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**CCC4LABPREP FOR THE QUATTRO CCC™ AND ADVANCED COUNTER CURRENT CHROMATOGRAPHY
SEPARATION OF PYRIDINE DERIVATIVES WITH ANTIOXIDANT ACTIVITY FROM LANTANA
TRIFOLIA BY THE USE OF STEP GRADIENT HSCCC**

Collaborative CCC / bioactivity research between Universidade Federal do Rio de Janeiro, Brazil;
Unversita Cattolica del Sacro Cuore, Italy and AECS (Quattro Instruments), UK

**SEPARATION OF PYRIDINE
DERIVATIVES WITH ANTIOXIDANT
ACTIVITY FROM *LANTANA TRIFOLIA*
BY THE USE OF STEP GRADIENT
HSCCC**

**Suzana G. Leitão^{1,*}, Leslie Brown², Gilda G. Leitão³,
Samira El-Adji³ and Franco Delle Monache⁴,**

**¹Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Rio de
Janeiro, Brazil, sgleitao@ufrj.br**

²AECS, PO Box 80, Bridgend, South Wales, UK,

³Núcleo de Pesquisas de Produtos Naturais-UFRJ,Brazil,

⁴Università Cattolica del Sacro Cuore, Roma, Italy.

L. trifolia is a plant regularly used in folk medicine which on examination was found to contain bioactive anti-oxidants.

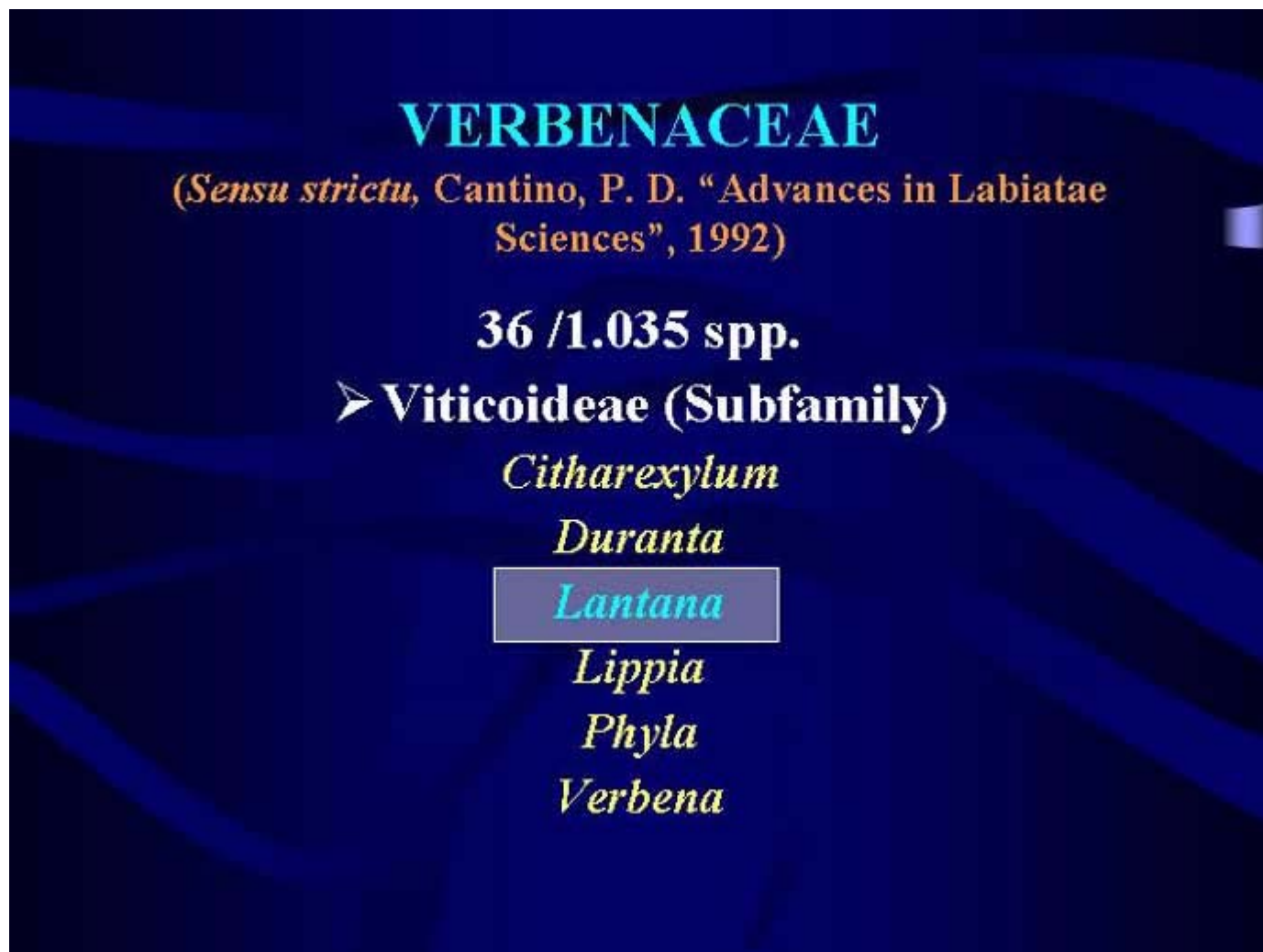
Lantana trifolia

- *Lantana* species are popularly known in Brazil as “Cambará”.
- They are used in the folk medicine for the treatment of innumerable conditions such as colds, rheumatism and respiratory distress.
- *Lantana trifolia* is found in Brazil mainly in the States of São Paulo and Minas Gerais.



The genus *Lantana* belongs to the Verbenaceae family and is an example of the genera still included in the Verbenaceae sensu strictu, according to Cantino, 1992 (Zomlefer, 1994).

The remaining 2/3 of the conventional Verbenaceae sensu lato have been transferred to the Lamiaceae sensu lato.



In our screening project for the search for anti-oxidative agents from Brazilian natural sources we have studied 71 Brazilian plant extracts, belonging to 16 species of 5 different families.

In that study, the ethanolic extract from the leaves of *Lantana trifolia* was very active, as can be seen from the EC-50 values shown in the Table.

SCREENING OF BRAZILIAN PLANT EXTRACTS FOR THE ANTIOXIDANT ACTIVITY BY THE USE OF DPPH FREE RADICAL METHOD

Mensor, L.L.; Menezes, F. S.; Leitão, G. G.; Reis, A.S.; Santos, T.C.; Coube, C.S. and Leitão, S.G.

Phytother. Res. 15, 127-130, 2001.

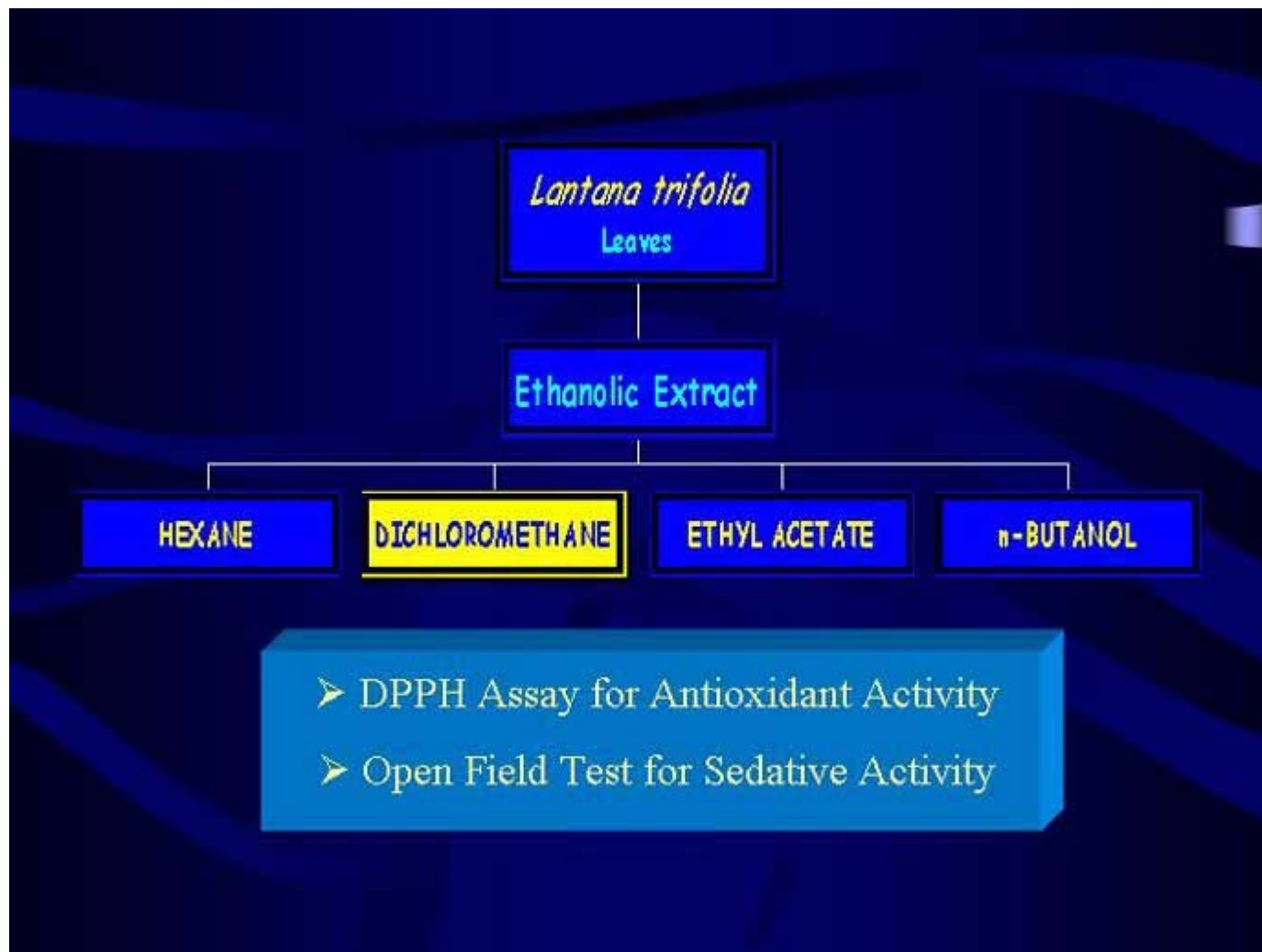
71 Plant Extracts → 16 spp. → 5 Families

TABLE 1 – EC50 Values of Ethanolic Plant Extracts. *Ginkgo biloba* was used as standard.

Family	Plant Species	Plant part/Organ	EC ₅₀ ± SD
Leguminosae	<i>Platypodium elegans</i>	Leaves	184.92 ± 2.69
	<i>Pseudopiptadenia contorta</i>	Leaves	13.84 ± 0.30
Moraceae	<i>Brosimum</i>	Leaves	119.31 ± 5.03
Verbenaceae	<i>guianense</i> <i>Lantana camara</i>	Leaves	40.99 ± 1.87
		Bark	114.40 ± 3.74
	<i>Lantana trifolia</i>	Leaves	25.84 ± 0.67
		Bark	123.00 ± 5.31
		Fruits	51.05 ± 3.61
		Flowerings	35.91 ± 0.71
	<i>Verbena litoralis</i>	Leaves	31.08 ± 0.29
	<i>Vitex cymosa</i>	Leaves	98.27 ± 2.15
	<i>Vitex polygama</i>	Leaves	21.94 ± 0.19
	<i>Ginkgo biloba</i>	Egb 761	40.72 ± 0.19

The ethanolic extract from the leaves of *L. trifolia* were partitioned between water and organic solvent to afford 4 new extracts. These were then assayed for anti-oxidative and sedative activity.

Combination of the results indicated that the CH₂Cl₂ was most active and this extract was therefore chosen for additional study to try to isolate novel potentially bioactive compounds.



Fractionation of the CH₂Cl₂ extract by traditional chromatographic methods, such as silica gel open column chromatography combined with preparative reversed phase HPLC, failed.

About 40% of the crude extract binds irreversibly to the silica, which means that potentially a significant loss of important bioactive substance could occur. Counter-Current Chromatography is a

technique where all components injected can be recovered and which only exposes compounds to aqueous / organic solvents and is thus less likely to destroy / irreversibly bond; labile / adsorptive compounds than would prep tlc, open tubular, hplc or flash chromatography techniques. Thus CCC was investigated after the failure of solid / liquid chromatographic techniques tested.

FRACTIONATION OF THE CH₂Cl₂ EXTRACT

➤ Traditional Chromatographic Methods:

- ~~Silica Gel Open Column Chromatography + Preparative RP-HPLC~~

FAILED



**ABOUT 40% OF THE CRUDE EXTRACT BINDS
IRREVERSIBLY TO THE SILICA,
WHICH MEANS THAT MAY BE WE ARE LOOSING
IMPORTANT BIOACTIVE SUBSTANCE**

Advantages of CCC

Counter-Current Chromatography

is...

...essentially, a liquid-liquid partition chromatography, where the stationary phase is retained with no solid support

ADVANTAGES:


- Versatility
- Fast and Efficient
- Economic
- Total sample recovery with retainment of biological activity

A comparison of the relative advantages of isocratic and gradient CCC

COUNTERCURRENT CHROMATOGRAPHY

ISOCRATIC X GRADIENT

- ✓ The majority of the work on CCC has dealt with **ISOCRATIC** analysis
- ✓ Little attention has been given to **GRADIENT** elution systems

ISOCRATIC CCC  **LIMITED**
Only a narrow window of polarity is viewed at one time

CRUDE EXTRACTS

Compounds either elute at the solvent front
or are infinitely retained at the stationary phase.

Description of the need to prescreen samples polarity by gradient reverse phase hplc to assist in the best choice of CCC biphasic solvents.

GRADIENT ELUTION IN CCC

SOME ATTEMPTS HAVE BEEN MADE TO DEVELOP
OPTIMIZATION STRATEGIES FOR CCC

Leslie Brown et al. *Journal of Chromatography*, 2001, in press.

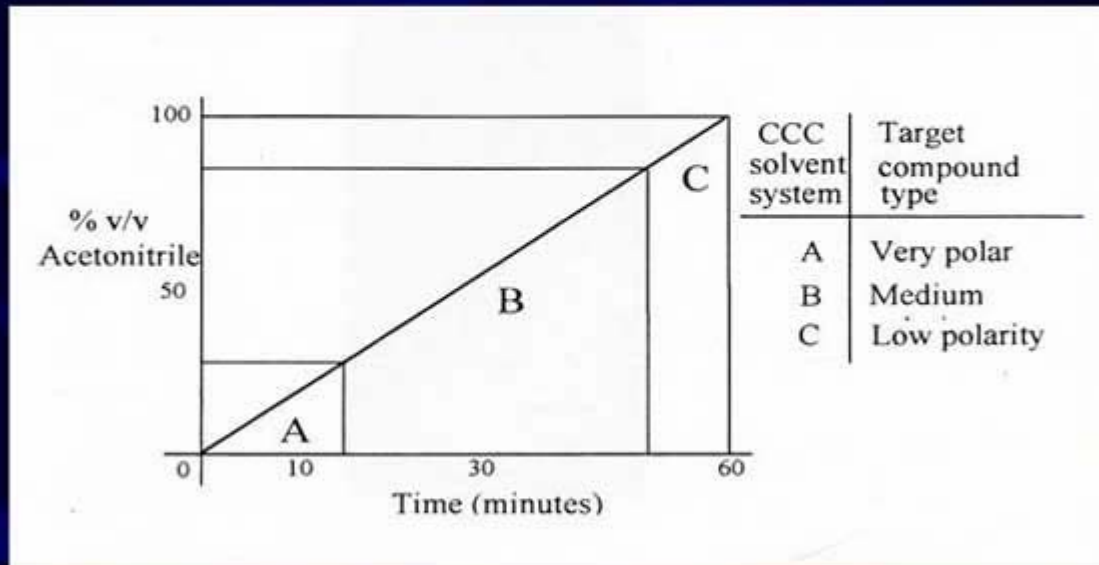
- FIRST STEP: Pre-screen the sample of interest with a simple HPLC gradient (water-acetonitrile 0 → 100%) to give an indication of the

POLARITY RANGE

of the compounds in the mixture

A description of the CCC solvent system chosen for *L. trifolia*

The chromatogram obtained allows to make choices as to which solvent system is suitable for the separation of the compounds



A description of the three main solvent systems which are suitable for gradient CCC separation of compounds.

Final choice of which solvent is to be used is based on their elution position on a 100% water (buffered to pH 6.8 with 10mM ammonium formate) to 100% acetonitrile; reverse phase hplc gradient. The L. trifolia extract corresponded to region B, which suggests mid-polarity compounds, and a preferred choice of a hexane: ethyl acetate: methanol: water biphasic solvent system. Next research was undertaken to find the best solvent ratios in order to develop a gradient system."

SOLVENT SYSTEM OPTIMIZATION

(Leslie Brown et al. *J. Chrom.* , 2001)

❑ REGION A: POLAR COMPOUNDS

Hexane/Butanol/Methanol/Water

❑ REGION B: MID-POLARITY COMPOUNDS

Hexane/Ethyl Acetate/Methanol/Water

❑ REGION C: NON-POLAR COMPOUNDS

Hexane/Acetonitrile

When optimizing the solvent system only one solvent is varied at a time.

In this optimization step, a small amount of the sample is dissolved in test tubes containing various ratios of the biphasic solvent system and then partitioned between the two phases in a test tube. Both

upper and lower phases are then examined by tlc etc. The partitioning results when examined and visualized by TLC to give a good indication of potentially successful CCC solvents. Since we chose the organic phase as the stationary phase (reverse phase operation of the CCC) the amount of methanol in the aqueous phase is varied, in order to pull the compounds from the organic phase into the mobile aqueous phase as the amount of methanol increases.

Note : Whilst this technique of tlc/test tube partitioning screening often proves to be accurate in predicting suitable CCC solvents, as we show for this example, it is not universally successful. Researchers should be aware that successful test tube partitioning / tlc does not guarantee successful CCC, plus a lack of resolution by tlc does not mean that this system may not be successful by CCC. Often it is best to do a generic CCC gradient to predict which solvent will be appropriate (Dr Les Brown, is available to Quattro users / potential purchasers to discuss any CCC applications).

OPTIMIZATION OF THE SOLVENT RATIOS

- Vary only 1 solvent at a time
- Test tube partitioning tests with a small amount of the sample
- View results by TLC

ON THE SOLVENT SYSTEM :

Hexane/Ethyl Acetate/Methanol/Water

Organic Phase → Stationary Phase

This slide shows the five different solvent systems utilized :- A, B, C, D and E; where the proportions of hexane, ethyl acetate and water were maintained and we varied only the methanol ratios, from 0.1 to 2.5. In order to ascertain whether this gradient will work, before going to the counter-current chromatograph itself, we performed test tube partitioning tests and TLC visualized the results.

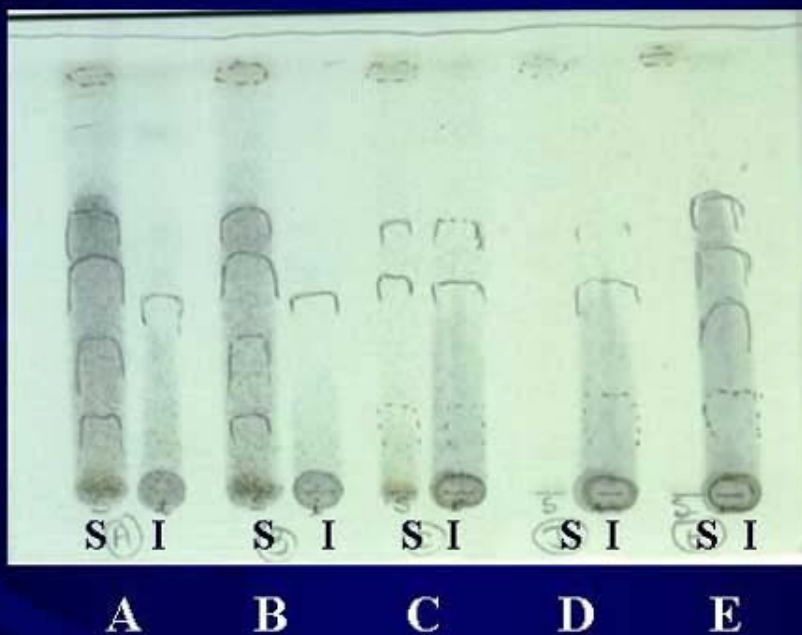
OPTIMIZATION OF THE SOLVENT RATIOS

	Hex.	EtOAc	MeOH	H ₂ O
A	1.0	1.0	0.1	1.0
B	1.0	1.0	0.5	1.0
C	1.0	1.0	1.0	1.0
D	1.0	1.0	1.75	1.0
E	1.0	1.0	2.5	1.0

This slide shows the TLC results from the partitioning test. For each solvent A, B, C, D, and E two spots are applied :- one for the organic phase (S = superior) and one for the aqueous phase (I= inferior).

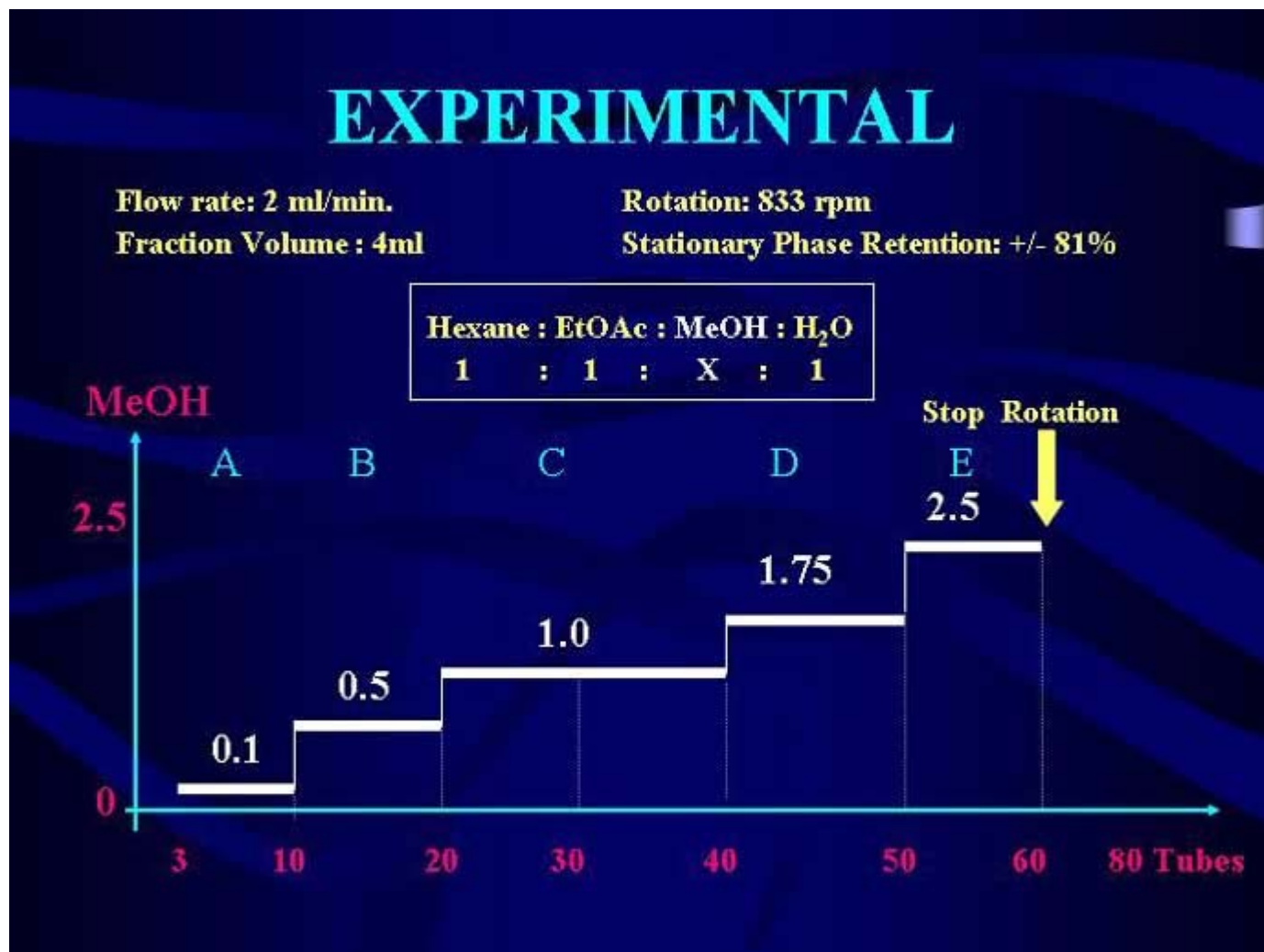
This TLC plate shows that as the amount of methanol increases in the systems, more compounds are extracted from the organic phase into the aqueous phase, which is to be the MOBILE PHASE. More polar compounds will thus elute first in the proposed reverse phase CCC chromatograph that will utilize the aqueous phase as the mobile phase. The less polar a compound the longer it will be retained. Infinitely non-polar compounds will not elute during the active gradient but will be eluted when the rotation is switched off and both the mobile and stationary phase in the column are then washed out together as the Archimedean Principle which previously retained the stationary phase is inactivated. Little band broadening occurs during washing out and the resolution of compounds in the coil is preserved. The stationary phase infinitely retained compounds will elute in the last drops of stationary phase washed out of the column.

TLC RESULTS FROM PARTITIONING TEST



This graphic summarizes the experimental conditions used for this separation. The flow rate was 2ml/min. and the rotation speed was approximately 830 rpm. 4 ml fractions were collected for 'off-line' analysis by tlc.

In this experiment, where the organic phase was the stationary phase, we achieved stationary phase retention of approximately 81%. A, B, C, D and E represent the five different solvent systems used in the step gradient, where methanol varied from 0.1 to 2.5 in the mixture. After 60 fractions (240 ml) were collected, we stopped the rotation and washed out both the mobile and stationary phase retained in the coil, these fractions (also 4 ml) were collected until all the stationary phase was washed out and contained the retained compounds inside the coil. The experiment lasted about 3 hours but fast gradients of 10 to 20 minutes are possible with alternate Quattro coil configurations.

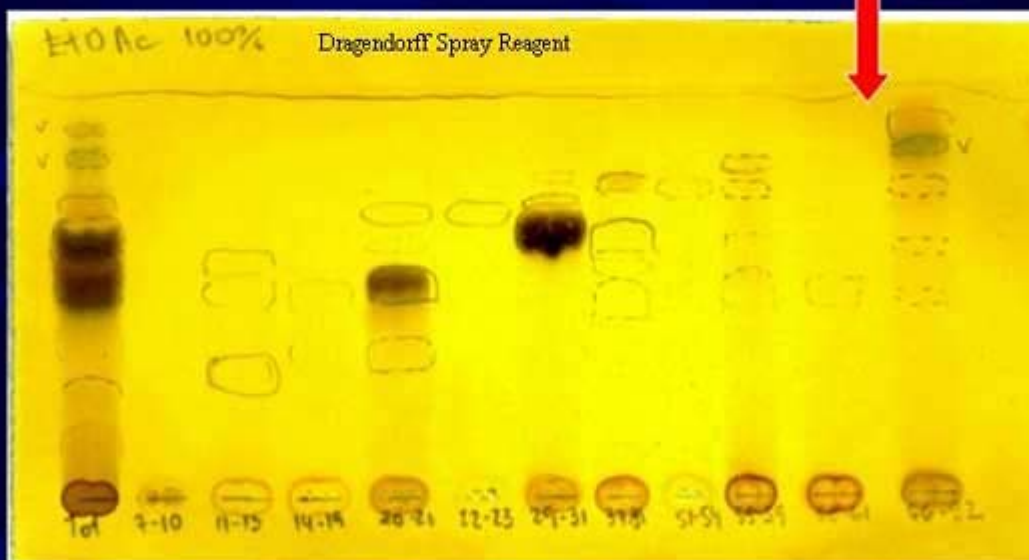


The shown tlc plate summarizes the results obtained in this experiment. The first spot refers to the total extract for comparison.

As discussed above, the more polar compounds elute first, leaving the less polar ones to elute later. It can be seen from the results, that the gradient system utilized allowed the separation of two alkaloid derivatives, from the multitude of other extracted components present. These compounds are selectively visualized by the development with Dragendorff reagent. Subsequent MS/NMR etc analysis showed that one of the components was in a highly pure form in the fraction from the CCC. It's important to point out that these two compounds were not separated when using solid / liquid chromatography preparative techniques

GRADIENT CCC RESULTS

Stop Rotation



CONCLUSIONS

- **HSCCC / Step Gradient**

Effective in the separation of pyridine derivatives from the CH_2Cl_2 extract from *Lantana trifolia* leaves.

2 PURE COMPOUNDS FROM A CRUDE
EXTRACT / \pm 3 hs.

➤FAST

➤ECONOMIC



EFFICIENT