HPTLC

Quantification validation et robustesse,

Quelques RAPPELS

7 JUILLET 2022
MONTPELLIER

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"Far better an approximate answer to the right question than an exact answer to the wrong one"

John TUKEY (thanks to Gerda MORLOCK for this suitable quote)

Quelques Rappels:

CCM HPTLC différences similitudes

Résolution Sensibilité Sélectivité

Qualitatif – quantitatif validation

Limite de quantification

Gamme de travail & linéarité

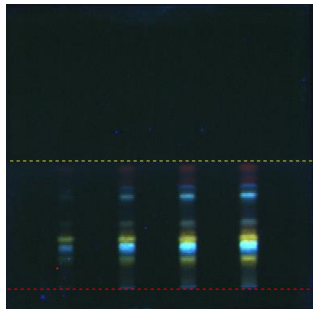
Robustesse / validations déjà publiées

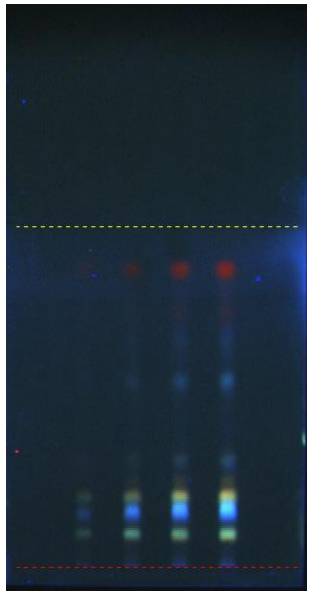
CCM HPTLC différences similitudes

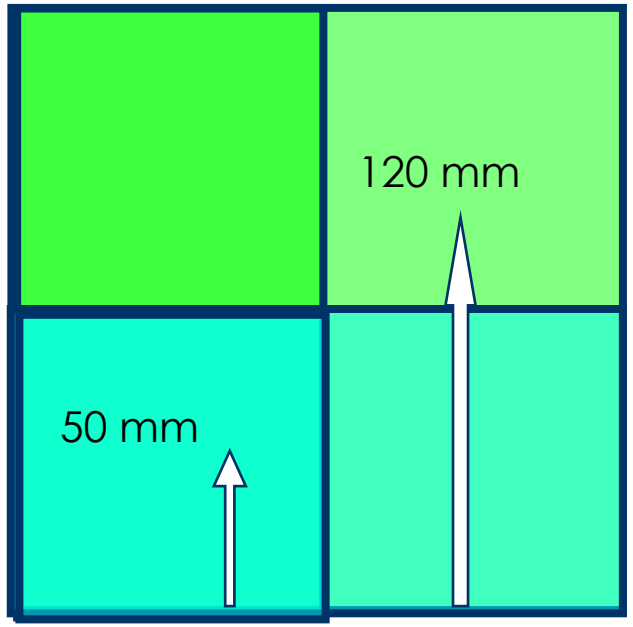
Épaisseur, taille et granulométrie ≠

Porosité identique

Seule l'HPTLC est quantitative
+ 5'/30' & 5mL/20mL
(aubépine)







Résolution Sensibilité Sélectivité

très peu de plateaux
sensibilité intrinsèque faible
MAIS quelle est la RÉALITÉ ?

HTC' P.SCHOENMAKERS A.MAKAROV P.SANDRA

RÉVÉLATION ⟹ ABSOLUMENT **TOUT VOIR**
SÉLECTIVITÉ ⟹ VOIR CE **QU'ON VEUT**
VOLUMES ⟹ OBTENIR DE LA **SENSIBILITÉ**

Qualitatif – quantitatif / validation

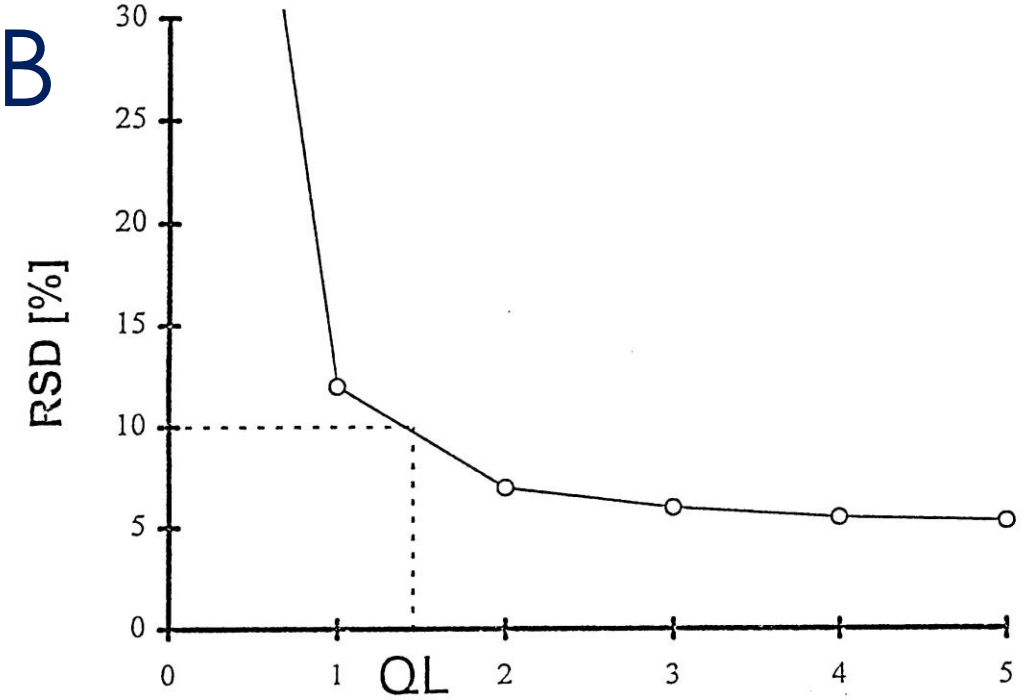
Il est simplement possible de valider une méthode pour garantir qu'elle est adaptée à l'objectif et qu'elle permet effectivement de répondre correctement à la question posée :
DOSAGE, ESSAI LIMITE ou IDENTIFICATION

ATTENTION AU TERME SEMI-QUANTITATIF!

Limite de quantification

Seule donnée valable
A déterminer comme décrit dans Eurachem entre autres
Se méfier du rapport S/B

Déposer des quantités décroissantes d'analytes avec la matrice (n ≥ 6) et determiner la plus petite quantité(pas concentration)permettant d'obtenir une RSD% acceptable.
En général inférieure à 5%.



Gamme de travail & linéarité

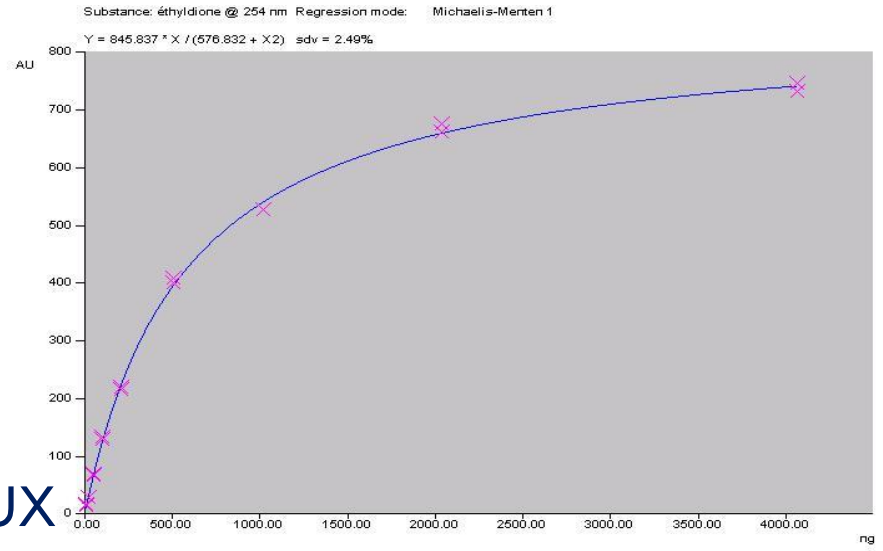
Détection>LOQ>gamme de travail

Il est primordial de suivre cette chronologie pour laquelle le choix de la sélectivité de détection est importante au départ. Toujours conserver la même gamme de référence, et déposer les échantillons dans cette gamme.

Linéarité

Tout le monde est maintenant censé ne plus faire la confusion entre la linéarité de la méthode et celle de la réponse d'un détecteur spécifique.

Sur plaque la détection se fait en **phase solide** et donc répond à des critères spécifiques (voir Prof.S.EBEL).
La réponse est linéaire sur un gamme étroite ou en fluorescence. Mais pas sur une gamme x10 ou x 100.



Choisir la fonction qui correspond le mieux

MAIS … ATTENTION
À l'incertitude sur les points de calibration !

Quelques illustrations:

Validation qualitative

Stéviosides dans les yaourts

Dextrines dans la pâte à pain

Détection d'activité antiradicalaire

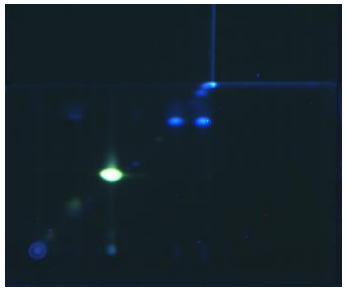
Ce qui est important

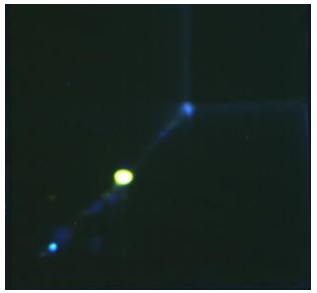
Validation qualitative

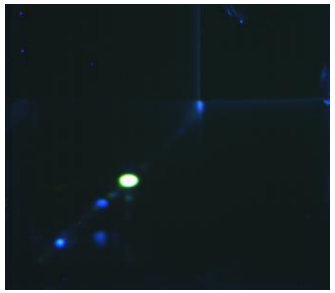
Exemple de test à réaliser

UTILISER UNE MIGRATION BIDIMENSIONNELLE afin de valider la stabilité des constituants sur la plaque (sinon retirer le MeOH, prendre une silice greffée ou tremper dans du BHT).

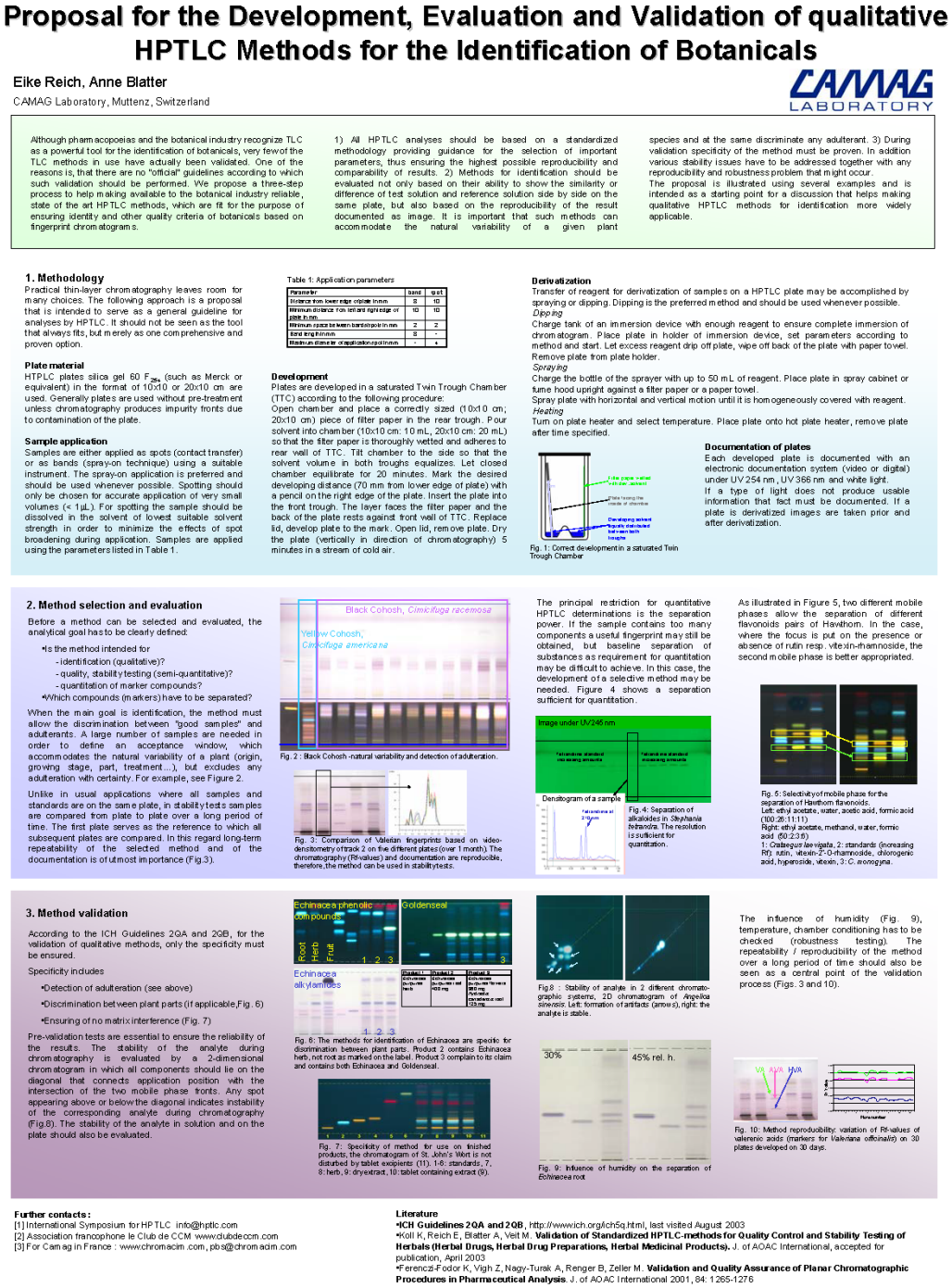
Ci-dessous *Hydrastis* avec CH Ph CAMAG et US Ph







C'est important



Proposal for the Development, Evaluation and Validation of qualitative HPTLC Methods for the Identification of Botanicals

Eike Reich, Anne Blatter

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CAMAG LABORATORY

Although pharmacopoeias and the botanical industry recognize TLC as a powerful tool for the identification of botanicals, very few of the TLC methods in use have actually been validated. One of the reasons is, that there are no "official" guidelines according to which such validation should be performed. We propose a three-step process to help making available to the botanical industry reliable, state of the art HPTLC methods, which are fit for the purpose of ensuring identity and other quality criteria of botanicals based on fingerprint chromatograms.

1) All HPTLC analyses should be based on a standardized methodology providing guidance for the selection of important parameters, thus ensuring the highest possible reproducibility and comparability of results. 2) Methods for identification should be evaluated not only based on their ability to show the similarity or difference of test solution and reference solution side by side on the same plate, but also based on the reproducibility of the result documented as image. It is important that such methods can accommodate the natural variability of a given plant species and at the same discriminate any adulterant. 3) During validation specificity of the method must be proven. In addition various stability issues have to be addressed together with any reproducibility and robustness problem that might occur.

The proposal is illustrated using several examples and is intended as a starting point for a discussion that helps making qualitative HPTLC methods for identification more widely applicable.

1. Methodology

Practical thin-layer chromatography leaves room for many choices. The following approach is a proposal that is intended to serve as a general guideline for analyses by HPTLC. It should not be seen as the tool that always fits, but merely as one comprehensive and proven option.

Plate material

HPTLC plates silica gel 60 F_{254} (such as Merck or equivalent) in the format of 10×10 or 20×10 cm are used. Generally plates are used without pre-treatment unless chromatography produces impurity fronts due to contamination of the plate.

Sample application

Samples are either applied as spots (contact transfer) or as bands (spray-on technique) using a suitable instrument. The spray-on application is preferred and should be used whenever possible. Spotting should only be chosen for accurate application of very small volumes (< 1µL). For spotting the sample should be dissolved in the solvent of lowest suitable solvent strength in order to minimize the effects of spot broadening during application. Samples are applied using the parameters listed in Table 1.

Table 1: Application parameters

Parameter	band	spot
Distance from lower edge of plate in mm	8	10
Minimum distance from left and right edge of plate in mm	10	10
Minimum space between band/spot in mm	2	2
Band length in mm	8	-
Maximum diameter of application spot in mm	-	4

Development

Plates are developed in a saturated Twin Trough Chamber (TTC) according to the following procedure:

Open chamber and place a correctly sized (10×10 cm; 20×10 cm) piece of filter paper in the rear trough. Pour solvent into chamber (10×10 cm: 10 mL, 20×10 cm: 20 mL) so that the filter paper is thoroughly wetted and adheres to rear wall of TTC. Tilt chamber to the side so that the solvent volume in both troughs equalizes. Let closed chamber equilibrate for 20 minutes. Mark the desired developing distance (70 mm from lower edge of plate) with a pencil on the right edge of the plate. Insert the plate into the front trough. The layer faces the filter paper and the back of the plate rests against front wall of TTC. Replace lid, develop plate to the mark. Open lid, remove plate. Dry the plate (vertically in direction of chromatography) 5 minutes in a stream of cold air.

Derivatization

Transfer of reagent for derivatization of samples on a HPTLC plate may be accomplished by spraying or dipping. Dipping is the preferred method and should be used whenever possible.

Dipping

Charge tank of an immersion device with enough reagent to ensure complete immersion of chromatogram. Place plate in holder of immersion device, set parameters according to method and start. Let excess reagent drip off plate, wipe off back of the plate with paper towel. Remove plate from plate holder.

Spraying

Charge the bottle of the sprayer with up to 50 mL of reagent. Place plate in spray cabinet or fume hood upright against a filter paper or a paper towel.

Spray plate with horizontal and vertical motion until it is homogeneously covered with reagent.

Heating

Turn on plate heater and select temperature. Place plate onto hot plate heater, remove plate after time specified.

Fig. 1: Correct development in a saturated Twin Trough Chamber

Documentation of plates

Each developed plate is documented with an electronic documentation system (video or digital) under UV 254 nm, UV 366 nm and white light.

If a type of light does not produce usable information that fact must be documented. If a plate is derivatized images are taken prior and after derivatization.

2. Method selection and evaluation

Before a method can be selected and evaluated, the analytical goal has to be clearly defined:

- Is the method intended for
  - identification (qualitative)?
  - quality, stability testing (semi-quantitative)?
  - quantitation of marker compounds?
- Which compounds (markers) have to be separated?

When the main goal is identification, the method must allow the discrimination between "good samples" and adulterants. A large number of samples are needed in order to define an acceptance window, which accommodates the natural variability of a plant (origin, growing stage, part, treatment…), but excludes any adulteration with certainty. For example, see Figure 2.

Unlike in usual applications where all samples and standards are on the same plate, in stability tests samples are compared from plate to plate over a long period of time. The first plate serves as the reference to which all subsequent plates are compared. In this regard long-term repeatability of the selected method and of the documentation is of utmost importance (Fig.3).

Fig. 2: Black Cohosh -natural variability and detection of adulteration.

Fig. 3: Comparison of Valerian fingerprints based on video-densitometry of track 2 on five different plates (over 1 month). The chromatography (Rf-values) and documentation are reproducible, therefore, the method can be used in stability tests.

The principal restriction for quantitative HPTLC determinations is the separation power. If the sample contains too many components a useful fingerprint may still be obtained, but baseline separation of substances as requirement for quantitation may be difficult to achieve. In this case, the development of a selective method may be needed. Figure 4 shows a separation sufficient for quantitation.

Fig. 4: Separation of alkaloides in *Stephania tetrandra*. The resolution is sufficient for quantitation.

As illustrated in Figure 5, two different mobile phases allow the separation of different flavonoids pairs of Hawthorn. In the case, where the focus is put on the presence or absence of rutin resp. vitexin-rhamnoside, the second mobile phase is better appropriated.

Fig. 5: Selectivity of mobile phase for the separation of Hawthorn flavonoids.
Left: ethyl acetate, water, acetic acid, formic acid (100:26:11:11)
Right: ethyl acetate, methanol, water, formic acid (50:2.3:6)
1: *Crataegus laevigata*, 2: standards (increasing Rf): rutin, vitexin-2''-O-rhamnoside, chlorogenic acid, hyperoside, vitexin, 3: *C. monogyna*.

3. Method validation

According to the ICH Guidelines 2QA and 2QB, for the validation of qualitative methods, only the specificity must be ensured.

Specificity includes

- Detection of adulteration (see above)
- Discrimination between plant parts (if applicable, Fig. 6)
- Ensuring of no matrix interference (Fig. 7)

Pre-validation tests are essential to ensure the reliability of the results. The stability of the analyte during chromatography is evaluated by a 2-dimensional chromatogram in which all components should lie on the diagonal that connects application position with the intersection of the two mobile phase fronts. Any spot appearing above or below the diagonal indicates instability of the corresponding analyte during chromatography (Fig.8). The stability of the analyte in solution and on the plate should also be evaluated.

Fig. 6: The methods for identification of Echinacea are specific for discrimination between plant parts. Product 2 contains Echinacea herb, not root as marked on the label. Product 3 complain to its claim and contains both Echinacea and Goldenseal.

Fig. 7: Specificity of method for use on finished products; the chromatogram of St. John's Wort is not disturbed by tablet excipients (11). 1-6: standards, 7, 8: herb, 9: dry extract, 10: tablet containing extract (9).

Fig.8 : Stability of analyte in 2 different chromatographic systems, 2D chromatogram of *Angelica sinensis*. Left: formation of artifacts (arrows), right: the analyte is stable.

Fig. 9: Influence of humidity on the separation of *Echinacea* root

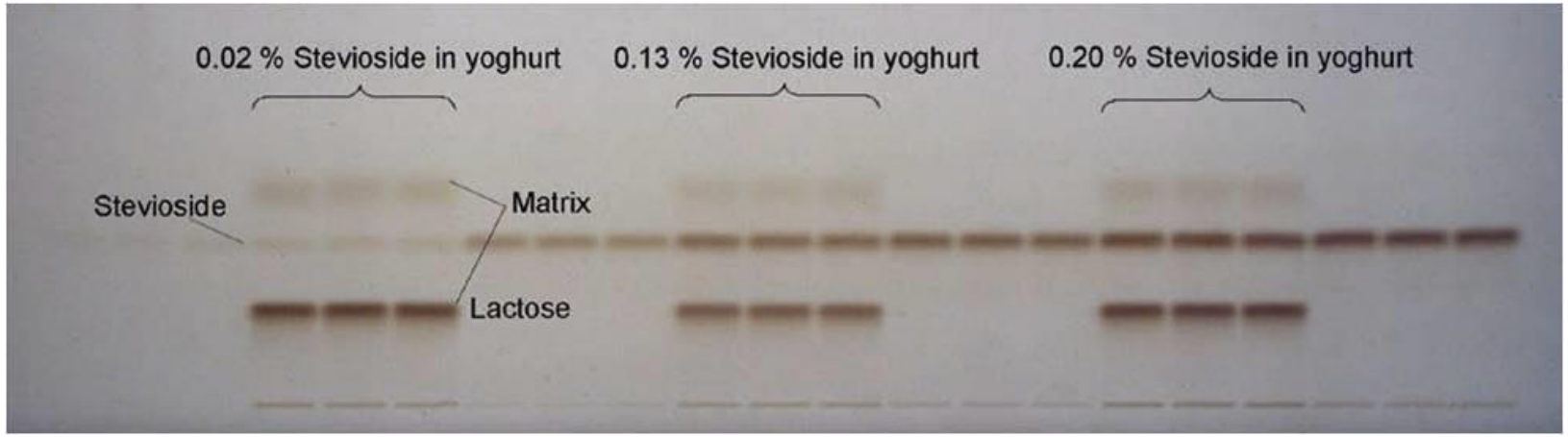
The influence of humidity (Fig. 9), temperature, chamber conditioning has to be checked (robustness testing). The repeatability / reproducibility of the method over a long period of time should also be seen as a central point of the validation process (Figs. 3 and 10).

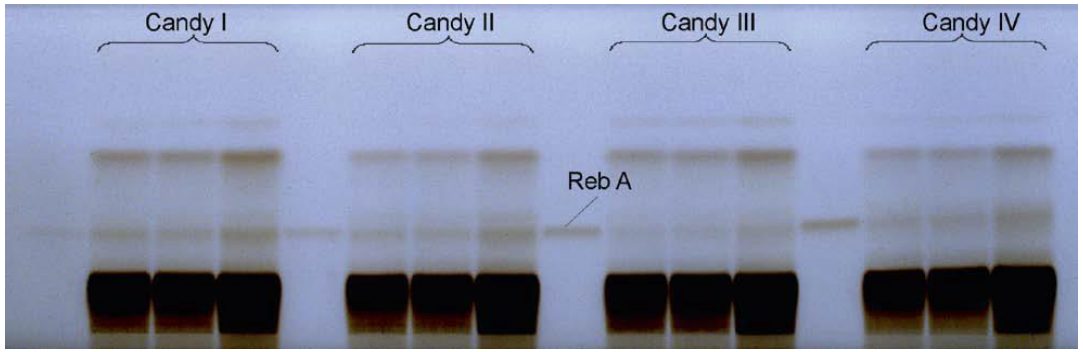
Fig. 10: Method reproducibility: variation of Rf-values of valerenic acids (markers for *Valeriana officinalis*) on 30 plates developed on 30 days.

Further contacts:
[1] International Symposium for HPTLC info@hptlc.com
[2] Association francophone le Club de CCM www.clubdeccm.com
[3] For Camag in France : www.chromacim.com, pbs@chromacim.com

Literature
- **ICH Guidelines 2QA and 2QB**, http://www.ich.org/ich5q.html, last visited August 2003
- Koll K, Reich E, Blatter A, Veit M. **Validation of Standardized HPTLC-methods for Quality Control and Stability Testing of Herbals (Herbal Drugs, Herbal Drug Preparations, Herbal Medicinal Products).** J. of AOAC International, accepted for publication, April 2003
- Ferenczi-Fodor K, Vigh Z, Nagy-Turak A, Renger B, Zeller M. **Validation and Quality Assurance of Planar Chromatographic Procedures in Pharmaceutical Analysis.** J. of AOAC International 2001, 84: 1265-1276

Stéviosides dans les yaourts





Fidélité adéquate pour ce type de matrices,

Tolérance sur les résultats adaptée aux faibles teneurs rencontrées dans les matrices alimentaires (0.02 à 0.2% en poids).

Grande spécificité par la dérivatisation post chromatographique,

Intervalle de mesure adapté aux produits alimentaires,

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High-performance thin-layer chromatography analysis of steviol glycosides in *Stevia* formulations and sugar-free food products, and benchmarking with (ultra) high-performance liquid chromatography

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ABSTRACT

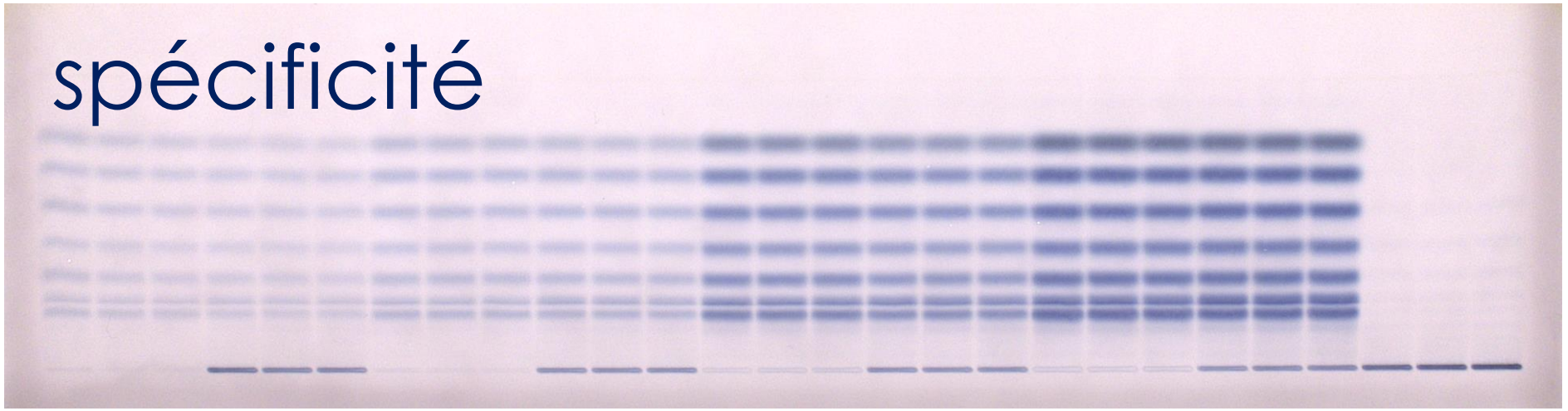
A high-performance TLC (HPTLC) method was newly developed and validated for analysis of 7 steviol glycosides in 6 different types of food and *Stevia* formulations. After a minimized one-step sample preparation, 21 samples were developed in parallel, allowing an effective food screening. Depending on the sample application volume, the method was suited to analyze food sample concentrations in the mg/kg range. LOQs of stevioside in natural yoghurt matrix spiked at 0.02, 0.13 and 0.2% were determined by the

Dextrines dans la pâte à pain

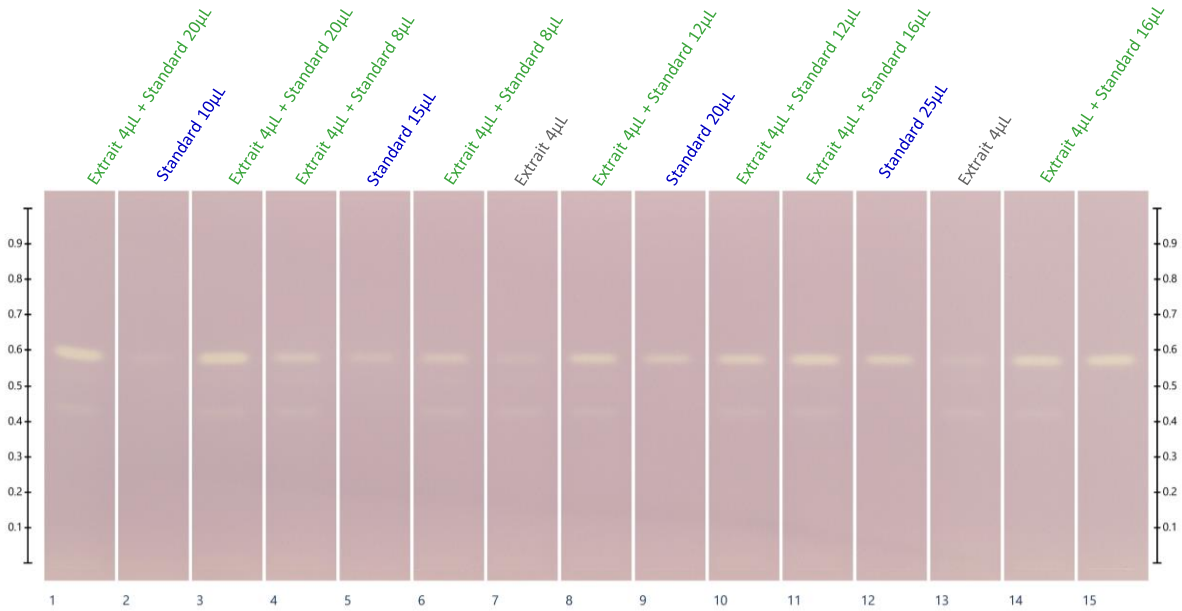
Méthode de dosages de DP1 à DP7

La performance de la méthode peut être évaluée avec SEULEMENT 5 PLAQUES !
Méthode de validation puissante avec calcul de l'erreur totale
Standards NF V03-110, USP <1210> (PF42(5)), ISO 21748…
Les profiles (accuracy + uncertainty) sont simples à obtenir et éclairent bien la performance analytique

"Application of prediction intervals to dextrine profiles of enzymatic digestion of starch and baking products" *J-M Roussel, HPTLC'17, BERLIN*



Détection d'activité antiradicalaire



Méthode quantitative validée sur un dosage d'actif
Dans un extrait végétal en utilisant une détection biologique directe d'activité sur la plaque
6 plaques pour validation de la méthode
3 plaques (sur 5) pour l'étude de **l'effet matrice**
Moins de deux semaines de temps passé en cumul traitement statistique inclus

(1er travail sérieux sur le sujet
validation détection activité sur plaque »)

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Check for updates

Application of the Life Cycle Management of Analytical methods concept to a HPTLC-DPPH assay method for acteoside content in industrial extracts of *Plantago lanceolata* L

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Plantago lanceolata L.
DPPH
HPTLC
Life Cycle Management of Analytical Methods
Total Analytical Error
Target Measurement Uncertainty

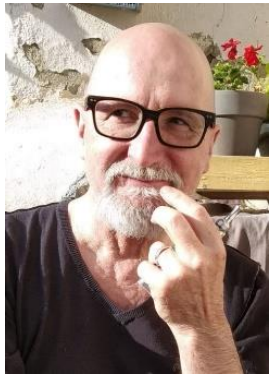
ABSTRACT

Analytical methods used for quality control of plants and plant extracts are based on the identification and quantification of chemical markers to manage batch reproducibility and efficacy.

The aim of this work was to assess the performance of a High Performance Thin Layer Chromatography (HPTLC) method developed for quality control of industrial dry extracts of ribwort plantain (*P. lanceolata* L.), using 2,2-diphenyl 1-picrylhydrazyle (DPPH) effect directed chemical reaction for antioxidant activity of acteoside, a phenylethanoid glycoside commonly used as a marker for *P. lanceolata* L., and to demonstrate the applicability of the Life Cycle Management of Analytical Methods concept to quantitative HPTLC-DPPH methods.

The first step was the determination of the Analytical Target Profile (ATP) and Target Measurement Uncertainty (TMU), taking into account the quality control requirements for such extracts and the detection method

Un peu de lecture



Livre à paraître de Colin POOLE

Chapter 13

Validation of Thin-layer Chromatographic Methods

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13.1 Introduction

There is no longer any discussion about the need to validate analytical methods. It is an obligation in the sense of the quality requirements of all testing [1], biomedical analysis [2] or pharmaceutical industry [3] laboratories. Many guidelines exist, describing with more or less details the concept and methodology of validation of analytical methods, as well as in the field of pharmaceutical products [4-6], food industry [7, 8], botanicals [9] cosmetic products [10], environmental analysis [11, 12] or bioanalysis [13-15]. Unfortunately, this abundance of texts and guidelines ultimately brings more confusion than solutions to the analyst when validating his methods.

A comparative study [16], carried out on nearly forty guidelines related to the validation of analytical methods, showed a certain number of inconsistencies between these guidelines, even though most of them were published by international organizations. For example, the definition of accuracy may vary between guidelines, linearity of the standard response may or may not be required, non-linear response functions may or may not be considered, and the method validation experimental designs may differ from a guideline to another.

Robustesse

Il est simple et assez rapide d'évaluer la robustesse d'une méthode d'HPTLC car une vingtaine de plaques suffisent.
(ex :saturation, conditions de révélation)

ET 5 PLAQUES SEULEMENT POUR UNE VALIDATION

Avis aux amateurs de robustesse

Project : HPTLC - Rosmarinic acid in Melissa Officinalis
Robustness: multi responses (with pred. Int.)
Directory : Rosmarinic_chromacim_2021

Page: 1/1

08/31/2021

Num.	ID.	Experimental conditions	Sample A			Sample B			Sample C		
			Rf Acide rosmarinique	Rs Ac. Ros/UNK	Ac. Rosm. Amount	Rf Acide rosmariniqu	Rs Ac. Ros/UNK	Ac. Rosm. Amount	Rf Acide rosmariniqu	Rs Ac. Ros/UNK	Ac. Rosm. Amount
1	Y3/1	Activation duration (min)=8 Saturation time (min)=22 HCOOH volume (ml)=5 H2O volume (ml)=5 Ethyl acetate volume (ml)=95 Staining reagent vol. (ml)=2.5 Heating conditions=no									
2	Y16/1	Activation duration (min)=12 Saturation time (min)=22 HCOOH volume (ml)=7 H2O volume (ml)=7 Ethyl acetate volume (ml)=95 Staining reagent vol. (ml)=2.5 Heating conditions=yes									
3	Y14/1	Activation duration (min)=12 Saturation time (min)=18 HCOOH volume (ml)=7 H2O volume (ml)=7 Ethyl acetate volume (ml)=85 Staining reagent vol. (ml)=1.5 Heating conditions=yes									
		Activation duration (min)=12 Saturation time (min)=22									
21	Y17/5	Saturation time (min)=20 HCOOH volume (ml)=6 H2O volume (ml)=6 Ethyl acetate volume (ml)=90 Staining reagent vol. (ml)=2 Heating conditions=no									
22	Y1/1	Activation duration (min)=8 Saturation time (min)=18 HCOOH volume (ml)=5 H2O volume (ml)=5 Ethyl acetate volume (ml)=85 Staining reagent vol. (ml)=1.5 Heating conditions=no									

Ce qui est important

Préciser la question, la bonne question

Bien définir son objectif

Réfléchir avant de commencer

Choisir la détection, penser sélectivité

Suivre la méthode simplement

Se former s'informer communiquer

La formation c'est important

Laboratoire CHROMACIM
CHROMACIM Laboratory

AGENDA :

Niveau 1 25-26 Octobre
Niveau 2 12-13 Octobre
AMD 22-24 Novembre
EDA 4-6 Octobre
avec le professeur MORLOCK

LÂCHEZ VOUS : ESSAYEZ L'HPTLC

MERCI

et
maintenant
passons aux
choses
sérieuses