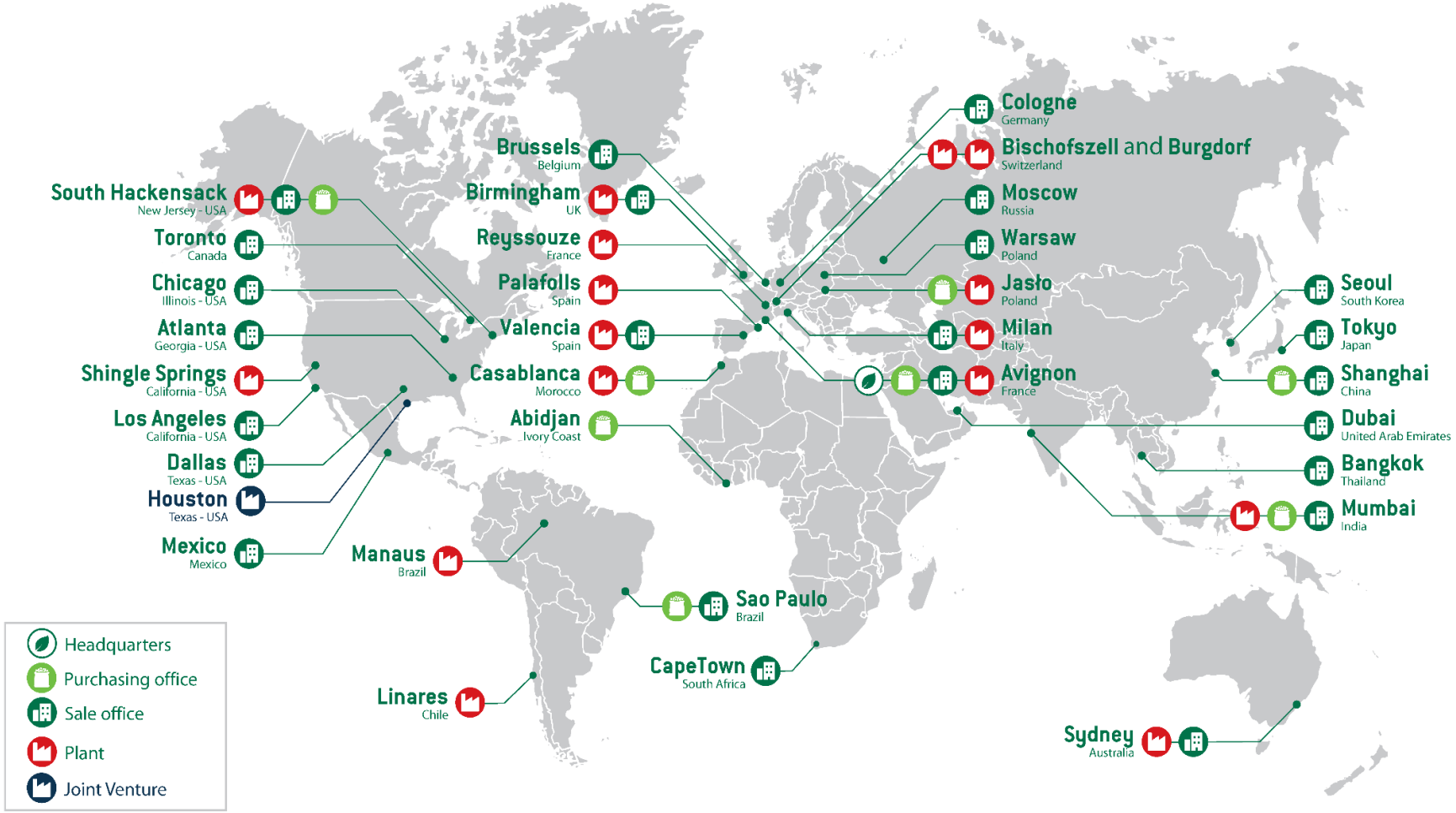
Admixture of Peanut skin on Pinus spp extracts : Use of HPTLC-densitometry to ensure Pinus spp extracts quality

-

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

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

Naturex overview
Locations



Naturex overview

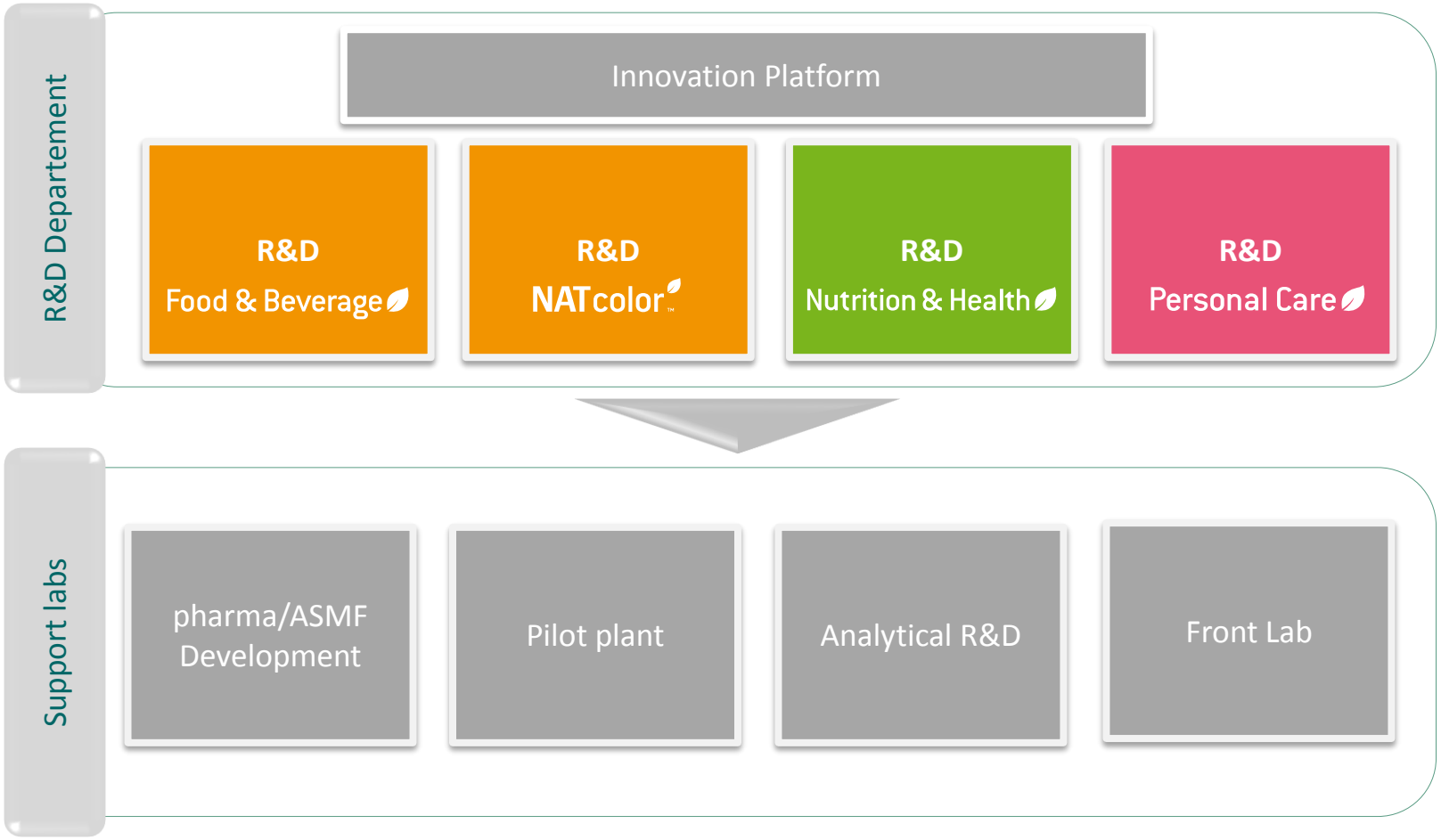
Research & Development

75
17 Ph. D
30 Eng/bandineers
28 Technicians

2 000
Developments / year

5 to 10
Scientific publications / year

54
Patent families



Naturex overview

Strategy for botanical identity testing

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

Introduction

Pine bark

Family : *Pinaceae*
Genus : Pinus

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

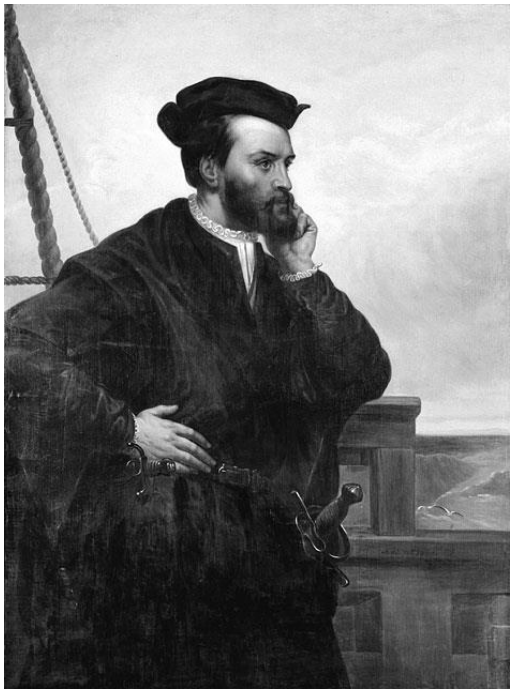
Introduction

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

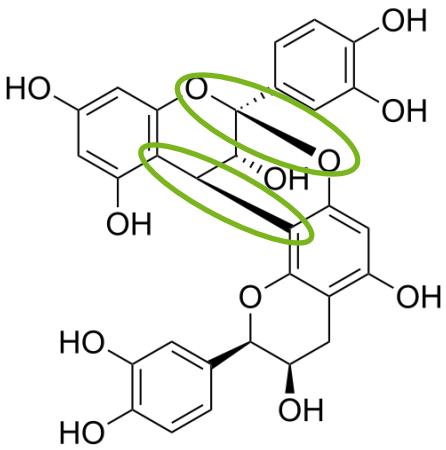
Procyanidins = Biopolymers of (+) - catechin and (-) - epicatechin subunits.

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

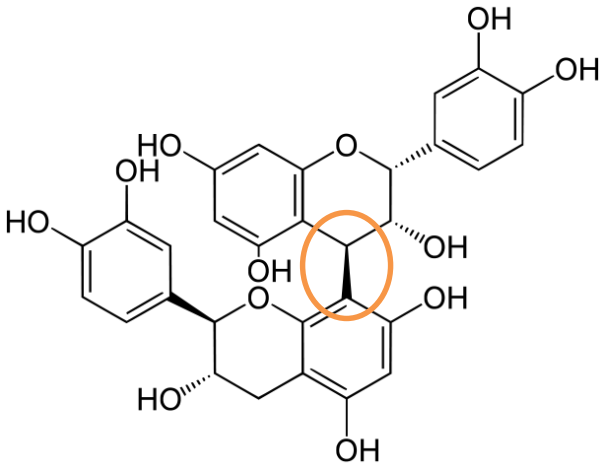
Ex : cinnamon bark, litchi pericarp, peanut, bilberry, grape seeds…

Protection against light, oxidation and predators

Various types of Procyanidins, based on linkage between monomeric units



A-type

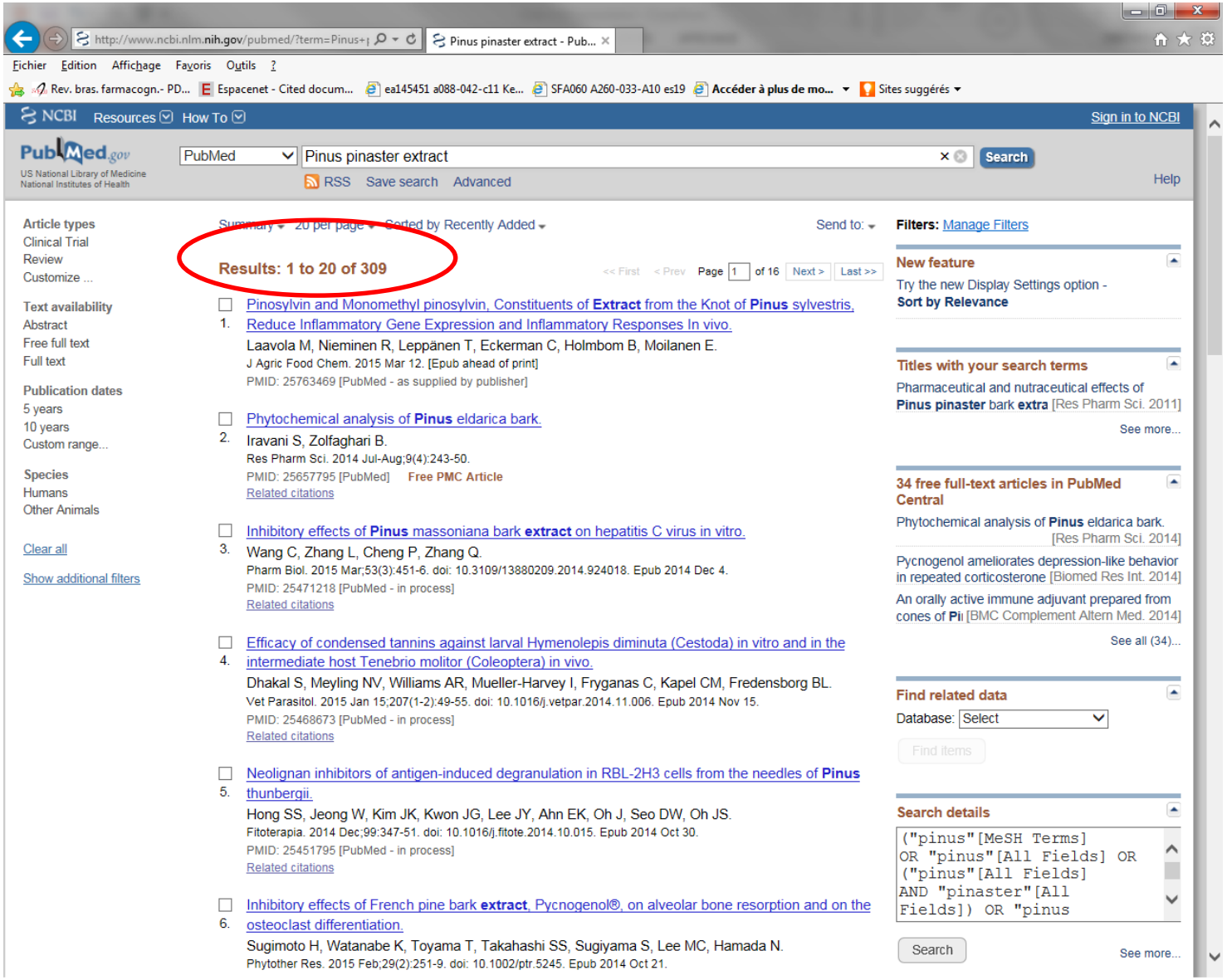


B-type
(4β→8)

Introduction

Pine bark

NATUREX



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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).

Introduction

Peanut skin

Family: Fabaceae
Genre: Arachis
Arachis hypogaea L.

Safety concern : allergen

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

Introduction

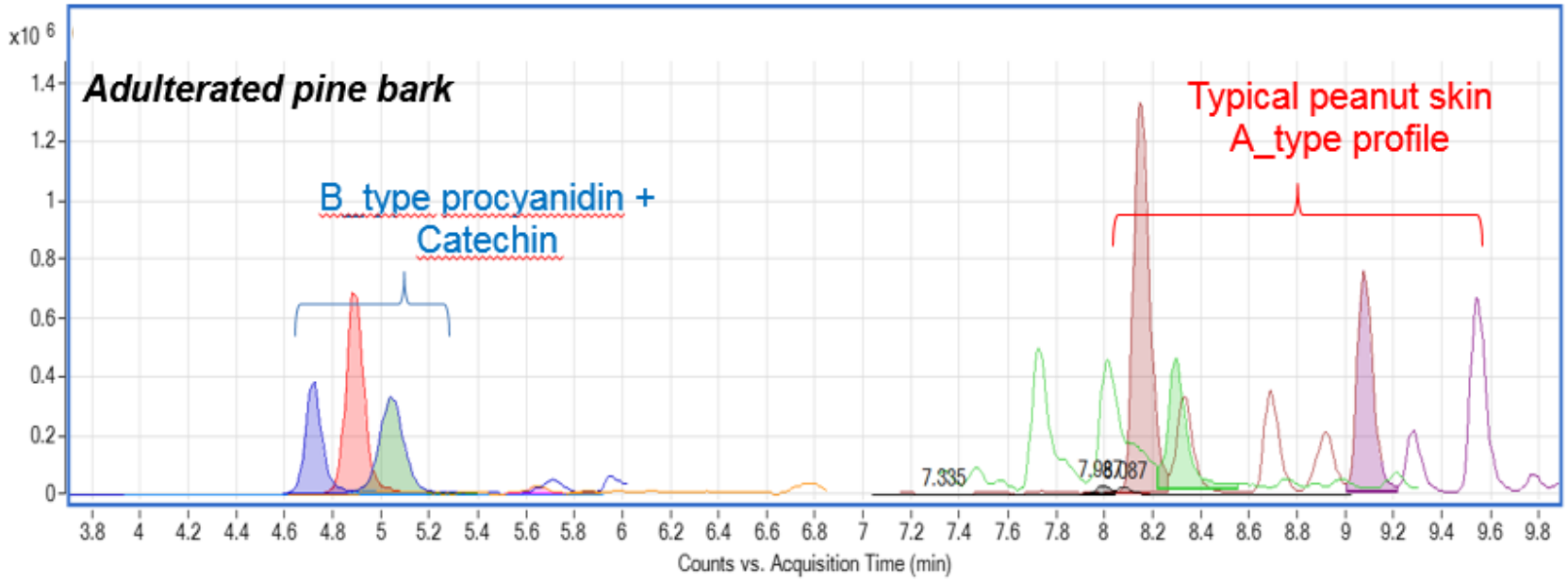
Procyanidins composition

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).

Examination by HPLC/MS_TOF



Motivation Research and Objectives

NATUREX

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.

Materials and methods

Materials

CAMAG Automatic TLC Sampler 4

CAMAG Automatic Developing Chamber 2

CAMAG TLC Vizualizer

CAMAG TLC Scanner 4

Materials and methods

NATUREX

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

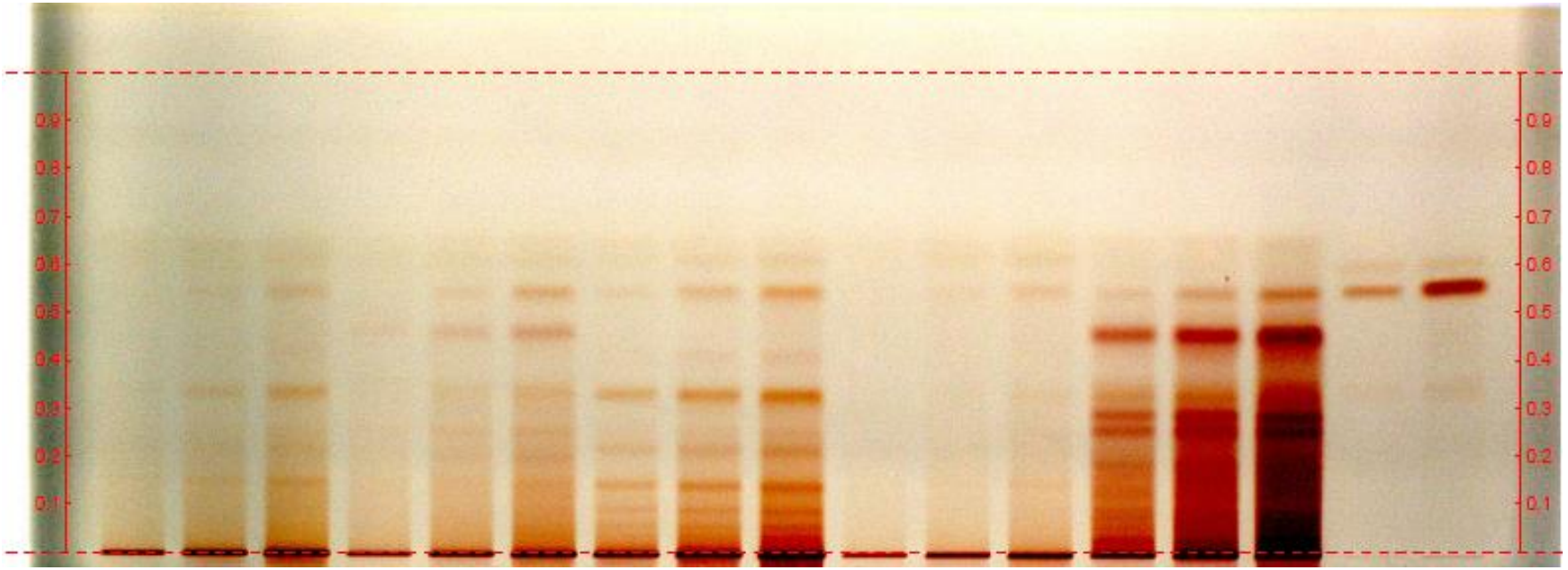
Parameters:

- **Sample preparation**: 1g of powdered raw material/extracts with 10 ml of EtOH/H_20 (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 F_{254}
- **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:

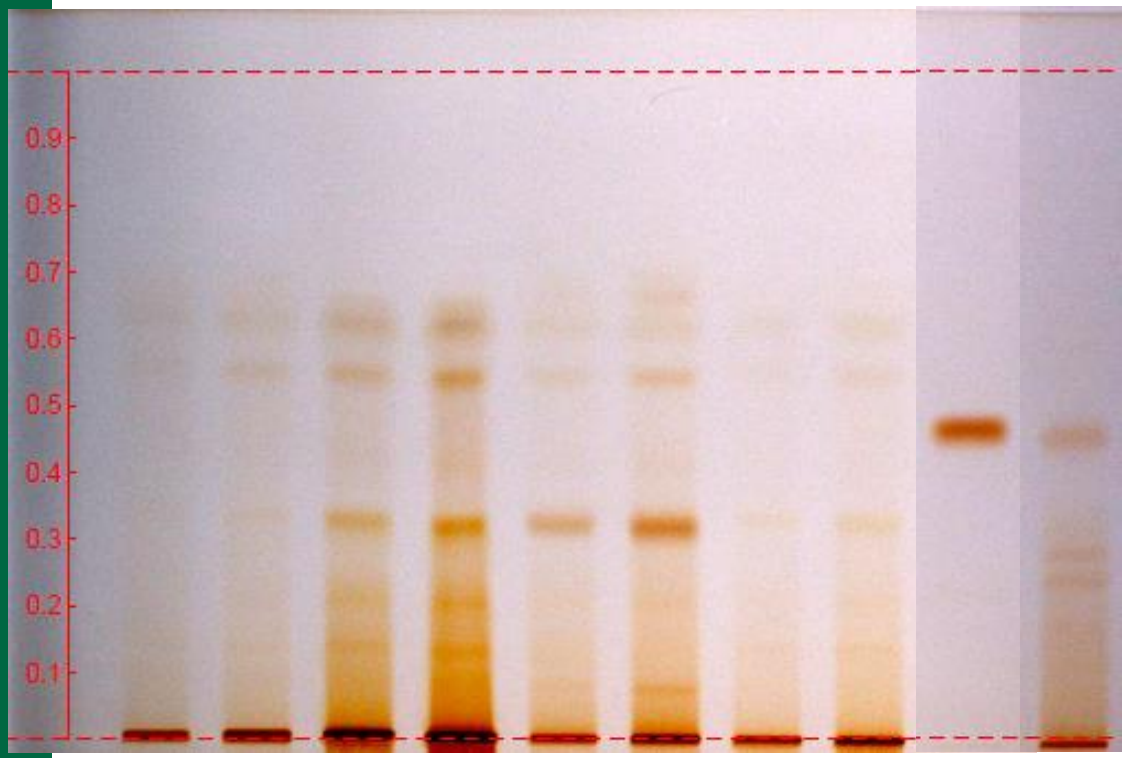


Humidity control: 39 %

Results and discussion

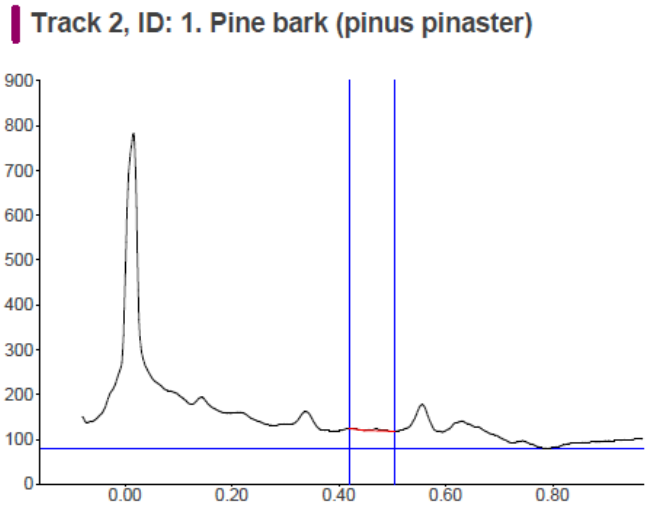
Preliminary results:

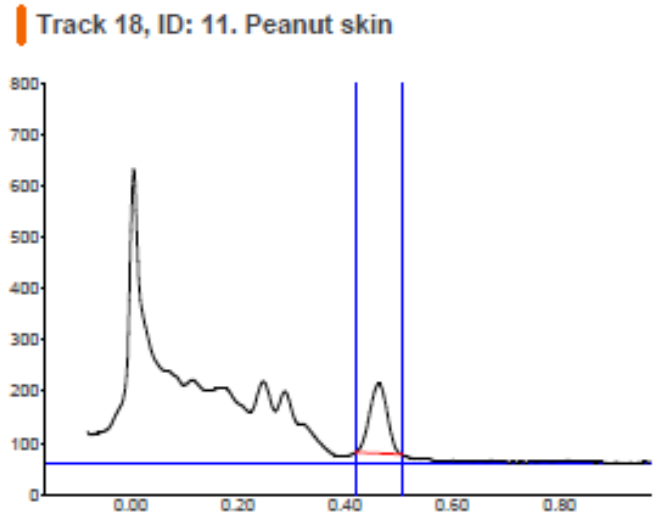
PA2 = Procyanidin A2



Pinus pinaster | Pinus spp | Pinus strobus | Pinus massoniana | PA2 | Peanut skin

Profiles UV at 486 nm

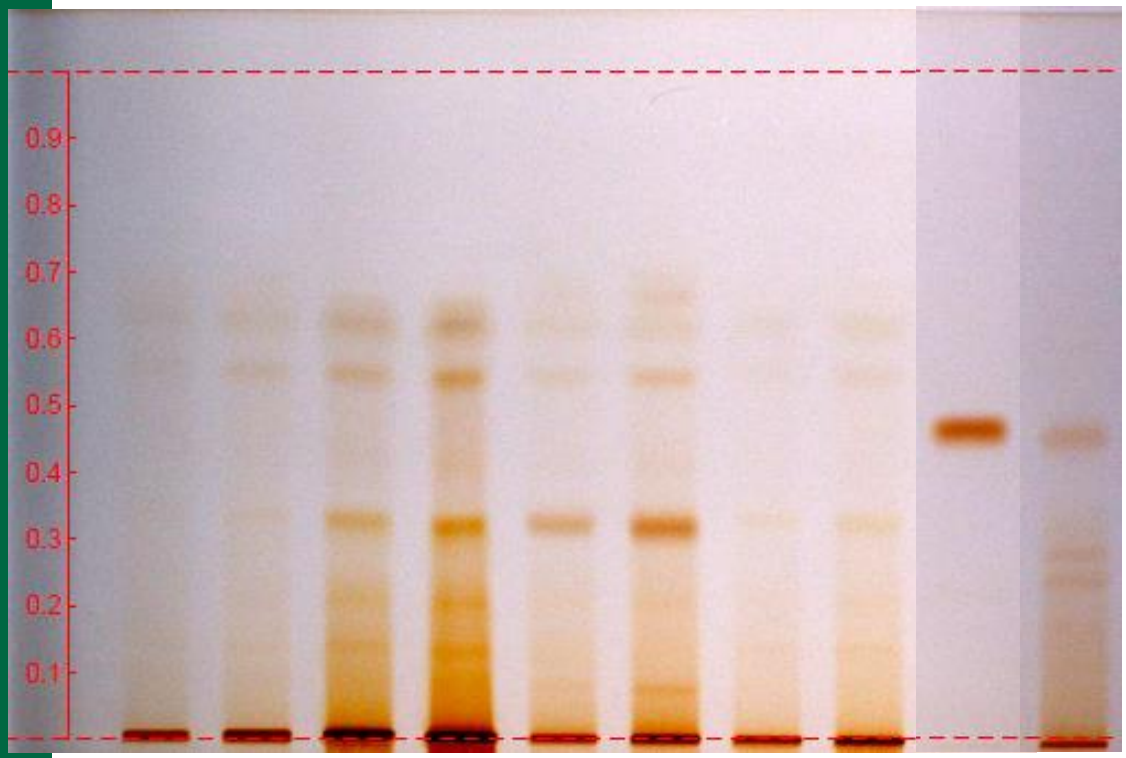




Results and discussion

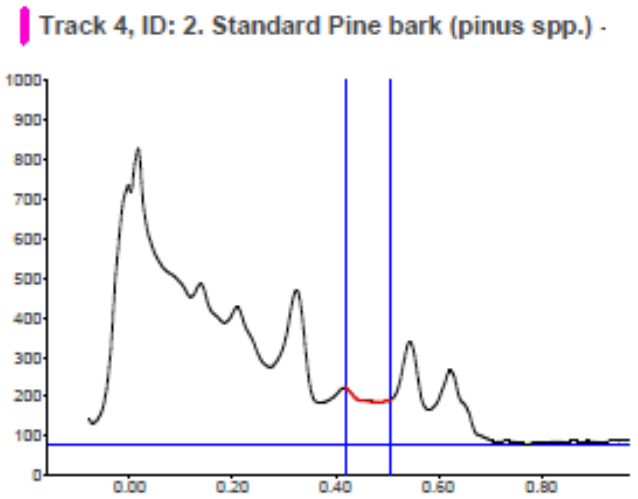
Preliminary results:

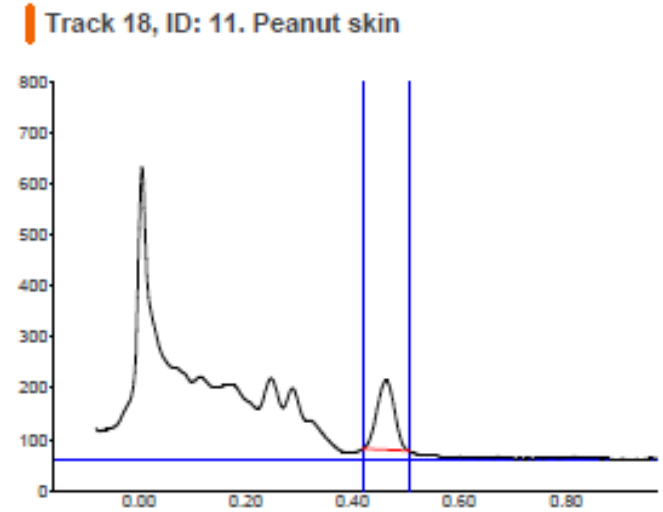
PA2 = Procyanidin A2



Pinus pinaster | Pinus spp | Pinus strobus | Pinus massoniana | PA2 | Peanut skin

Profiles UV at 486 nm





Results and discussion

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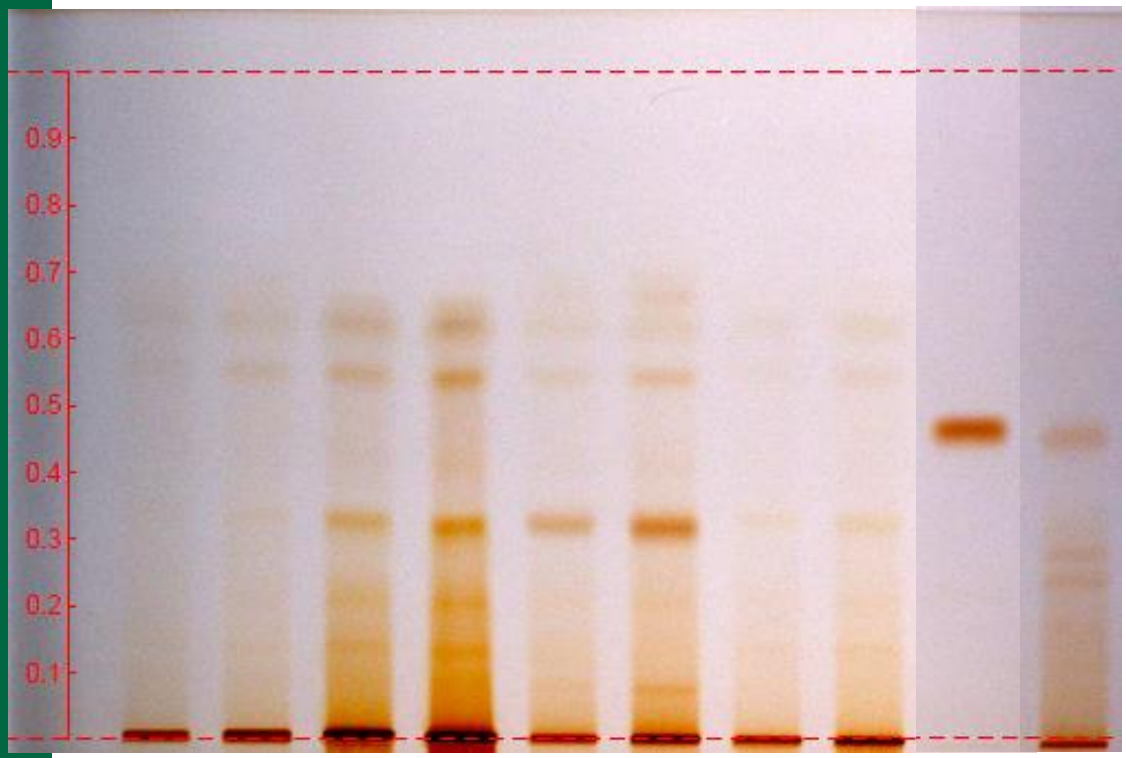
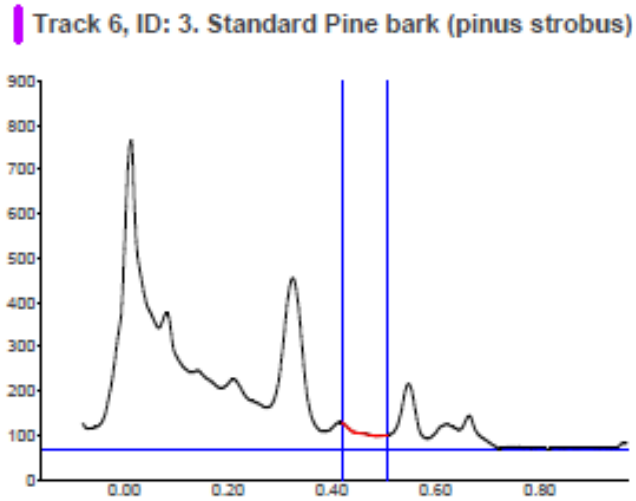
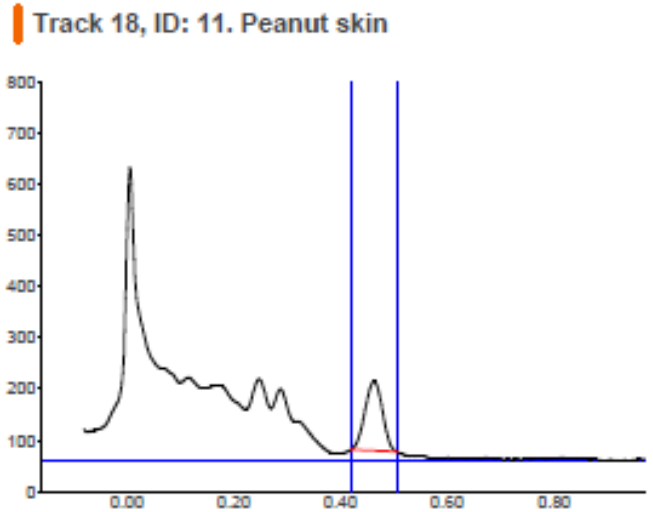
PA2 = Procyanidin A2



Pinus pinaster | Pinus spp | Pinus strobus | Pinus massoniana | PA2 | Peanut skin

Profiles UV at 486 nm





Results and discussion

Quantification method Validation as per ICH guidelines:

NATUREX

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

Results and discussion

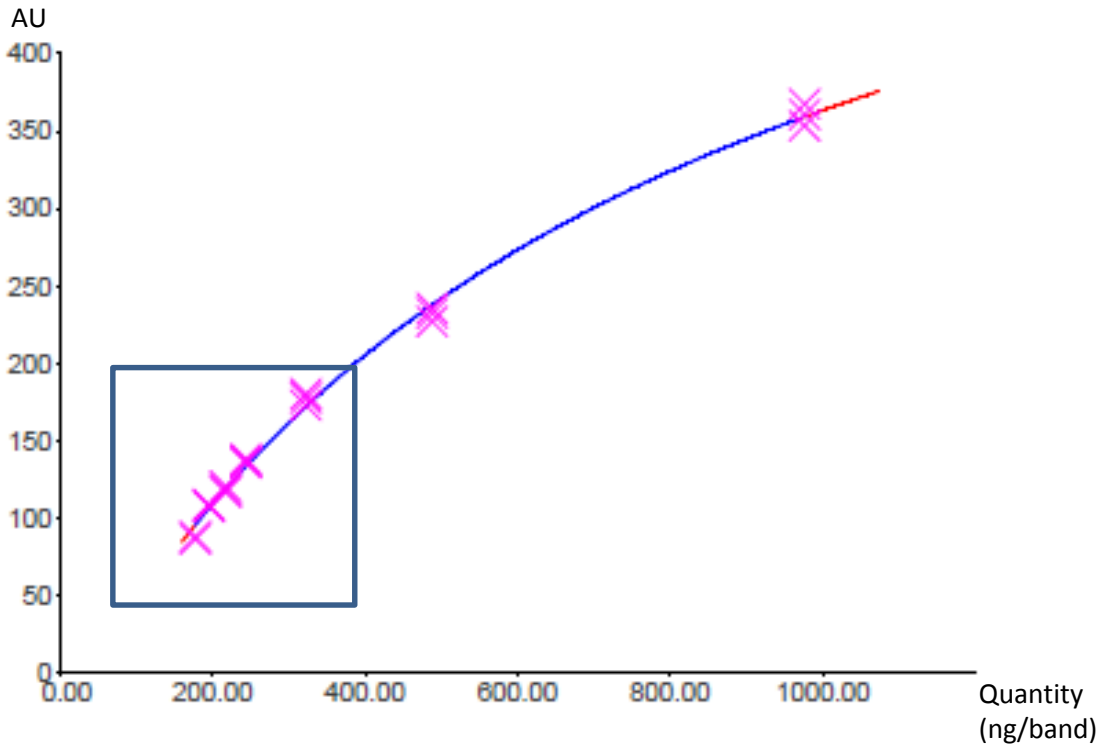
Quantification method Validation as per ICH guidelines:

NATUREX

Linearity

Substance: Procyanidin A2 @ 486 nm

Regression via height: Michaelis-Menten 2 Y = -36.25 + (708.4 * X) / (772.2 + X) sdv = 3.16

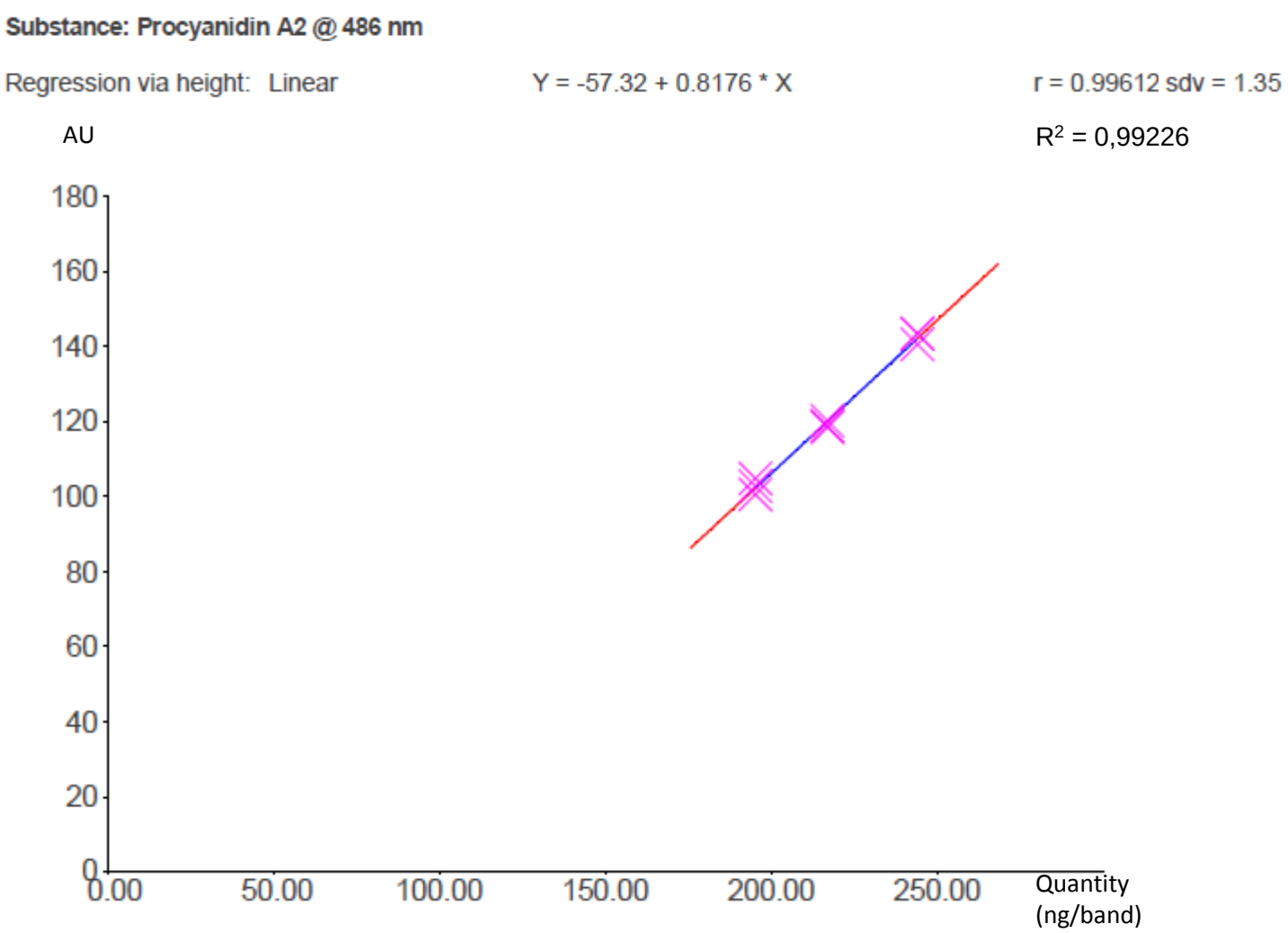


Results and discussion

Quantification method Validation as per ICH guidelines:

NATUREX

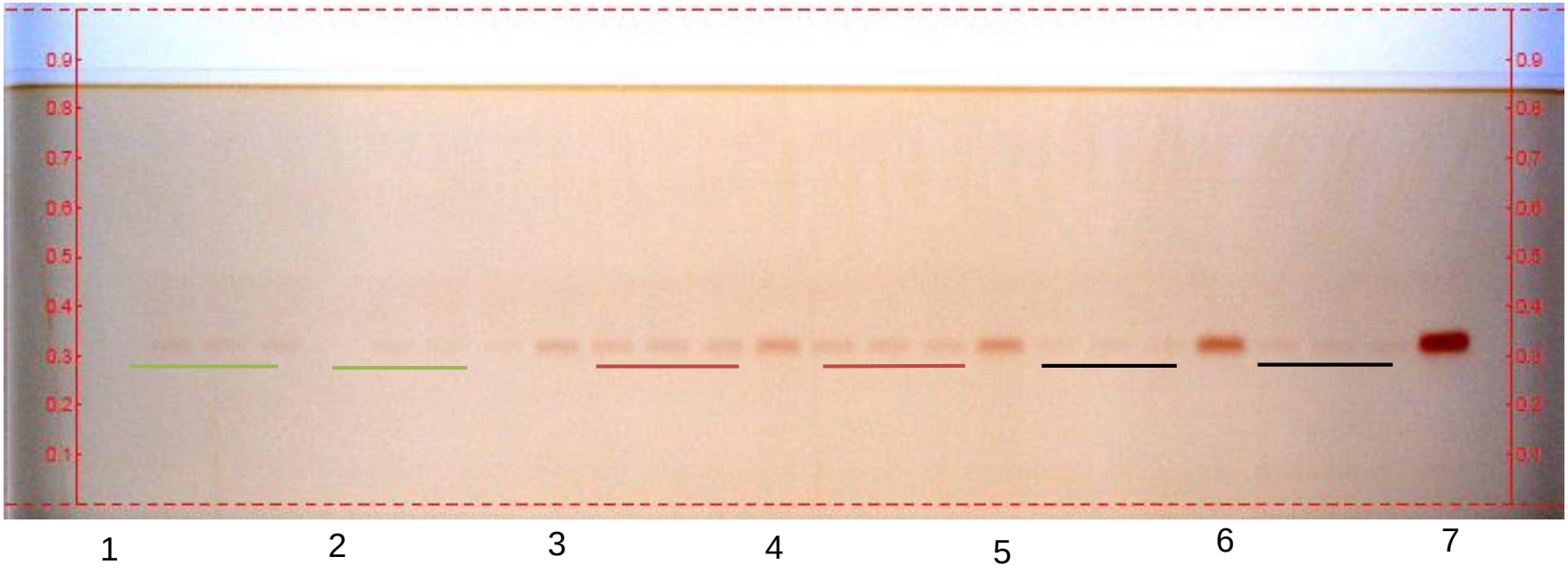
Linearity



Results and discussion

NATUREX

Limit of detection (LOD)



123,6 ng/band

161,5 ng/band

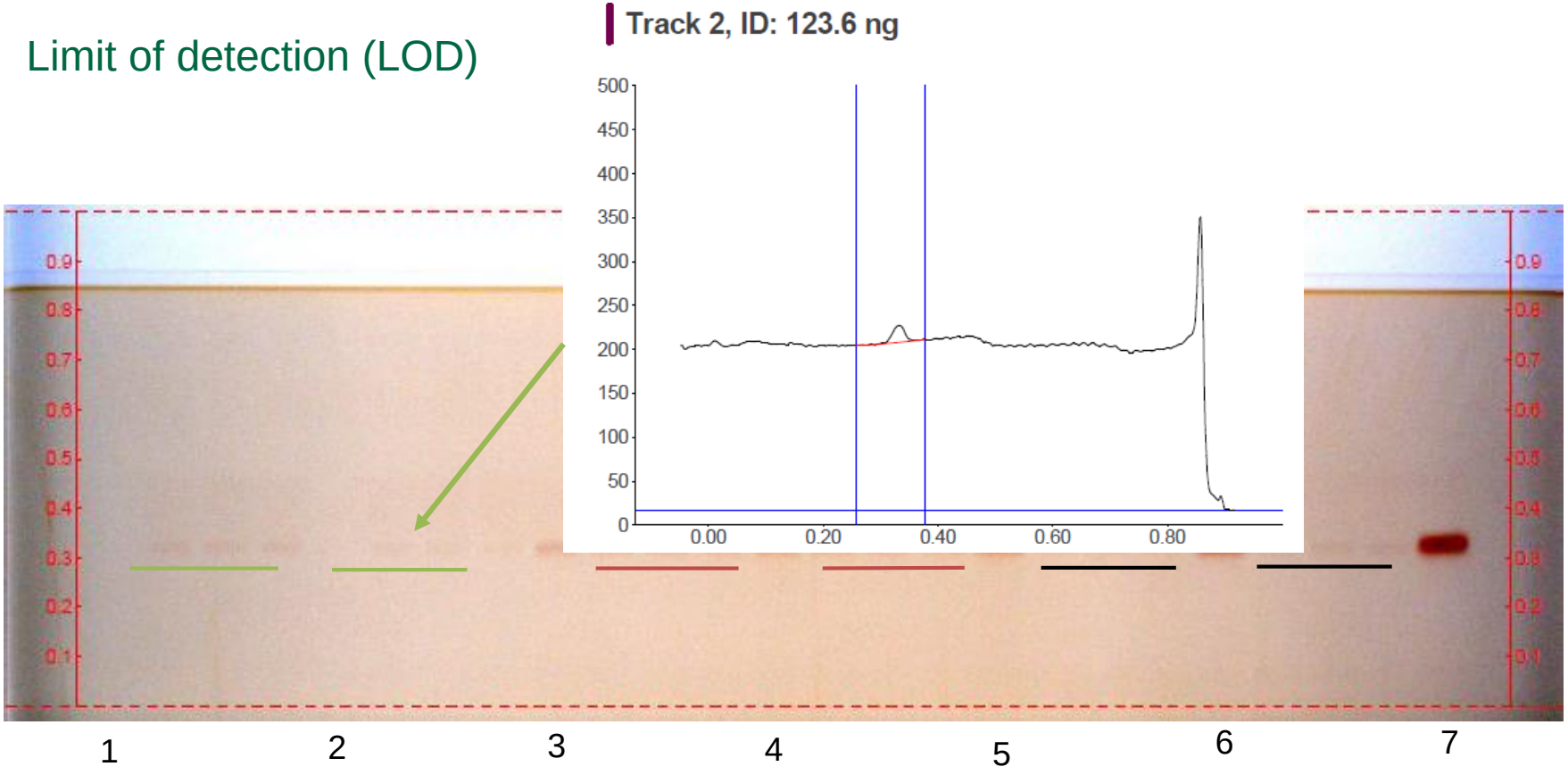
136 ng/band

→ LOD approximately at 120 ng/band

Results and discussion

NATUREX

Limit of detection (LOD)



123,6 ng/band

161,5 ng/band

136 ng/band

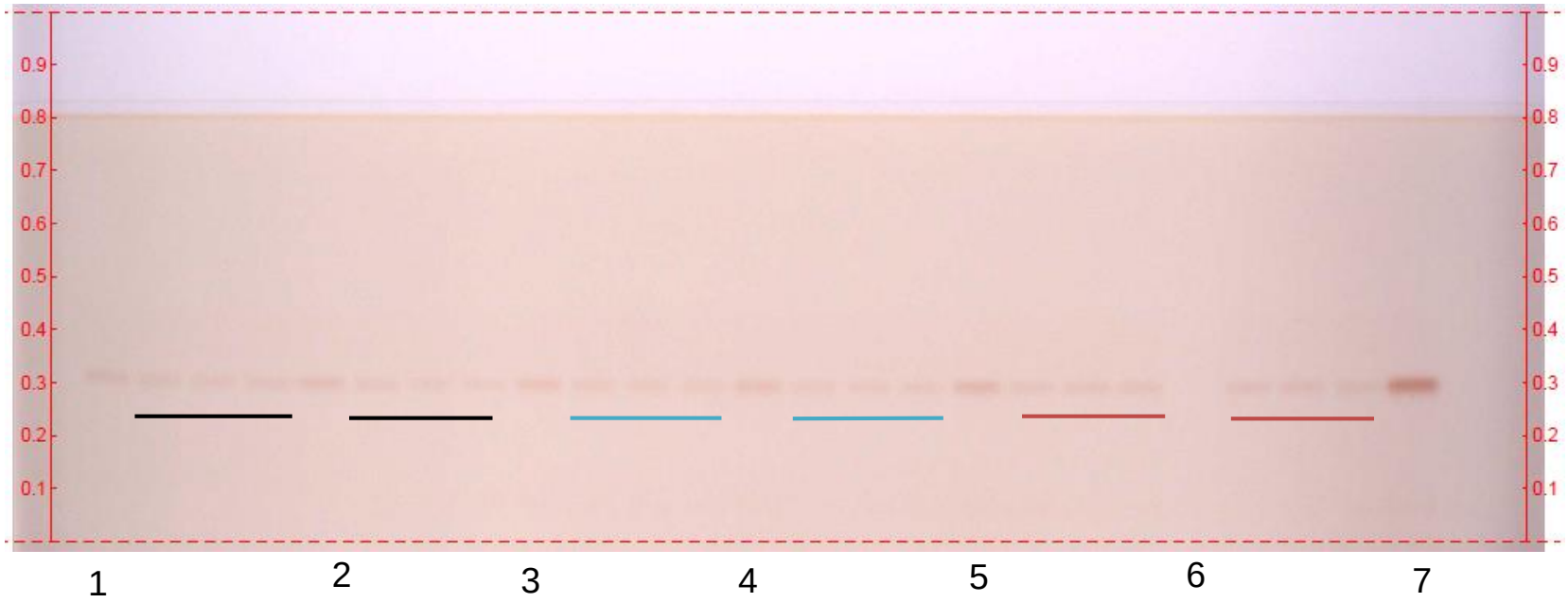
→ LOD approximately at 120 ng/band

Results and discussion

Quantification method Validation as per ICH guidelines:

NATUREX

Limit of quantification (LOQ)



136 ng/band

151,1 ng/band

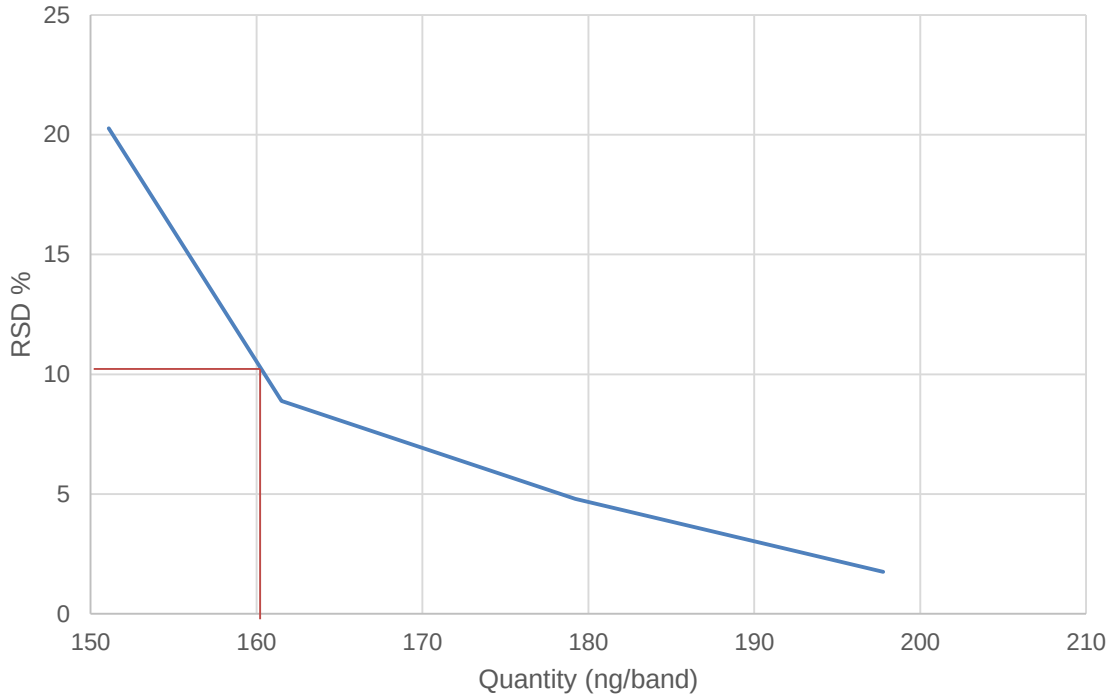
161,5 ng/band

Results and discussion

Quantification method Validation as per ICH guidelines:

NATUREX

Limit of quantification



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

Results and discussion

Quantification method Validation as per ICH guidelines:

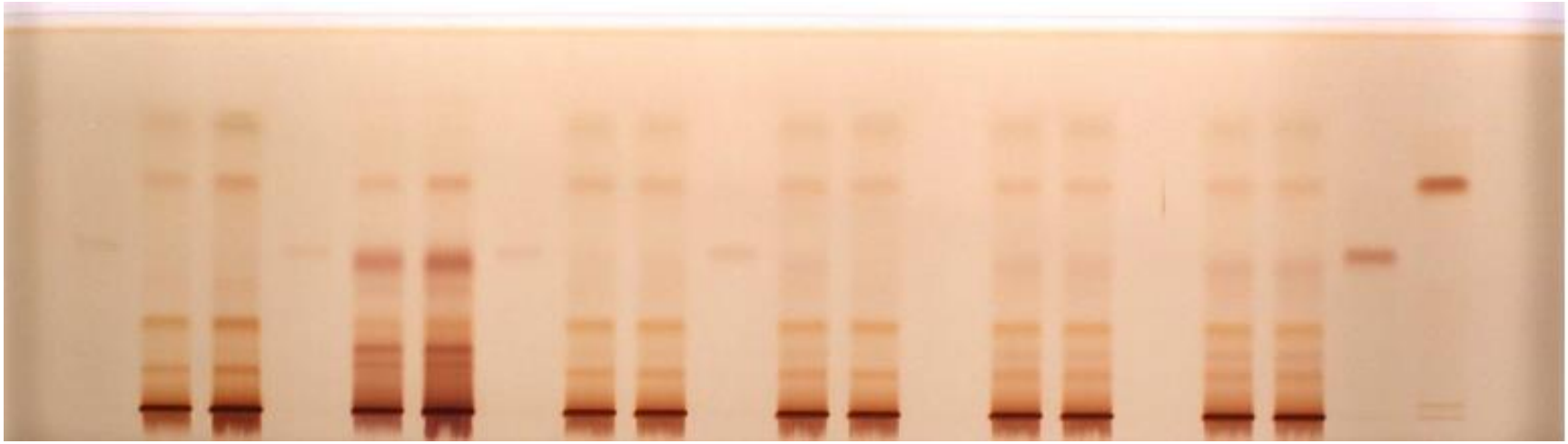
NATUREX

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

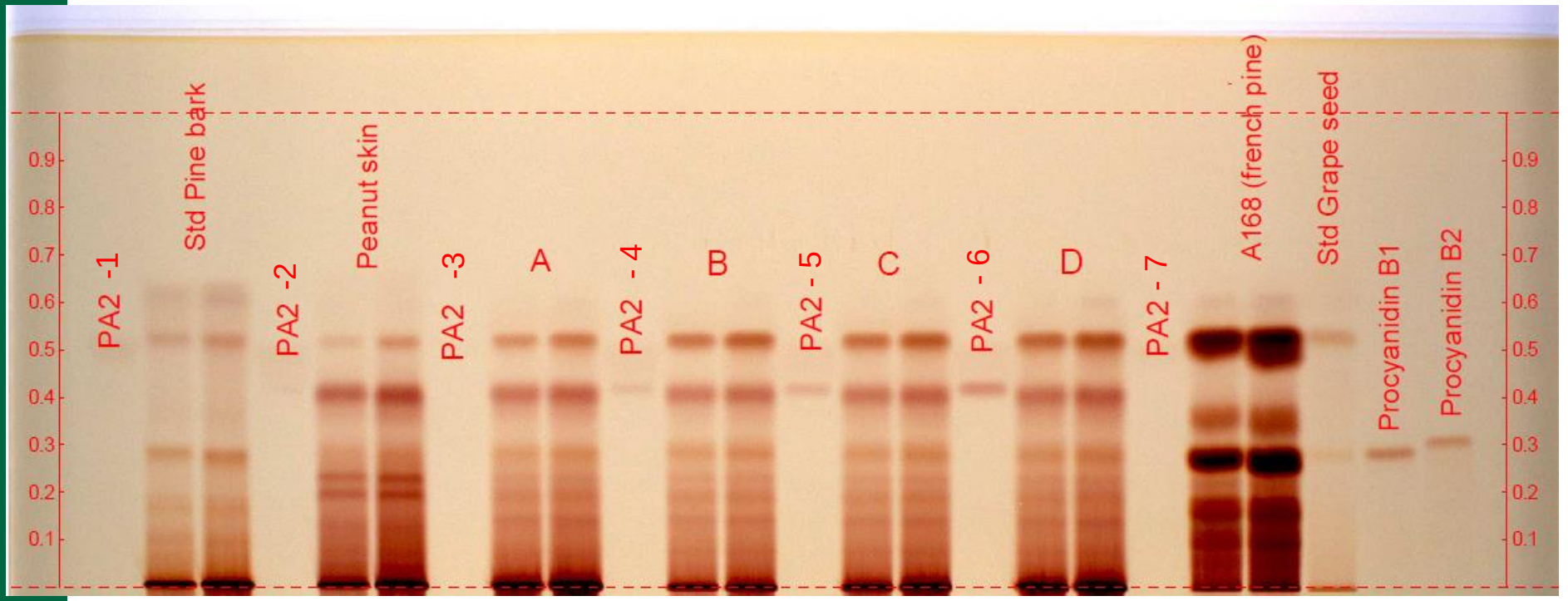
Sample from vial C5: 90% Pine bark KIR0411NTX + 10% Peanut skin

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:



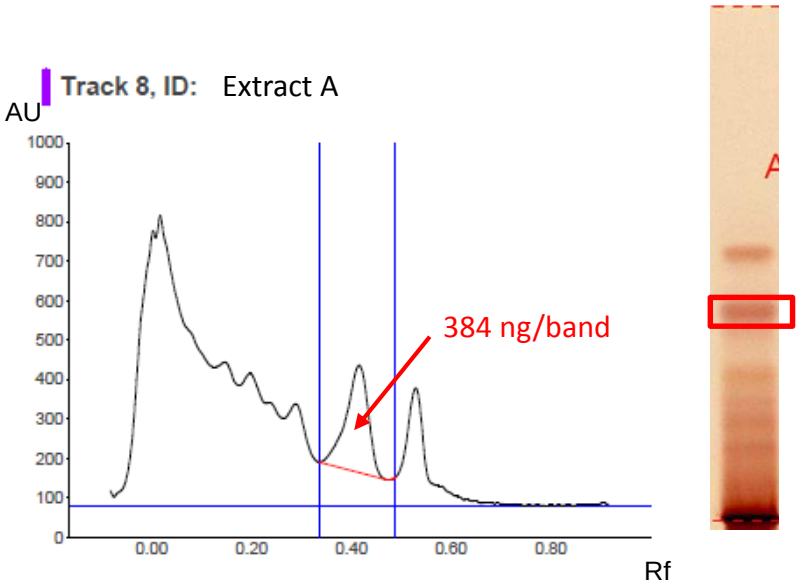
PA2 = Procyanidin A2

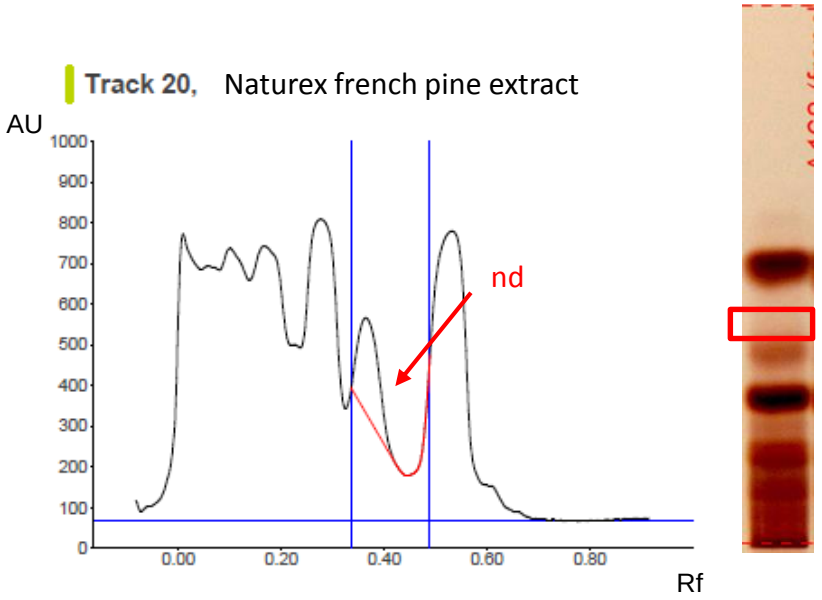
- A, B, C and D = competitor extracts adultered
- PA2 absent on Naturex french pine extract

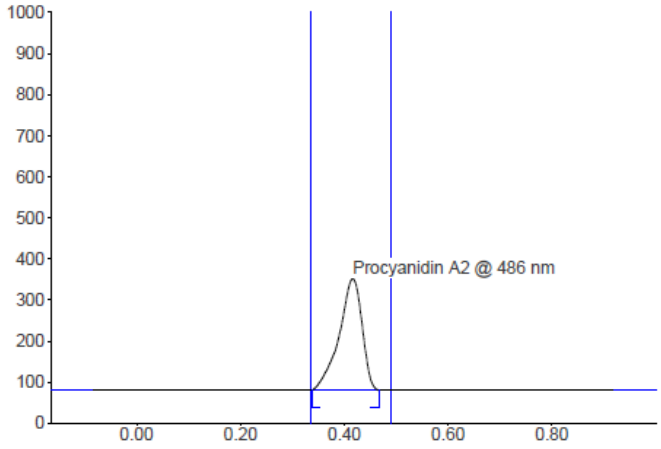
	Gamme étalon (ng/band)
PA2 - 1	179,220
PA2 - 2	197,200
PA2 - 3	219,095
PA2 - 4	246,500
PA2 - 5	325,570
PA2 - 6	493,000
PA2 – 7	986,000

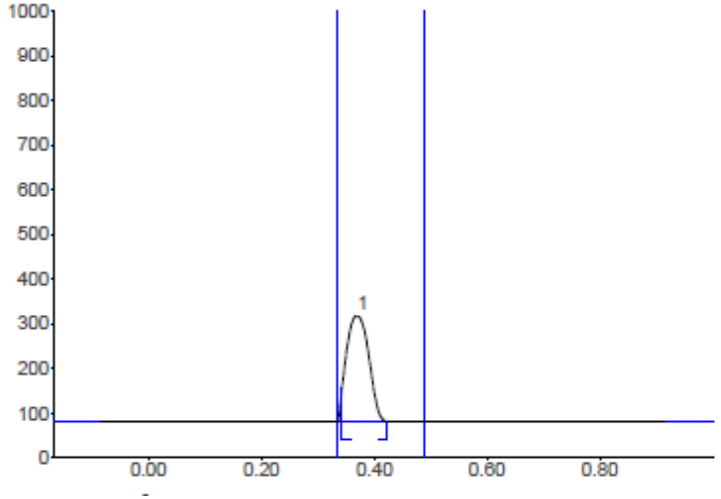
Results and discussion

Results on extracts:



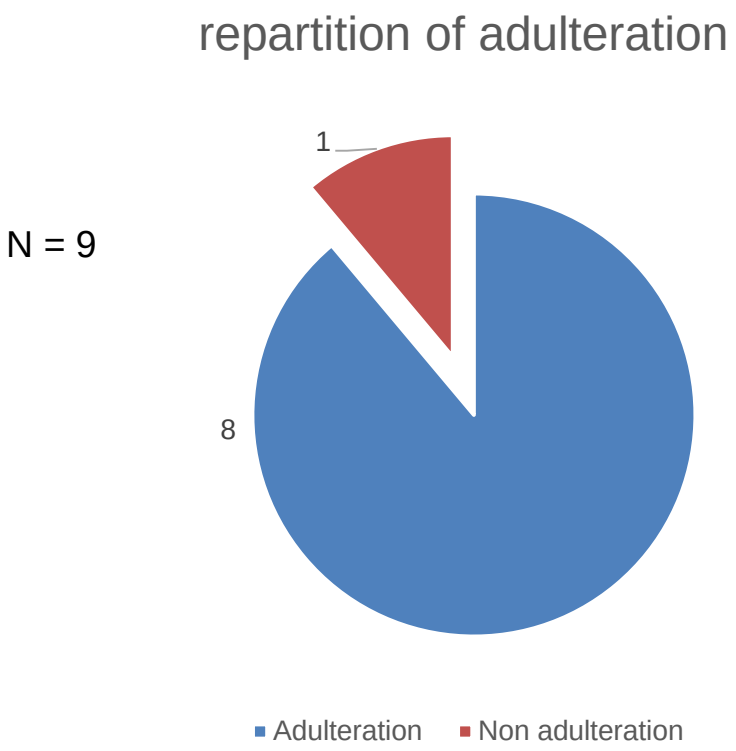






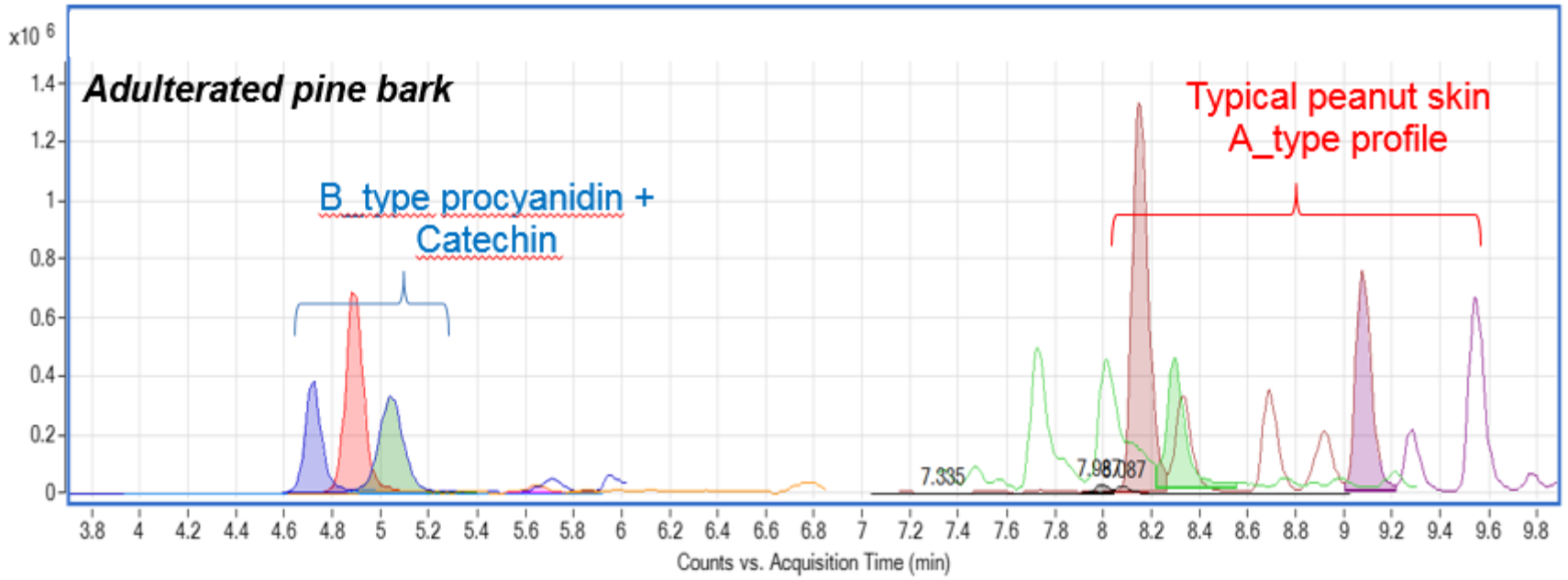
Results and discussion

Results on extracts:



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

Conclusion

- 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.

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Acknowlegdements!

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