
migration distance	80 mm
drying time	10 min
Detection-Scanner3	
slit dimension	5.00 x 045 mm
scanning speed	10 mm/s
data resolution	100 µm/step
wavelength	330 nm

HPTLC METHOD

Optimized Parameters of Method

STABILITY

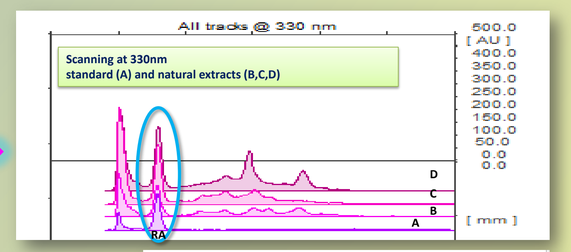




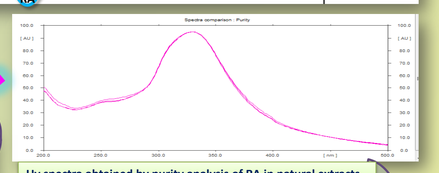
Separation of rosmarinic acid on HPTLC plate by Camag ADC2

SELECTIVITY

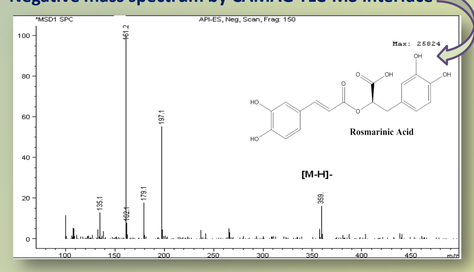
Densitogram



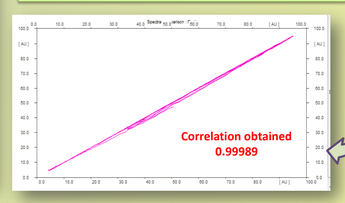
Peak purity



Negative mass spectrum by CAMAG TLC-MS interface



Uv spectra obtained by purity analysis of RA in natural extracts



PRE-VALIDATION AND VALIDATION

Validation is a requirement to demonstrate the reliability and the suitability of a quantitative method, integrated in the development process. In the last years some papers dealing with TLC validation have been published [9,10]; notwithstanding two critical steps are to be stressed especially in herbal drug analysis:

LINEARITY AND MATRIX EFFECT

Here we report on the usefulness of the pre-validation step based on the accuracy profiles.

Pre-validation is a procedure proposed by Société Francaise des Sciences et Techniques Pharmaceutiques (SFSTP) on the basis of regulatory guidelines, aiming to identify the model to use for the calibration curves and evaluate the matrix effects before designing the 'validation' phase.

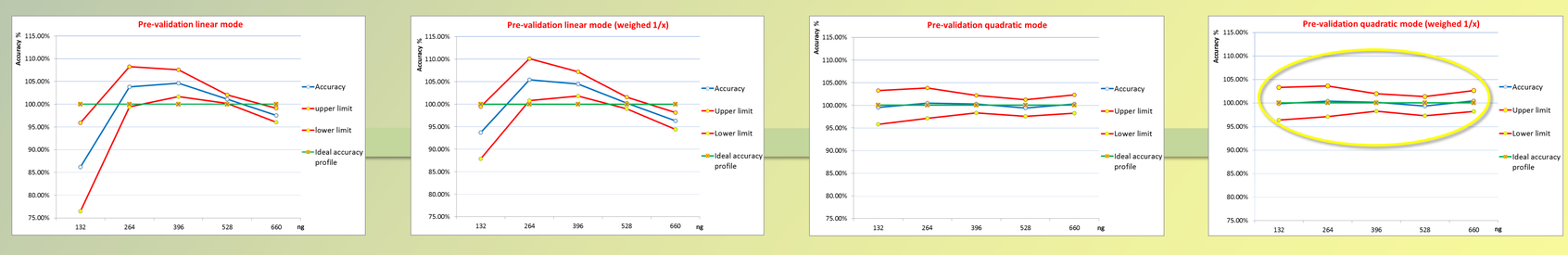
Total error / confidence interval

Bias+ Precision / $LC_b = \bar{b} \pm t_{Risk}^{N-p} \sqrt{S_b^2}$

Selection of the right calibration model

Five calibration levels were obtained in triplicate on three different days over a range of 132 - 660 ng of rosmarinic acid. Four regression functions were calculated:
linear model, weighed linear (1/x) model, quadratic model and weighed (1/x) quadratic model.
The bias, the repeatability and the intermediate precision were back-calculated for each level using the four regression models.

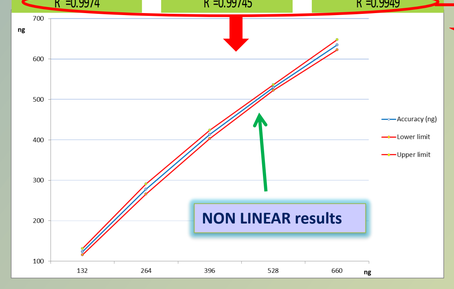
Accuracy profiles



Linearity

LINEAR signal response function

Day 1	Day 2	Day 3
Linear Fit: y=a+bx	Linear Fit: y=a+bx	Linear Fit: y=a+bx
Coefficient Data:	Coefficient Data:	Coefficient Data:
a = 60.43	a = 178.90	a = 245.34
b = 10.8	b = 10.80	b = 10.25
R^2=0.9974	R^2=0.99745	R^2=0.9949

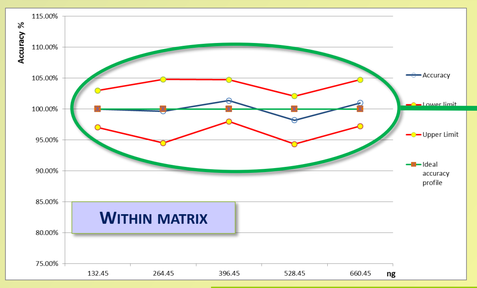


NON LINEAR signal response function

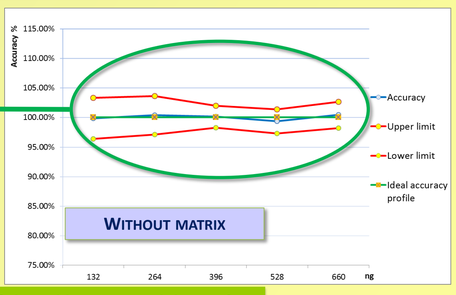
Day 1	Day 2	Day 3
Quadratic Fit: y=a+bx+cx^2	Quadratic Fit: y=a+bx+cx^2	Quadratic Fit: y=a+bx+cx^2
Coefficient Data:	Coefficient Data:	Coefficient Data:
a = -519.9503	a = -400.20452	a = -583.60734
b = 14.5690	b = 14.55804	b = 15.63843
c = -0.0048	c = -0.00475	c = -0.00680
R^2=0.99977	R^2=0.99978	R^2=0.99956

Matrix effect evaluation

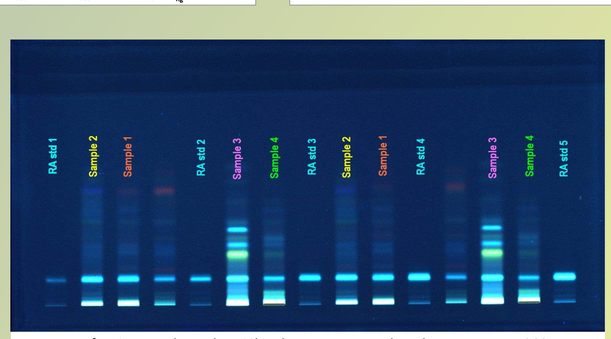
A possible matrix effect must be taken into account.
No blank matrix being available, the method of standard addition was used.



Accuracy profiles



Calibration data are in agreement . The validity of the calibration model out of matrix is then assessed



Assay of RA in natural samples. LiChrosher F_{254s} D: natural product reagent, UV 366 nm

Assay of RA in real samples

Sample	ng found (n=6)	RSD
Leaves of one year old (1)	431.25	1.83
Leaves of the current year (2)	488.04	1.84
Hydroalcholic extract A (3)	317.60✖	1.31
Hydroalcholic extract B (4)	326.43✖	0.7
Rosmarino ERBAVITA® capsules	756.01*	6.0
Salvia ERBAVITA® capsules	370.43*	6.7
Oleoresin GIOTTI®	450.07*	2.0

✖ referred to 1 mL of commercial sample
*referred to 100 mg of powder

References
[1] M. Petersen, M.S. Simmonds, Phytochemistry 62 (2003) 121.
[2] T. Hamaguchi, K. Ono, A. Murase, M. Yamada, Am. J. Pathol. 175 (2009) 2557.
[4] I. Bros, M.-L. Soran, R.D. Briciu, S.C. Cobzac, J. Planar Chromatogr. 22 (2009) 25.
[5] I. Fecka, D. Raj, M. Krauze-Baranowska, Chromatographia 66 (2007) 87.
[6] J. Nowakowska, J. Planar Chromatogr. 17 (2004) 149.
[7] G. Janicsak, I. Mathe, Acta Pharm. Hung. 68 (1998) 259.
[8] I. Fecka, A. Kowalczyk, W. Cisowski, J. Planar Chromatogr. 17 (2004) 22.
[9] K. Ferenczi-Fodor, B. Renger, Z. Vegh, J. Planar Chromatogr. Mod. TLC 23 (2010) 173.
[10] B. Renger, Z. Vegh, K. Ferenczi-Fodor, J. Chromatogr. A 1218 (2011) 2712.

Acknowledgements
The authors are grateful to Dr. Sandra Gallori for her graphic design of the poster

Grafica S. Gallori
sandra.gallori@unifi.it

P10d