

Admixture of Peanut skin on Pinus spp extracts : Use of HPTLC-densitometry to ensure Pinus spp extracts quality

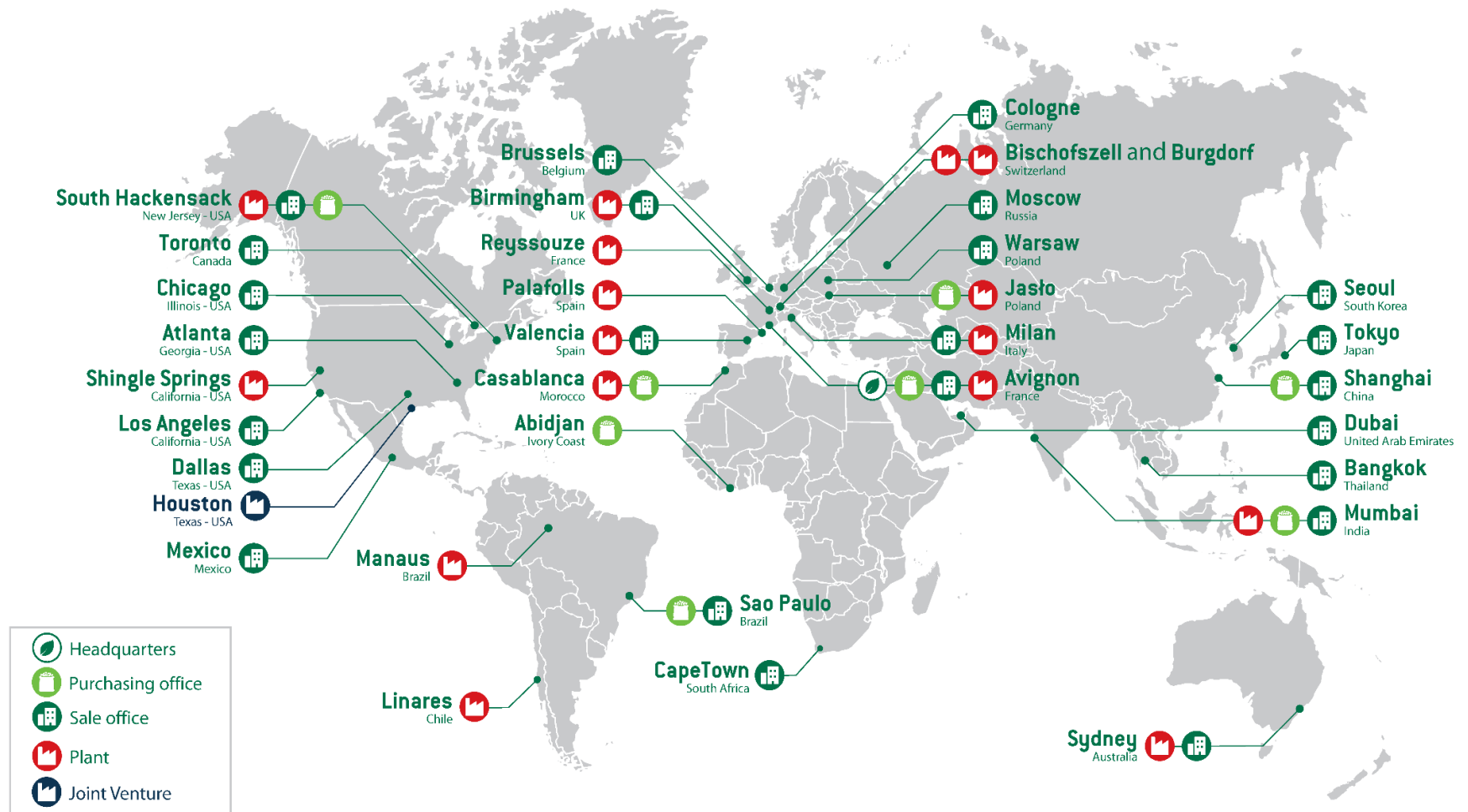
Mélange de peau de cacahuète aux extraits de Pins :
Utilisation de l'HPTLC-densitométrie pour assurer la
qualité des extraits de Pins

A photograph of a hummingbird in flight, hovering near a cluster of bright pink flowers. The bird is positioned in the center-right of the frame, facing left. Its wings are spread, and its long beak is pointed towards the flowers. The background is a soft, out-of-focus green.

FEUILLATRE, M.; LE, T. N.; BILY A.; FALCAO, L.
R&D Nutrition and Health

Naturex overview

Locations



- Headquarters
- Purchasing office
- Sale office
- Plant
- Joint Venture

Naturex overview

Research & Development

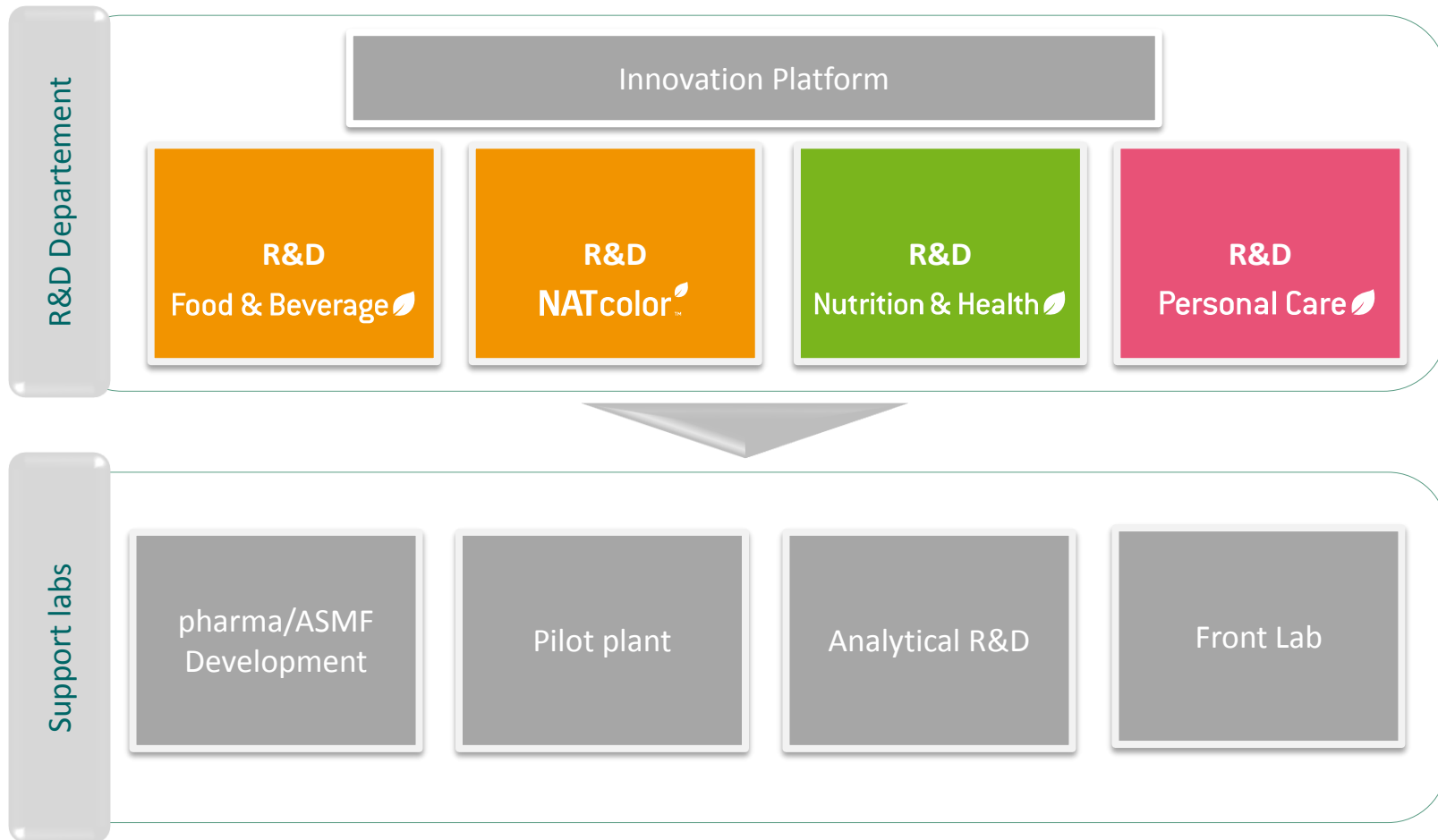


75
17 Ph. D
30 Eng/bandineers
28 Technicians

2 000
Developments
/ year

5 to 10
Scientific
publications
/ year

54
Patent families





370 HPTLC methods developed, 200 HPLC methods
Collection of authenticated plant specimens

Naturex Integrated platform for identification

Level 1 : Macroscopy/ microscopy, DNA and use of validated botanical standard

Level 2 : Phytochemical levels (HPTLC, HPLC, GC, NMR...)

Level 3 : Process knowledge and impact on profile interpretation



Family : *Pinaceae*

Genus : *Pinus*

Pinus pinaster, is a maritime pine native to southwest France that also grows in countries along the western Mediterranean.



Introduction

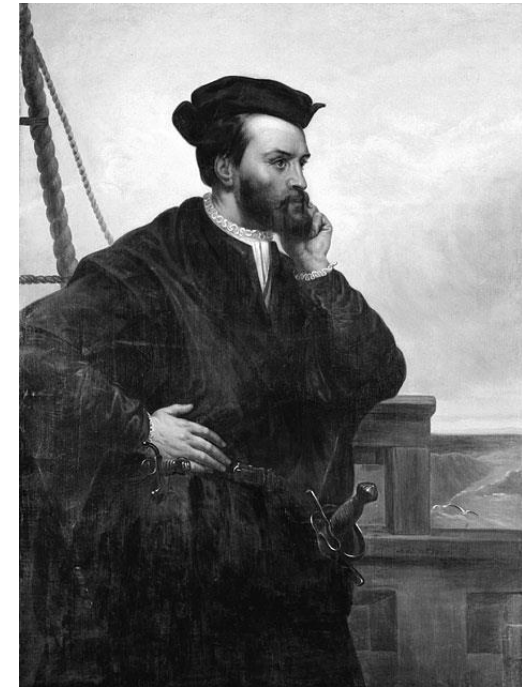
Pine bark

Jacques Cartier landed in Quebec, Canada.

Ship crew became ill and began dying from what we now know as scurvy, a deficiency of vitamin C.

Natives taught explorers to use local tree bark as a tea for the scurvy

Cartier brought back seed to Fontainebleau (Durzan, 2009).



Jacques Cartier
(1491 – 1557).



Dr. J. Masquelier read Jacques Cartier's account and this turned his attention to the antioxidant procyanidins of conifer bark

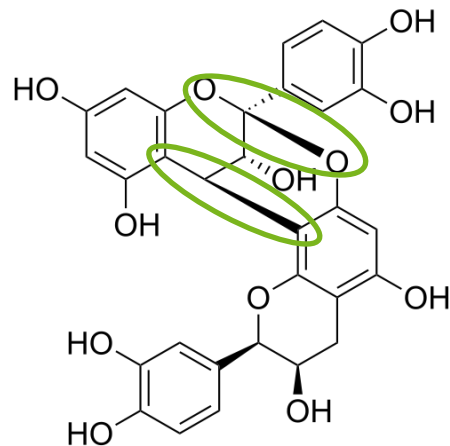
Procyanidins = Biopolymers of (+) - catechin and (-) - epicatechin subunits.

Present abundantly in plant kingdom (fruits, barks, leaves, seeds)

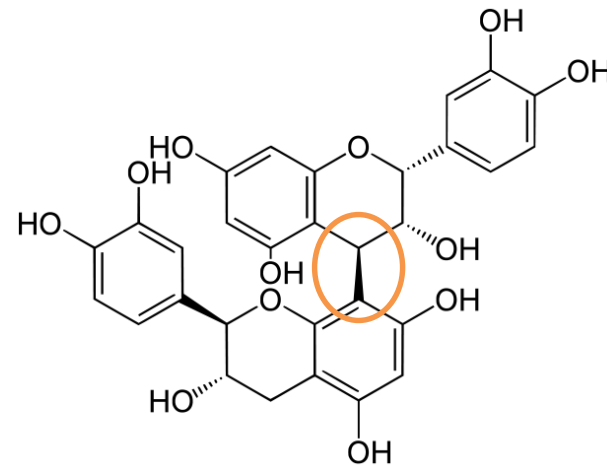
Ex : cinnamon bark, litchi pericarp, peanut, bilberry, grape seeds...

Protection against light, oxidation and predators

Various types of Procyanidins, based on linkage between monomeric units



A-type



B-type
(4 β →8)

Introduction

Pine bark



http://www.ncbi.nlm.nih.gov/pubmed/?term=Pinus+... Pinus pinaster extract - Pub... X

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[Pinosylvin and Monomethyl pinosylvin, Constituents of Extract from the Knot of *Pinus sylvestris*, Reduce Inflammatory Gene Expression and Inflammatory Responses In vivo.](#)
1. Laavola M, Nieminen R, Leppänen T, Eckerman C, Holmbom B, Moilanen E.
J Agric Food Chem. 2015 Mar 12. [Epub ahead of print]
PMID: 25763469 [PubMed - as supplied by publisher]

[Phytochemical analysis of *Pinus eldarica* bark.](#)
2. Iravani S, Zolfaghari B.
Res Pharm Sci. 2014 Jul-Aug;9(4):243-50.
PMID: 25657795 [PubMed] **Free PMC Article**
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[Inhibitory effects of *Pinus massoniana* bark extract on hepatitis C virus in vitro.](#)
3. Wang C, Zhang L, Cheng P, Zhang Q.
Pharm Biol. 2015 Mar;53(3):451-6. doi: 10.3109/13880209.2014.924018. Epub 2014 Dec 4.
PMID: 25471218 [PubMed - in process]
[Related citations](#)

[Efficacy of condensed tannins against larval *Hymenolepis diminuta* \(Cestoda\) in vitro and in the intermediate host *Tenebrio molitor* \(Coleoptera\) in vivo.](#)
4. Dhakal S, Meyling NV, Williams AR, Mueller-Harvey I, Frygasas C, Kapel CM, Fredensborg BL.
Vet Parasitol. 2015 Jan 15;207(1-2):49-55. doi: 10.1016/j.vetpar.2014.11.006. Epub 2014 Nov 15.
PMID: 25468673 [PubMed - in process]
[Related citations](#)

[Neolignan inhibitors of antigen-induced degranulation in RBL-2H3 cells from the needles of *Pinus thunbergii*.](#)
5. Hong SS, Jeong W, Kim JK, Kwon JG, Lee JY, Ahn EK, Oh J, Seo DW, Oh JS.
Fitoterapia. 2014 Dec;99:347-51. doi: 10.1016/j.fitote.2014.10.015. Epub 2014 Oct 30.
PMID: 25451795 [PubMed - in process]
[Related citations](#)

[Inhibitory effects of French pine bark extract, Pycnogenol®, on alveolar bone resorption and on the osteoclast differentiation.](#)
6. Sugimoto H, Watanabe K, Toyama T, Takahashi SS, Sugiyama S, Lee MC, Hamada N.
Phytother Res. 2015 Feb;29(2):251-9. doi: 10.1002/ptr.5245. Epub 2014 Oct 21.

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An orally active immune adjuvant prepared from cones of **Pi** [BMC Complement Altern Med. 2014]
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("pinus"[All Fields]
AND "pinaster"[All
Fields]) OR "pinus
Search See more...

Some Pine Bark (*Pinus pinaster* Aiton) health properties

- **Skin cancer preventive action *in vivo*** (Kyriazi et al., 2006).
- **Protect skin for photoaging** : *Pinus pinaster* Aiton extract helps reduce ultraviolet radiation damage to the skin (Furumura et al., 2012)
- **Menstrual pain** were investigated on 116 women aged 18-48 using a supplement (60 mg/day) or a placebo. As results, women with dysmenorrhea had a significantly lower pain (Suzuki et al., 2008).
- **Erectile dysfunction (ED)** : patients were treated for 1 month with placebo or a combination of L-arginine aspartate and a commercial extract of bark of *Pinus Maritima* (Stanislavov et al., 2007). The erectile domain of the IIEF significantly improved with the formulation compared with placebo ($P < 0.05$) (Ledda et al., 2010).

Family: Fabaceae
Genre: Arachis
Arachis hypogaea L.

Safety concern : allergen



- Peanut skin is a high-volume by-product of peanut industry
- Already shown as adulterating agent for Grape seed extracts (Villani et al., 2015).
- Adulteration here is mainly for economic gain!

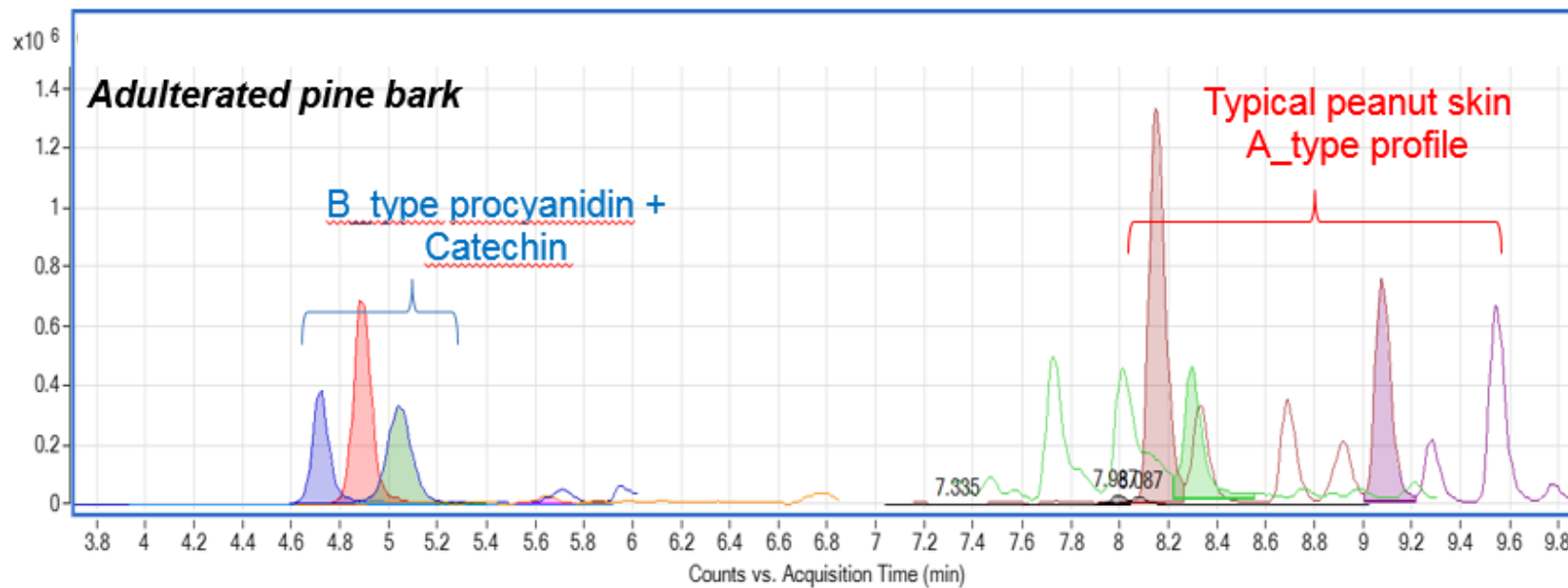


Pine bark extract : B-type dimers (B1, B2, B3 and B7), trimers and oligomers (5 to 7 units) as well as the monomers (Rimbach et al., 2000).

Traces of A-type on *Pinus sylvestris* L. (Karonen et al. 2004)

Peanut skin extract : principally of A-type PACs with negligible quantities of the monomers, catechin and epicatechin (Villani et al., 2015).





Motivation

Pine bark extracts (*Pinus pinaster* Aiton): High difference in price between Naturex and competitors' extracts (x 10)

The objectives of this study were:

- To evaluate competitors commercially available *Pinus* spp extracts by phytochemical profiling;
- To identify if admixture/adulteration are existing and if positive find a marker for control quality;
- To develop a simple, fast and accurate TLC qualitative and quantitative method for *Pinus* spp extracts quality control.



CAMAG Automatic TLC Sampler 4



CAMAG Automatic Developing Chamber 2



CAMAG TLC Visualizer



CAMAG TLC Scanner 4

- Chemicals:**
- Toluene, acetone, dichloromethane and methanol used are HPLC grade purchased from VWR International
 - Formic acid used are of ACS certified grade from VWR International
 - Fast Blue Salt B from Sigma-Aldrich
 - Procyanidin A2, B1, B2; catechin purchased from Extrasynthèse

Parameters:

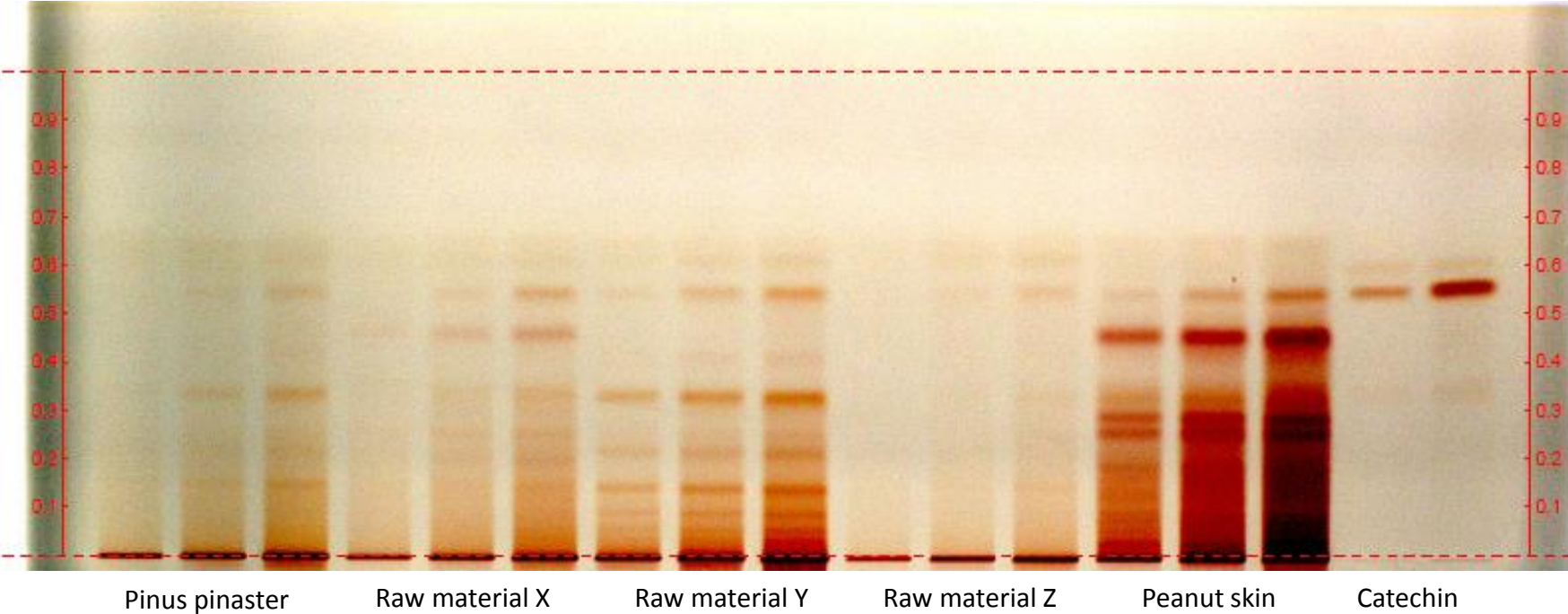
- **Sample preparation:** 1g of powdered raw material/extracts with 10 ml of EtOH/H₂O (v/v), sonicate for 10 min, heat at 50°C for 20 min, filter through 0,45 µm
- **Reference standard :** 1 mg of procyanidin A2, B1, B2 or catechin in 2 ml of MeOH
- **Stationary phase:** HPTLC plates 20 x10 cm Si 60 F₂₅₄
- **Sample application:** ATS4, 6mm bands, 2 or 4 µl for raw material, 0,5 or 0,7 µl for extracts
- **Mobile Phase:** Toluene/Acetone/ Formic Acid (4.5/4.5/1) (v/v/v)
- **Development:** ADC2, 20x10 cm Twin Trough Chamber (CAMAG) saturated. Developed to 70 mm for the bottom of the plate, dry for 5 min with cool air
- **Detection:** Fast blue B salt (immersion), detected with visible light (visualizer and densitometer at 486 nm)



(Sudberg et al.)

Results and discussion

Preliminary results:



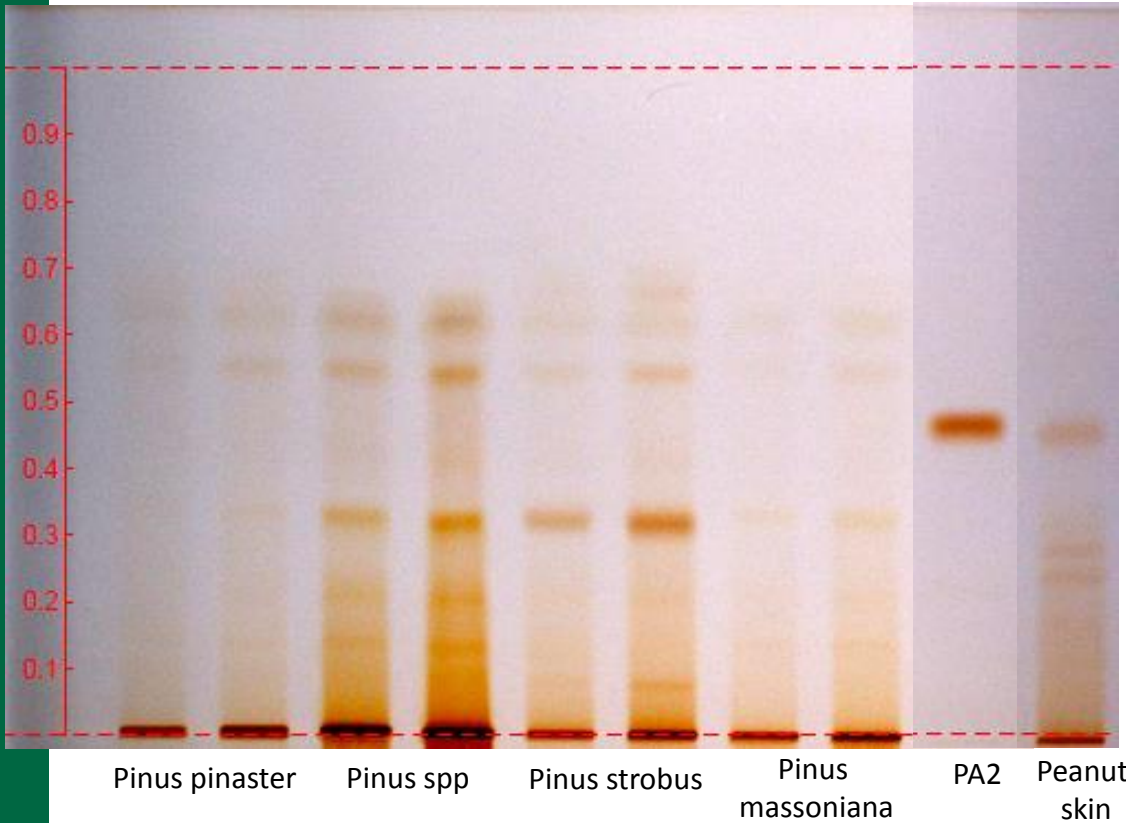
Humidity control: 39 %

Results and discussion

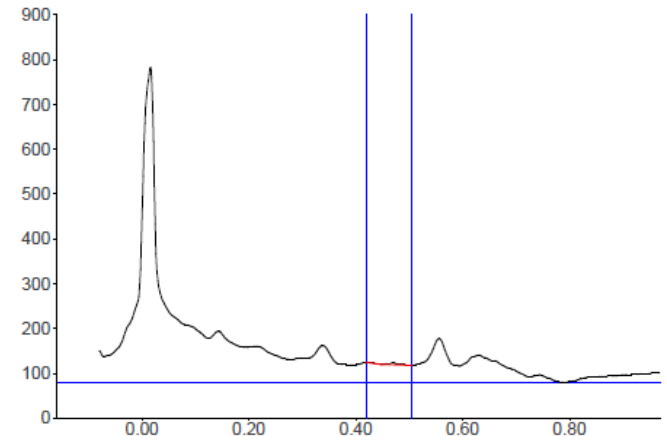
Preliminary results:

PA2 = Procyanidin A2

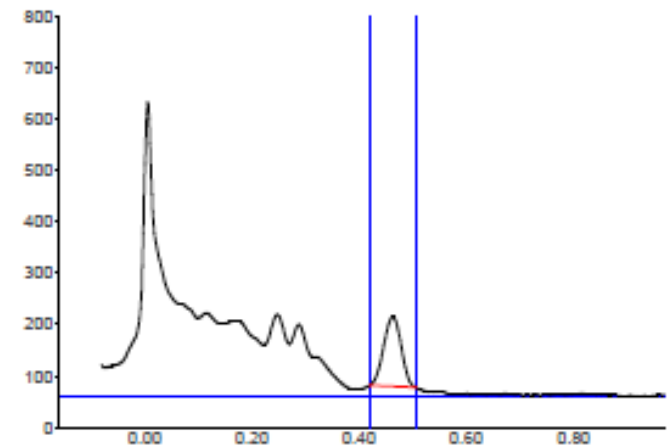
Profiles UV at 486 nm



Track 2, ID: 1. Pine bark (pinus pinaster)



Track 18, ID: 11. Peanut skin

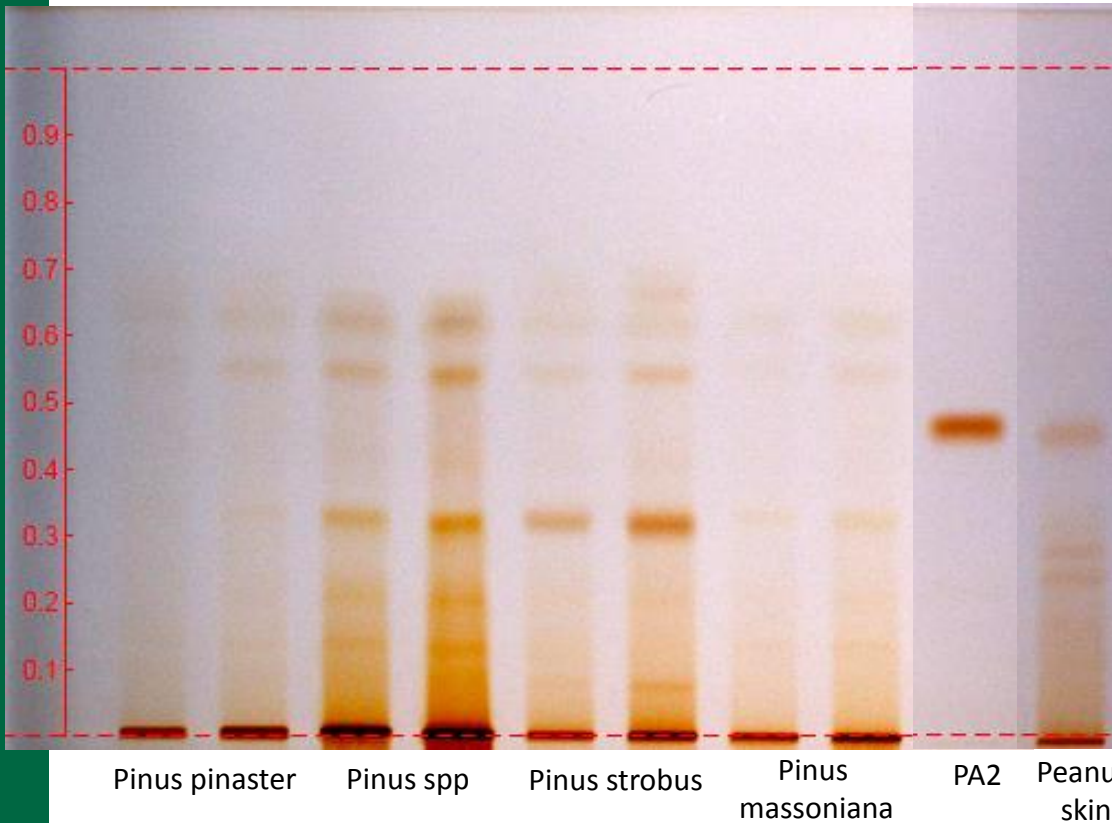


Results and discussion

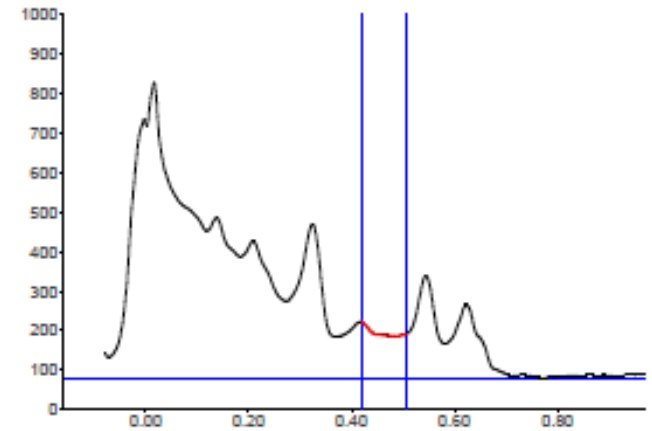
Preliminary results:

PA2 = Procyanidin A2

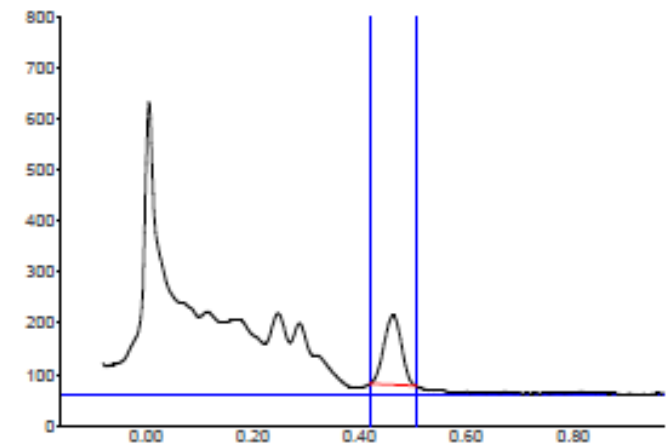
Profiles UV at 486 nm



Track 4, ID: 2. Standard Pine bark (pinus spp.) -



Track 18, ID: 11. Peanut skin

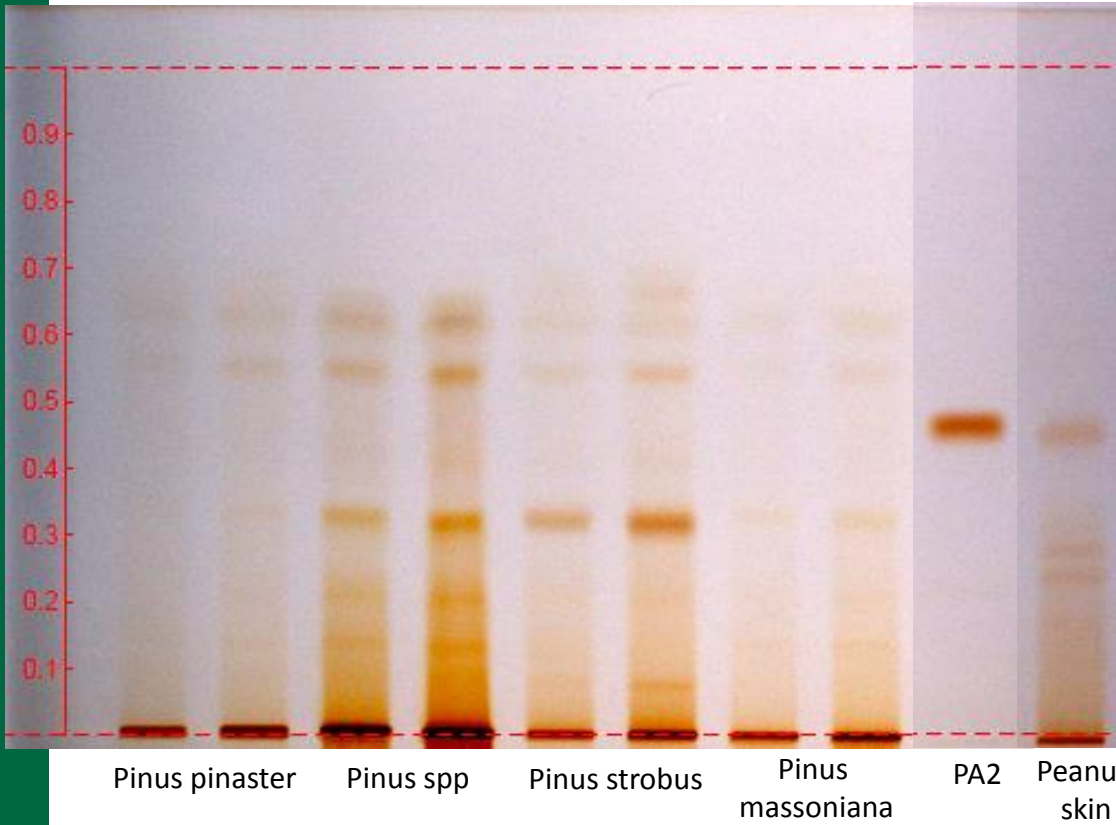


Results and discussion

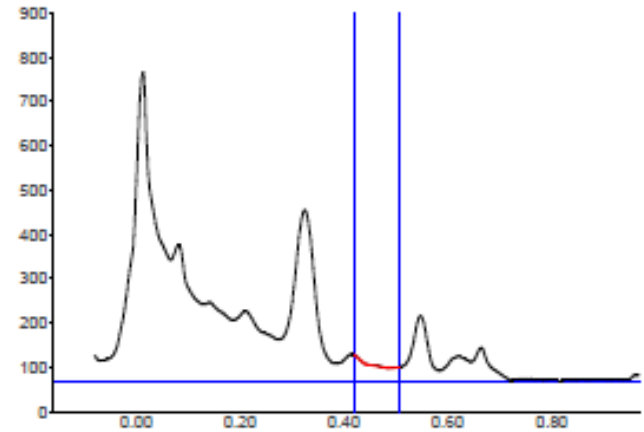
Preliminary results:

PA2 = Procyanidin A2

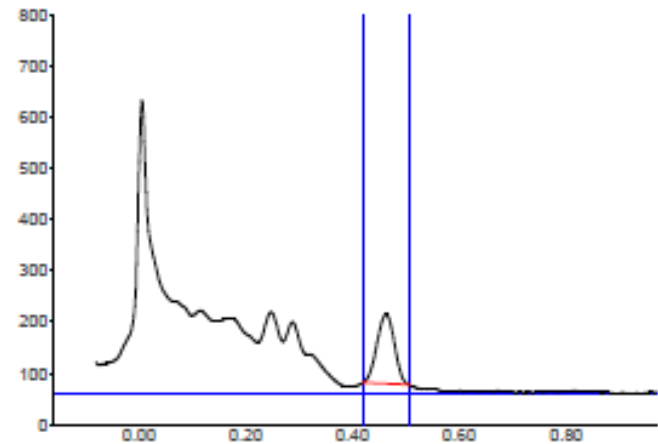
Profiles UV at 486 nm



Track 6, ID: 3. Standard Pine bark (pinus strobus)



Track 18, ID: 11. Peanut skin



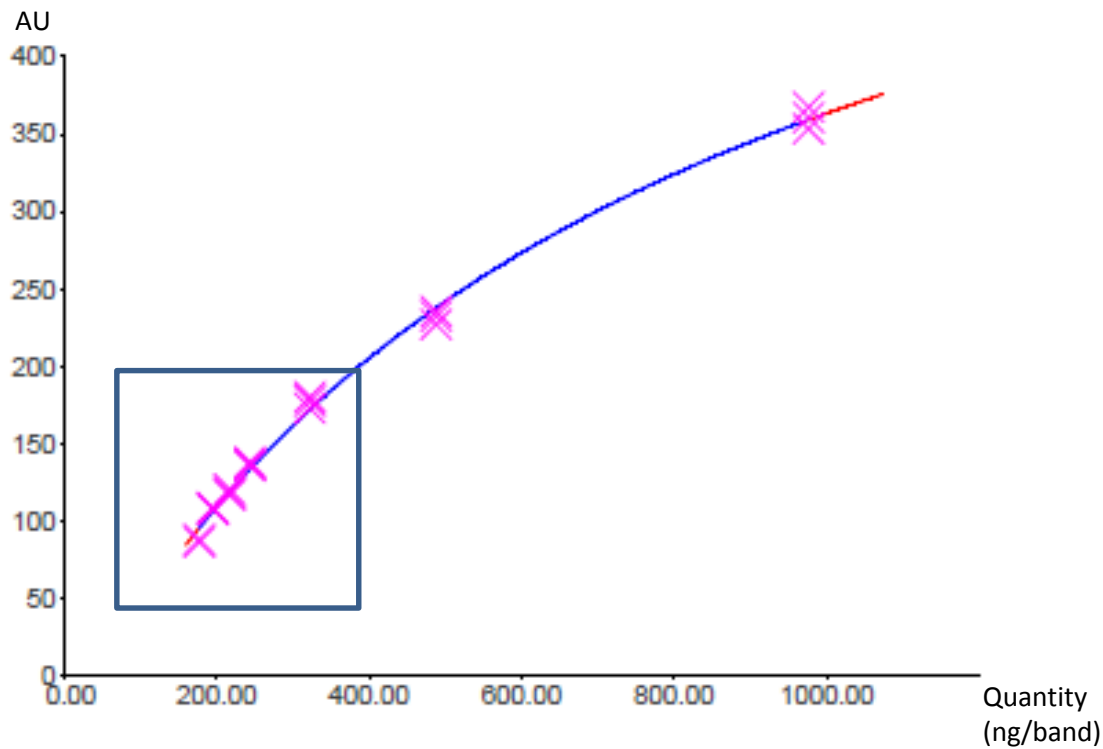
Parameters
Specificity (on going)
Linear range
Linear equations
R2
Precision (%RSD)
Repeatability
Intermediaire precision
LOD
LOQ
Accuracy (on going)
Average recovery (on going)

Linearity

Substance: Procyanidin A2 @ 486 nm

Regression via height: Michaelis-Menten 2 $Y = -36.25 + (708.4 * X) / (772.2 + X)$

sdv = 3.16



Quantification method Validation as per ICH guidelines:

Linearity

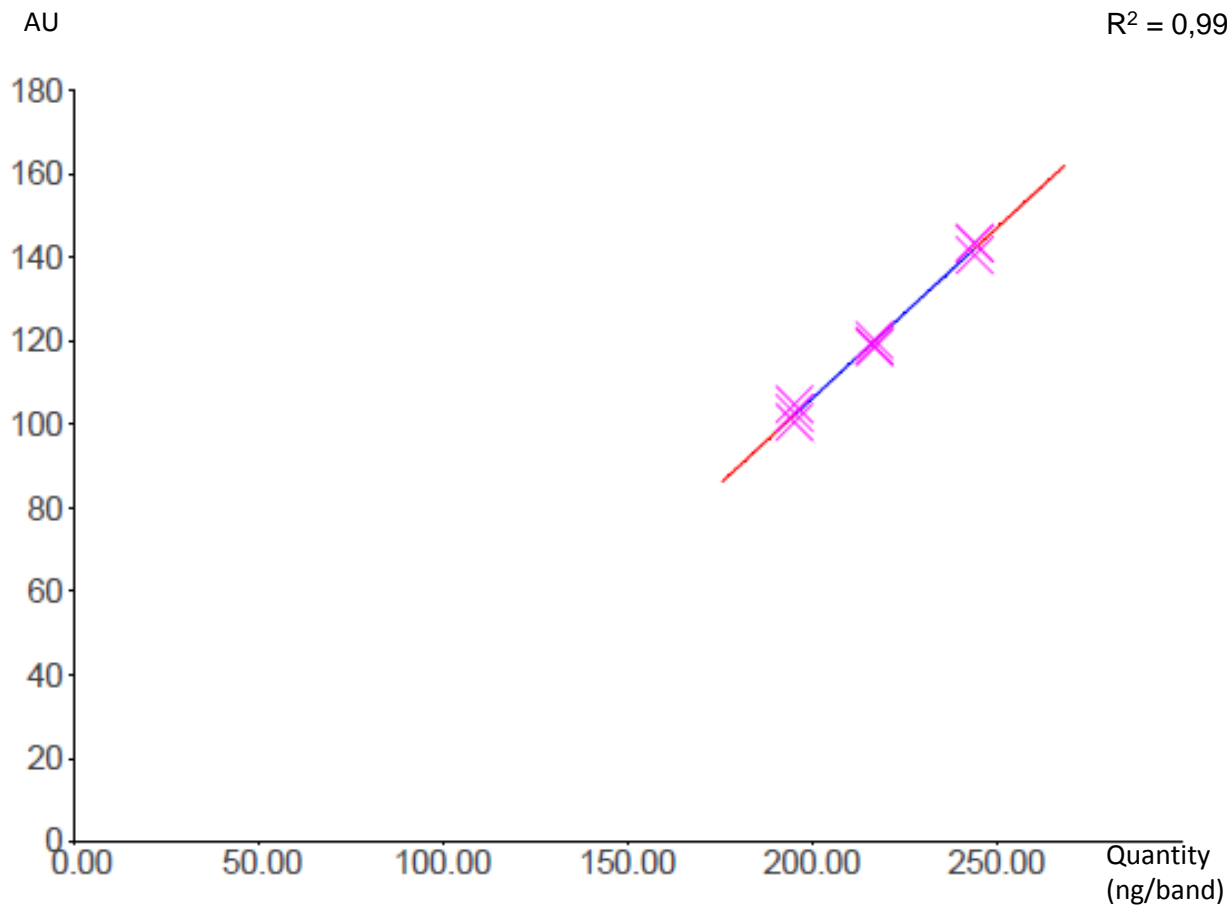
Substance: Procyanidin A2 @ 486 nm

Regression via height: Linear

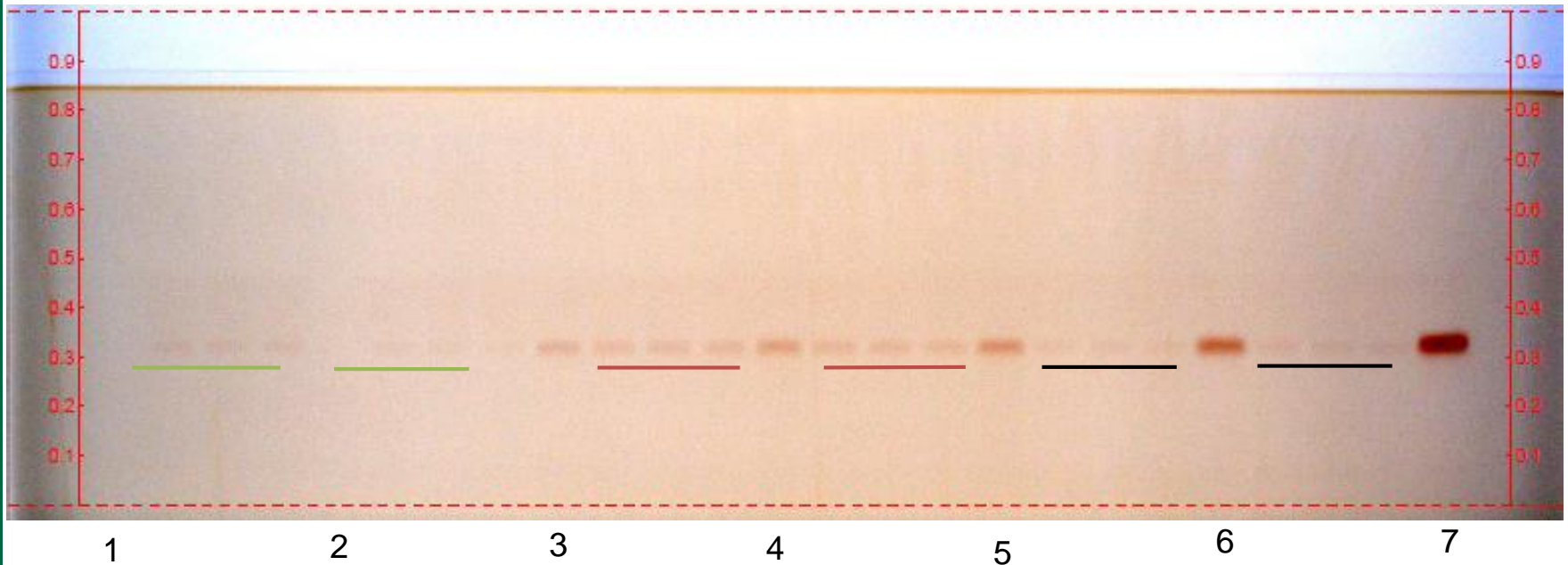
$$Y = -57.32 + 0.8176 * X$$

$r = 0.99612$ $sdv = 1.35$

$R^2 = 0,99226$



Limit of detection (LOD)

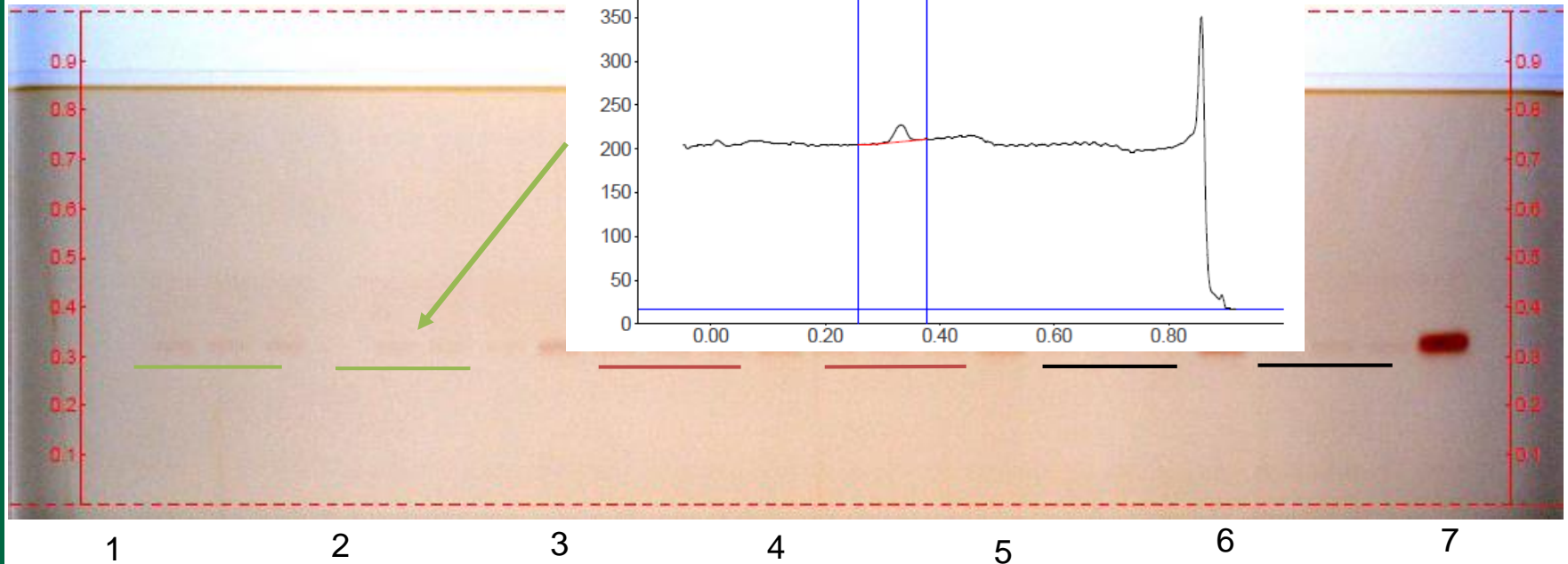


- 123,6 ng/band
- 161,5 ng/band
- 136 ng/band

→ LOD approximately at 120 ng/band

Limit of detection (LOD)

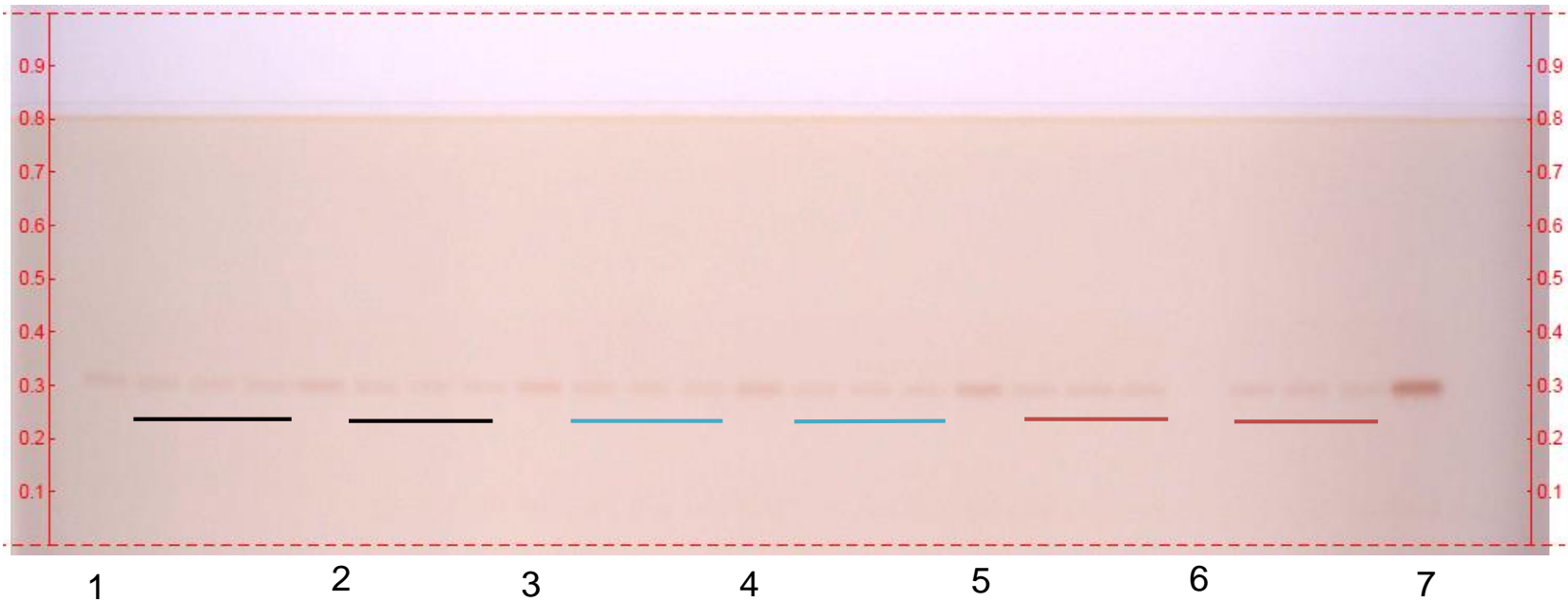
Track 2, ID: 123.6 ng



- 123,6 ng/band
- 161,5 ng/band
- 136 ng/band

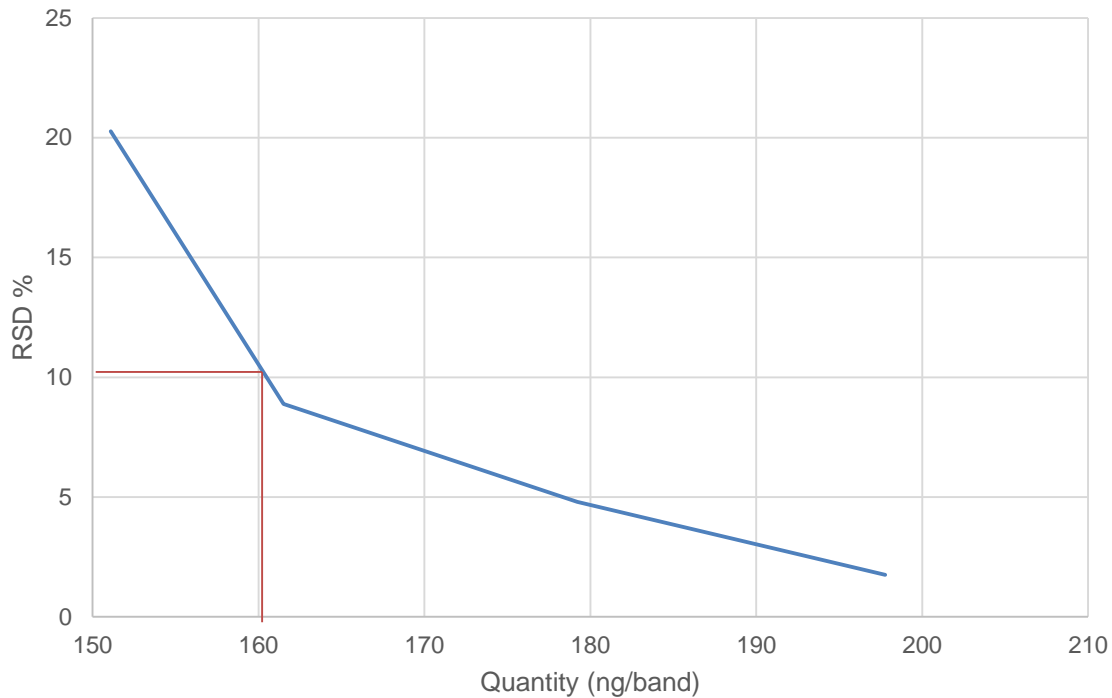
→ LOD approximately at 120 ng/band

Limit of quantification (LOQ)



- 136 ng/band
- 151,1 ng/band
- 161,5 ng/band

Limit of quantification



RSD acceptable : 10 % → LOQ ~ 160 ng/band

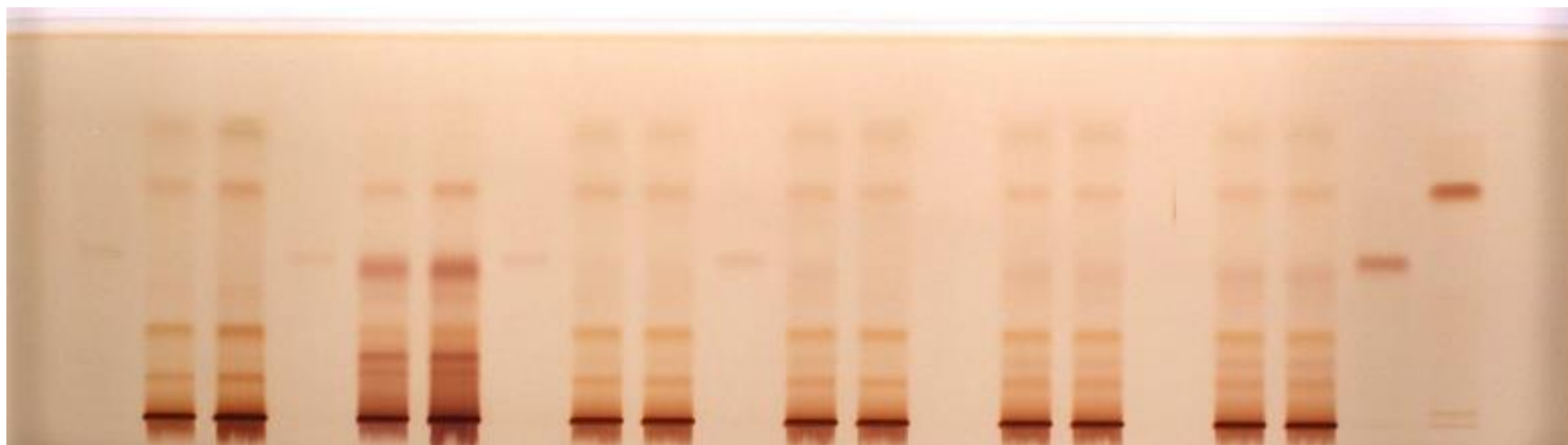
Quantification method Validation as per ICH guidelines:

Summary :

Parameters	TLC-densitometric method
Specificity	On going
Linear range	200 – 250 ng/band
Linear equations	0,8176 x – 57,32
R2	0,99226
Precision (%RSD)	
Repeatability	8,88
Intermediaire precision	On going
LOD	~ 120 ng/band
LOQ	~ 160 ng/band
Accuracy	On going
Average recovery	On going

Results and discussion

« Artificial » adulteration :



Standard Pine bark Standard Peanut skin 1% Peanut skin 5% Peanut skin 10% Peanut skin 15% Peanut skin Catechin

Substance	Rf	X(average)	CV [%]	n	Regression
Procyanidin A2	0.32	651.54 ng	3.349	2	MiMe 2

Sample from vial C3: 99% Pine bark KIR0411NTX + 1% Peanut skin

Result via height

Substance	Rf	X(average)	CV [%]	n	Regression
Procyanidin A2	0.31	135.72 ng	2.972	2	MiMe 2

LOD < Sample < LOQ

Sample from vial C4: 95% Pine bark KIR0411NTX + 1% Peanut skin

Result via height

Substance	Rf	X(average)	CV [%]	n	Regression
Procyanidin A2	0.31	164.27 ng	0.000	1	MiMe 2

Sample from vial C5: 90% Pine bark KIR0411NTX + 10% Peanut skin

Result via height

Substance	Rf	X(average)	CV [%]	n	Regression
Procyanidin A2	0.31	189.32 ng	0.519	2	MiMe 2

Results and discussion

Results on extracts:



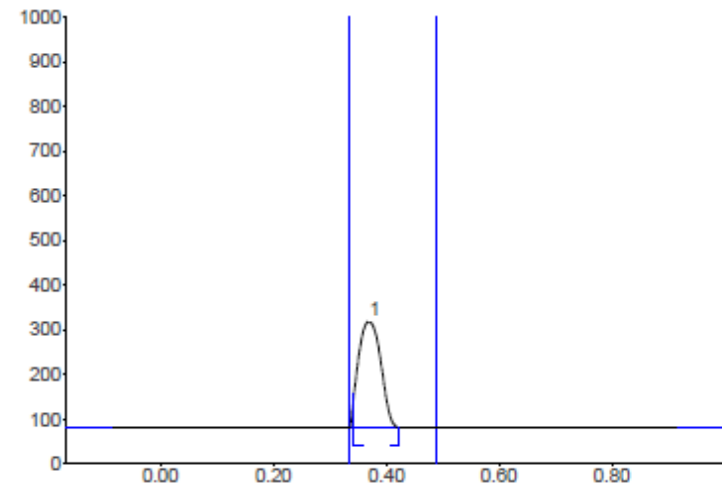
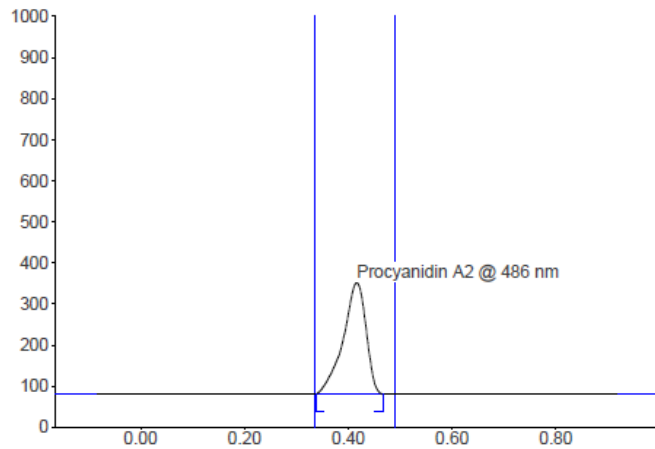
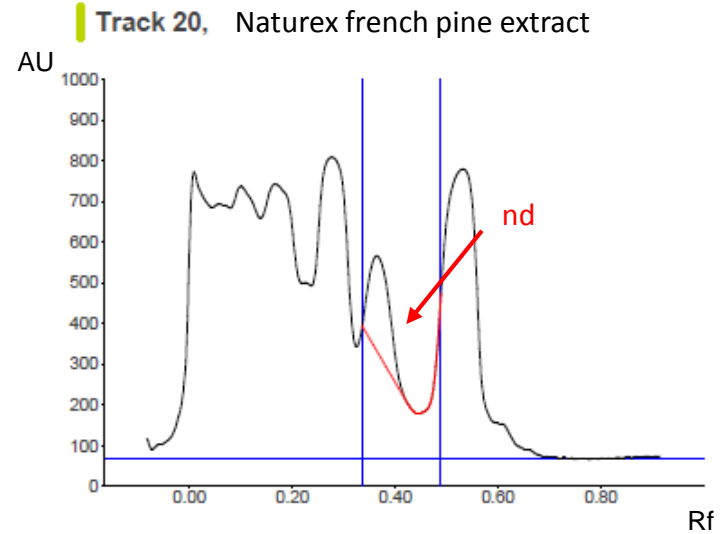
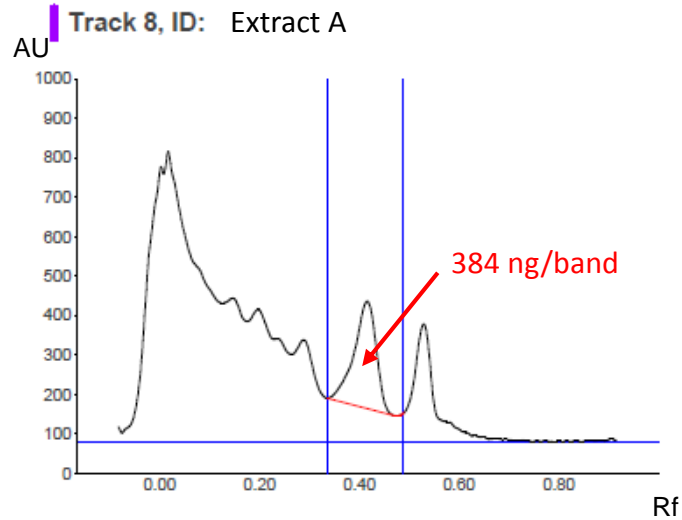
PA2 = Procyanidin A2

- A, B, C and D = competitor extracts adulterated
- PA2 absent on Naturex french pine extract

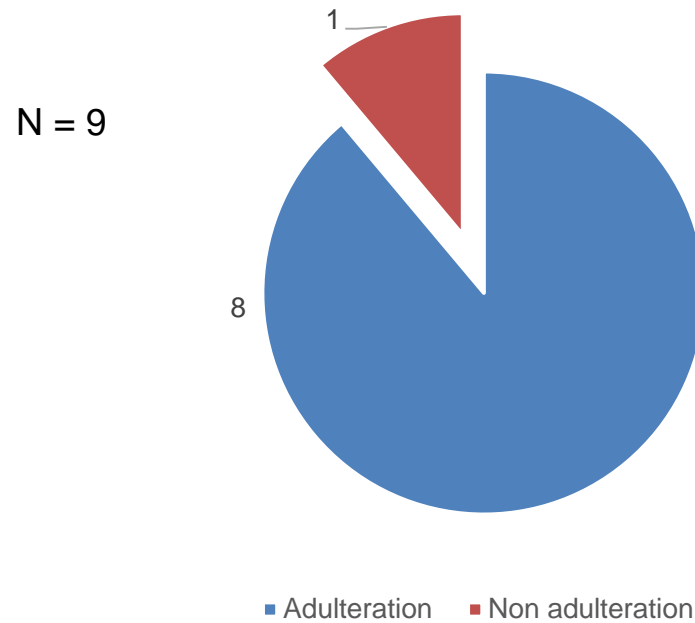
	Gamme étalon (ng/band)
PA2 - 1	179,220
PA2 - 2	197,200
PA2 - 3	219,095
PA2 - 4	246,500
PA2 - 5	325,570
PA2 - 6	493,000
PA2 - 7	986,000

Results and discussion

Results on extracts:

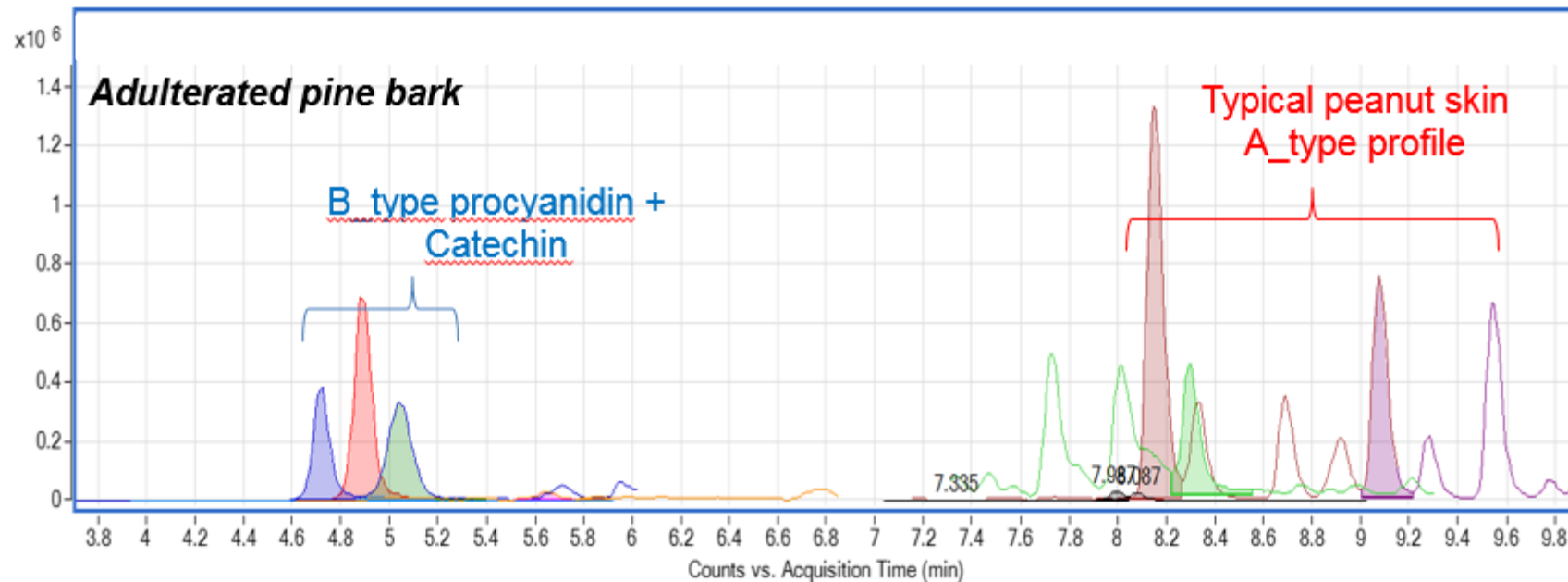


repartition of adulteration



Results and discussion

Examination by HPLC/MS_TOF



Extracts	HPLC-MS-TOF PA2	HPTLC PA2
A	d	d
B	d	d
French pine extract	nd	nd
Peanut skin	d	d

- Admixture of Pine bark products is a significant problem : 8 commercial products contained detectable quantities of peanut skin admixture;
- Procyanidin A2 was found as a good marker for control quality of Peanut skin addition on Pinus spp.
- HPTLC method validation is ongoing but we can already demonstrates simple, fast as with able of detect Peanut Skin in the Pinus spp extracts;
- Validation of the quantitative method of procyanidin A2 by HPTLC-densitometry is on going as per ICH guidelines and preliminary results are encouraging.

Don J. Durzan. Arginine, scurvy and Cartier's "tree of life." *Journal of Ethnobiology and Ethnomedicine* 2009, 5:5. Available on line <http://www.ethnobiomed.com/content/5/1/5>

Kyriazi, M., D.Yova, M.Rallis, and A.Lima. 2006. Cancer chemopreventive effects of *Pinus Maritima* bark extract on ultraviolet radiation and ultraviolet radiation-7,12, dimethylbenz(a)anthracene induced skin carcinogenesis of hairless mice. *Cancer Letters* 237:234-241.

Furumura, M., N.Sato, N.Kusaba, K.Takagaki, and J.Nakayama. 2012. Oral administration of French maritime pine bark extract (Flavang/bandenol((R))) improves clinical symptoms in photoaged facial skin. *Clin. Interv. Aging* 7:275-286.

Suzuki, N., K.Uebaba, T.Kohama, N.Moniwa, N.Kanayama, and K.Koike. 2008. French maritime pine bark extract significantly lowers the requirement for analgesic medication in dysmenorrhea: a multicenter, randomized, double-blind, placebo-controlled study. *J Reprod Med* 53.

Ledda, A., G.Belcaro, M.R.Cesarone, M.Dugall, and F.Schonlau. 2010. Investigation of a complex plant extract for mild to moderate erectile dysfunction in a randomized, double-blind, placebo-controlled, parallel-arm study. *BJU. Int.* 106:1030-1033.

Villani et al. Chemical investigation of commercial grape seed derived products to assess quality and detect adulteration. *Food Chemistry* 170 (2015) 271–280 .

Rimbach et al. Activity of monomeric, dimeric, and trimeric flavonoids on NO production, TNF- α secretion, and NF- κ B- dependent gene expression in RAW 264,7 macrophages. *Febs Letters*, 165 (2000) 93-97 .

Sudberg et al. Validation of a High Performance Thin-Layer Chromatographic Fingerprint Method for the Simultaneous Identification of Grape Seed and Peanut Skin and the Adulteration of Commercial Grape Seed Extract with Peanut Skin. Poster presentation.

Acknowledgements!

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l.falcao@naturex.com