Pharmacopoeia Monograph on Artemisinin

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Scope of Presentation

- Overview of artemisinin (ART) monograph methods
- Consider limitations and possible improvements based on a recent study funded by MMV/FSC

Monograph protocols for Artemisinin

Assay:

- HPLC-UV
- Chemical transformation of artemisinin-UV

Related substances:

- HPLC-UV
- TLC-Densitometer

ART quantification limits given for ASSAY methods only

http://apps.who.int/phint/en/p/docf/

Monograph protocols for Artemisinin

Assay method	ART content limits
HPLC-UV	97.0% and 102.0%
Chemical transformation-UV	98.0% and 102.0%

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HPLC-UV method

Column: 3µm C18, 10cm x 4.3mm
Detection: 216nm
Solvent: aqACN at 0.6ml per min

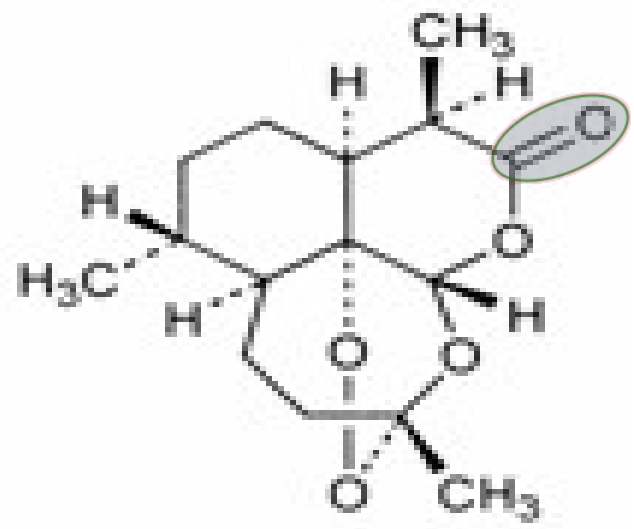
Time (min)	Mobile phase A % v/v of ACN	Mobile phase B % v/v of water
0 – 17	60	40
17 – 30	60 – 100	40 – 0
30 – 35	100 – 60	0 – 40
35 – 45	60	40

Nature of stationary phase not defined

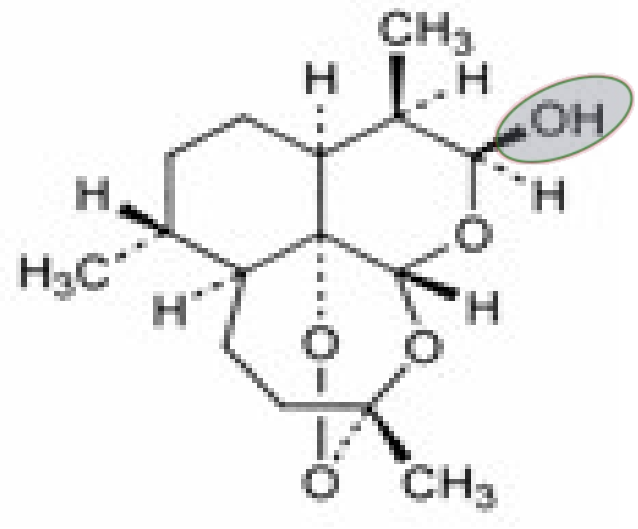
Quantification by HPLC-UV method

- System suitability checked by ensuring minimum resolution and relative retention times against artenimol.
- Use peak area from reference artemisinin sample to calculate relative purity of test sample and also of “related substances.”

Standards used in the Monograph



Artemisinin

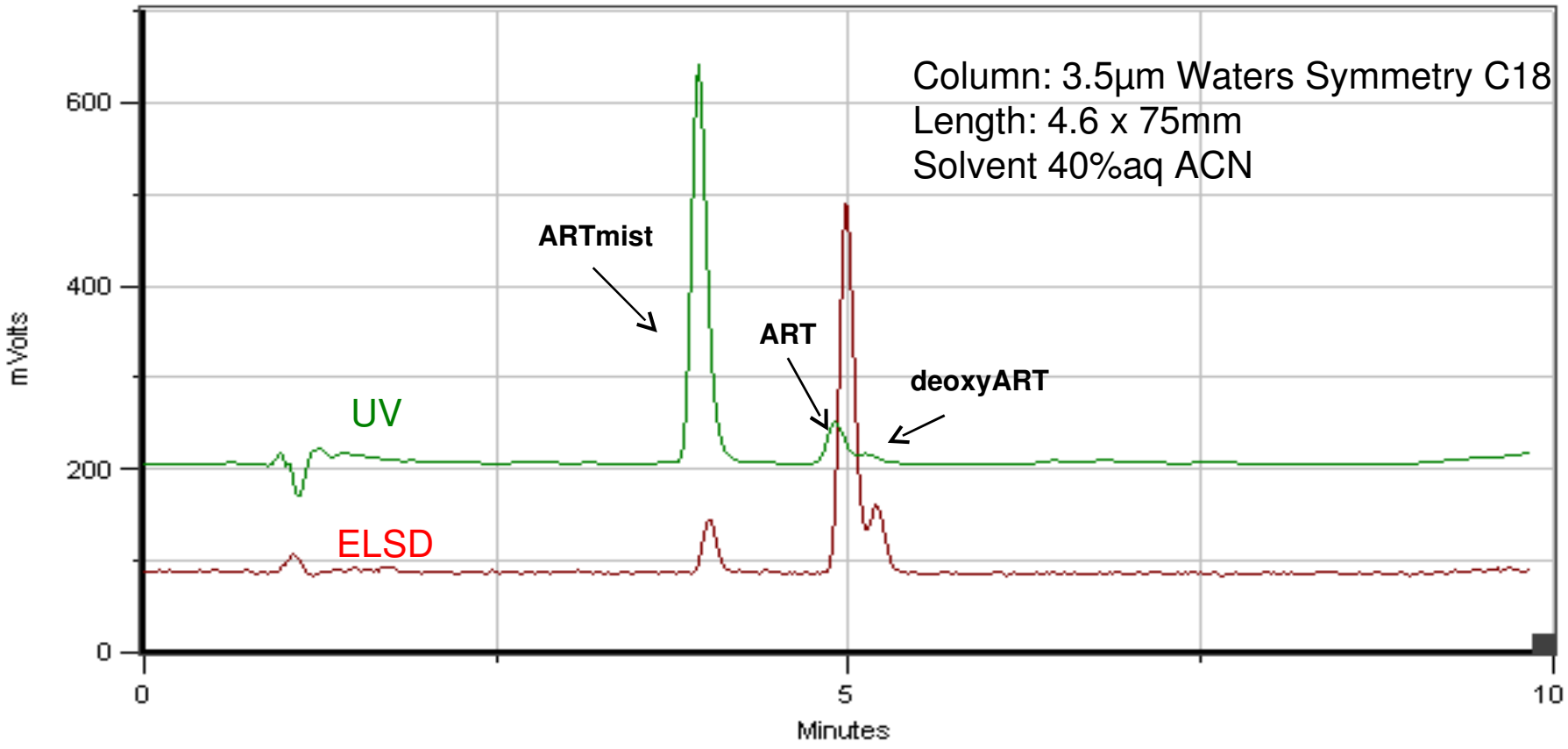


Artenimol

Monograph HPLC-UV method - LIMITATIONS

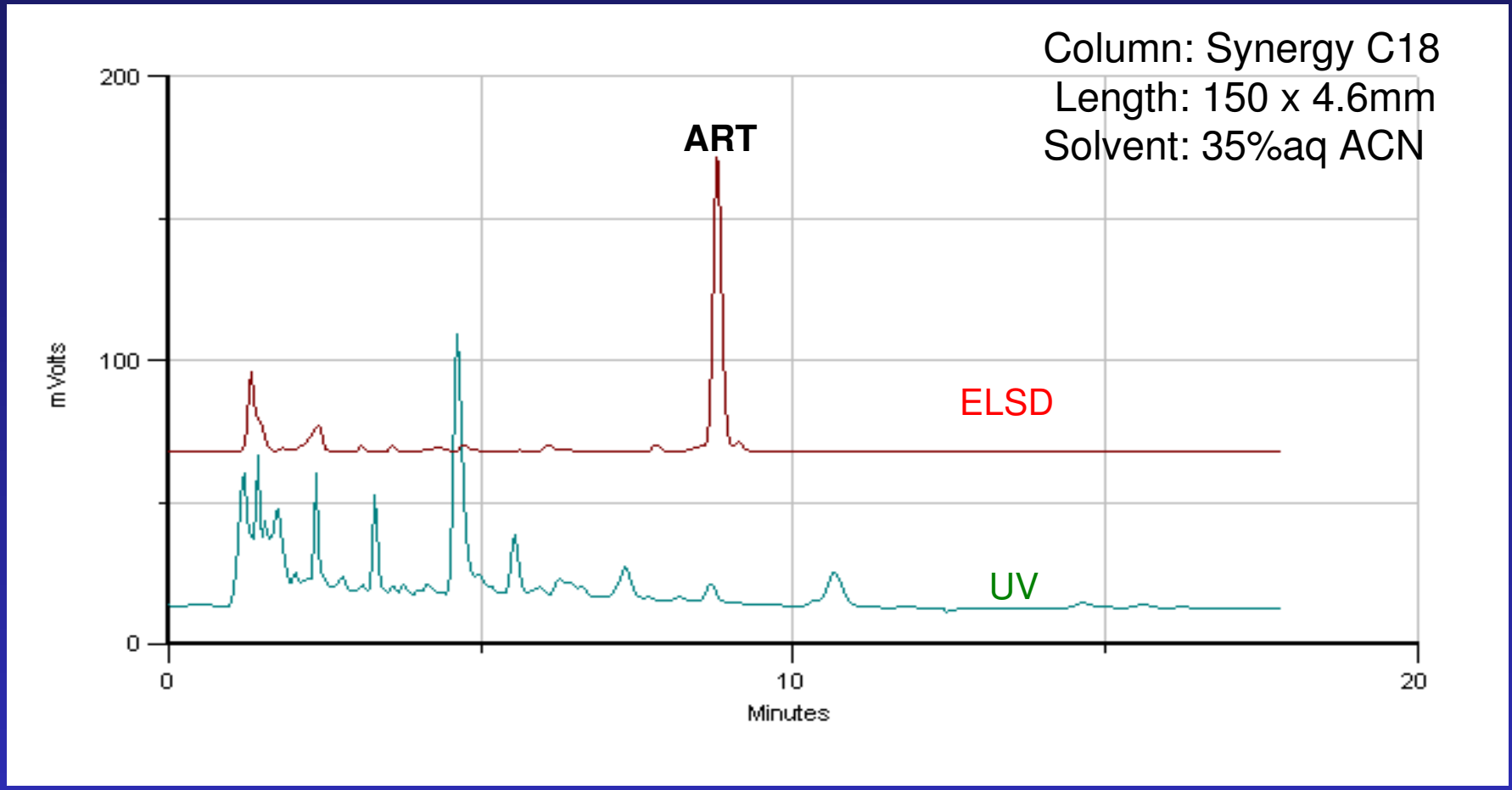
- Is UV the best detector for quantifying artemisinin?

Some key impurities in crystalline ART



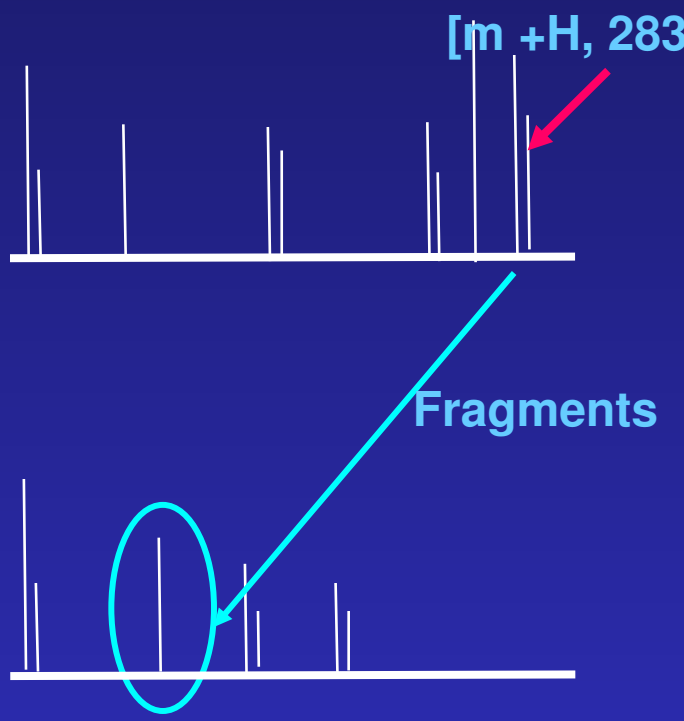
- Quantification using UV detector requires reference compounds

HPLC of hexane extract of *A. annua*



- For mixtures ELSD better than UV
- Estimation of impurities from combined UV peak areas is misleading

Mass spectroscopy

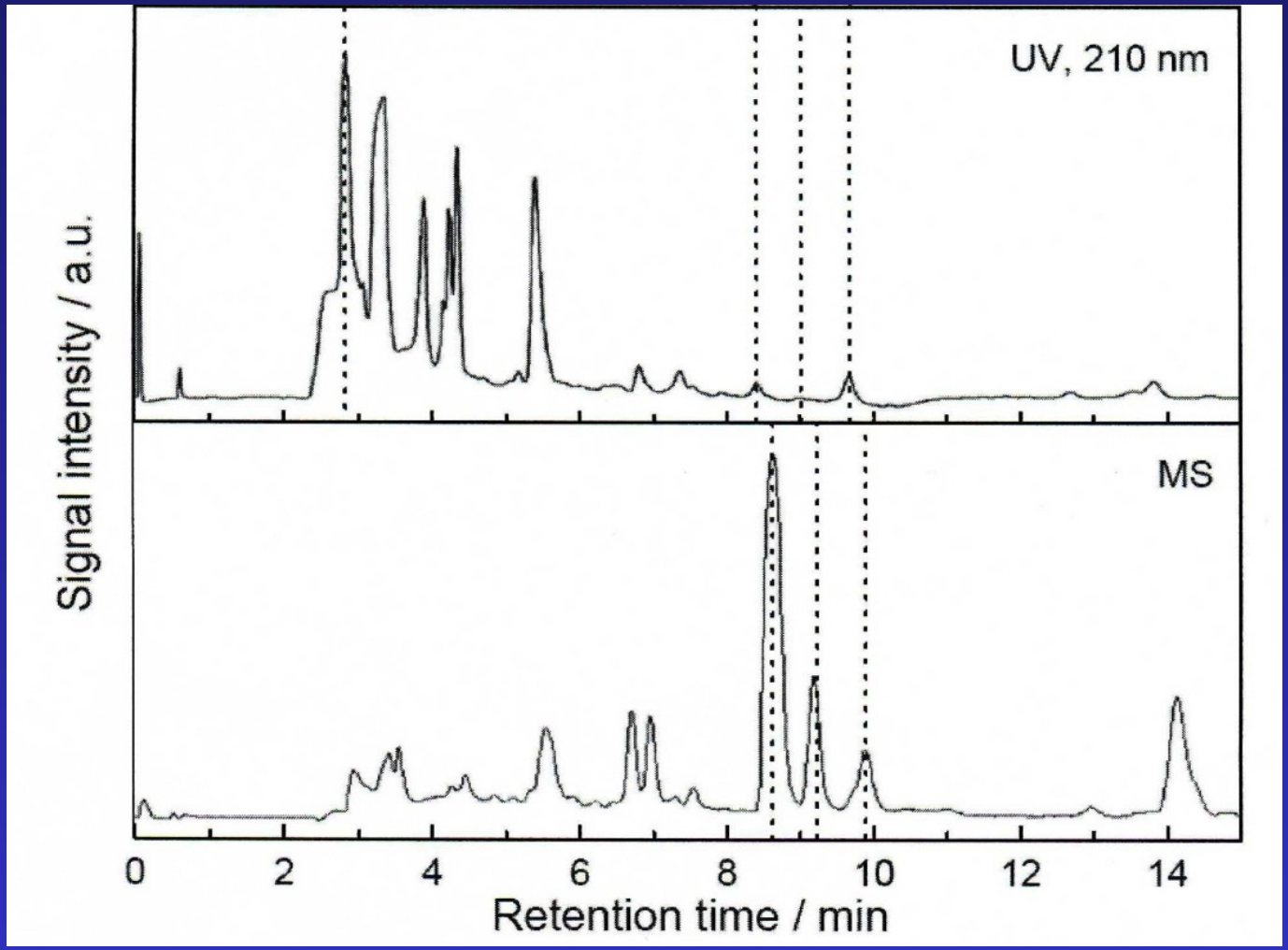


LC-MS requires HPLC separation

LC-MSMS – does not need HPLC separation

Comparison UV and MS detectors

Synergy Luna C18 (250mm) 30%aqMeOH



MS identified previously undetected compounds

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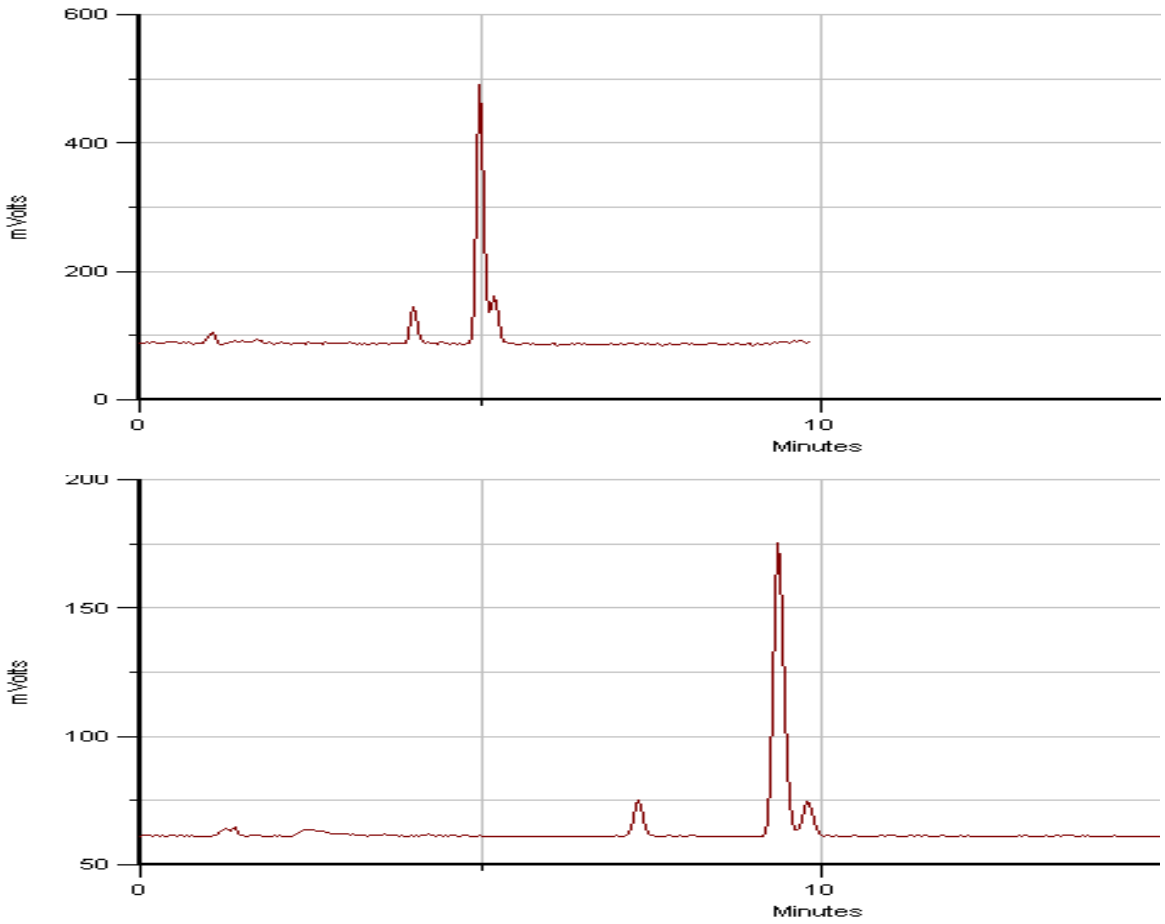
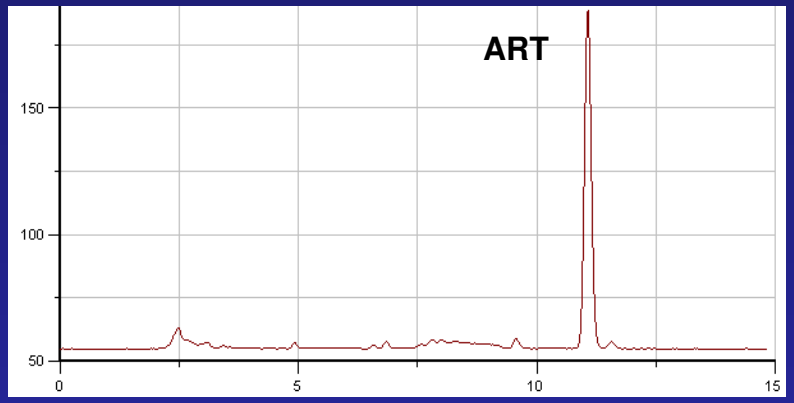


Column: 3.5µm Waters Symmetry C18
Length: 4.6 x 75mm
Solvent 40%aq ACN

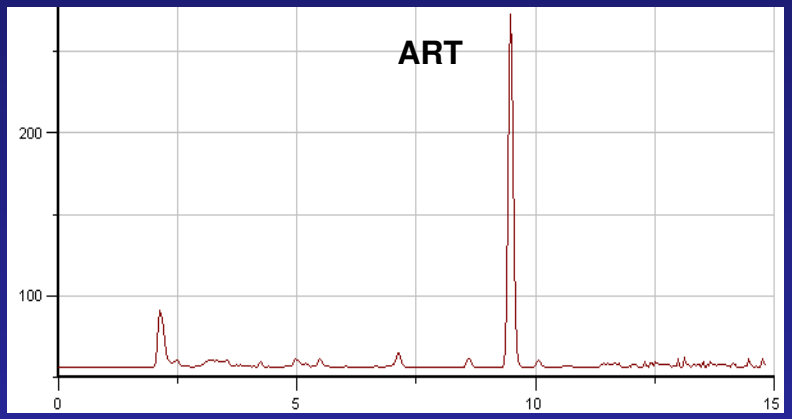
Column: 5µm Hichrom Symmetry 5C18
Length: 4.6 x 140mm
Solvent 40%aq ACN

- Column dimensions and type of stationary phase influence resolution

HPLC of *A. annua* extract – comparison of solvents

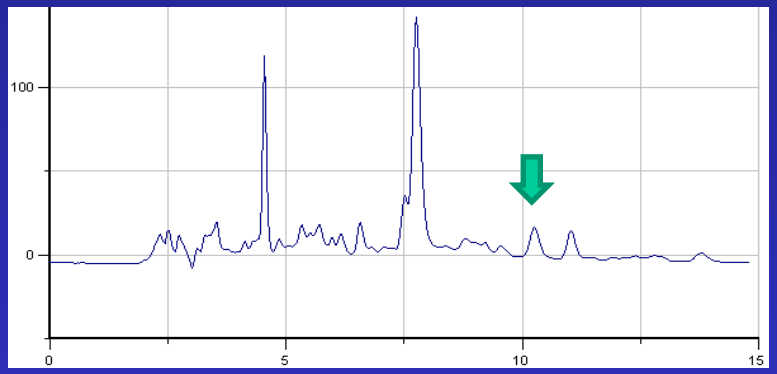


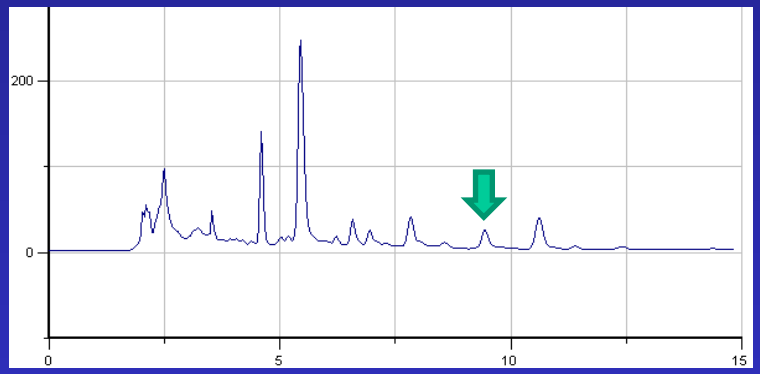
25%aq MeOH



30%aq ACN

ELSD



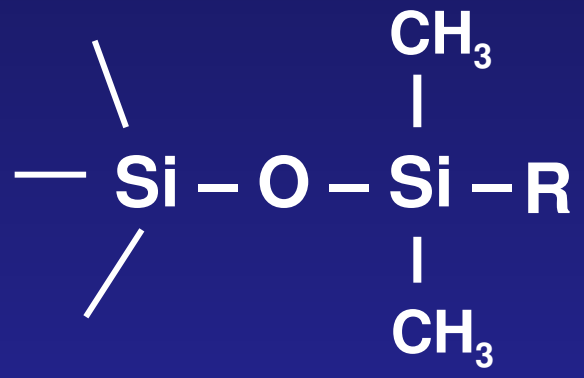


UV at 220nm

Betasil C18 (250mm) column

- **Major differences between ACN and MeOH**

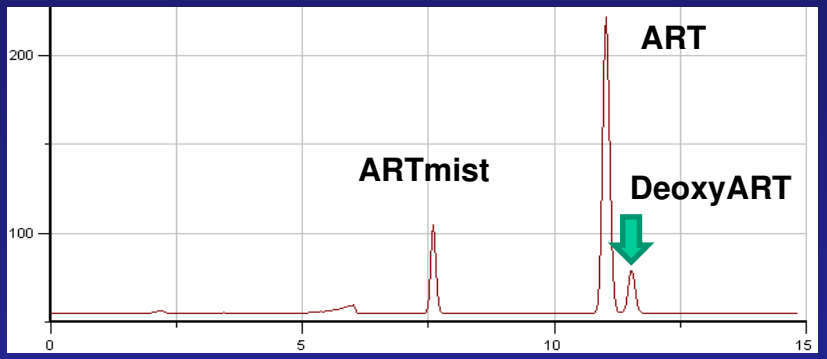
Stationary phases in HPLC columns



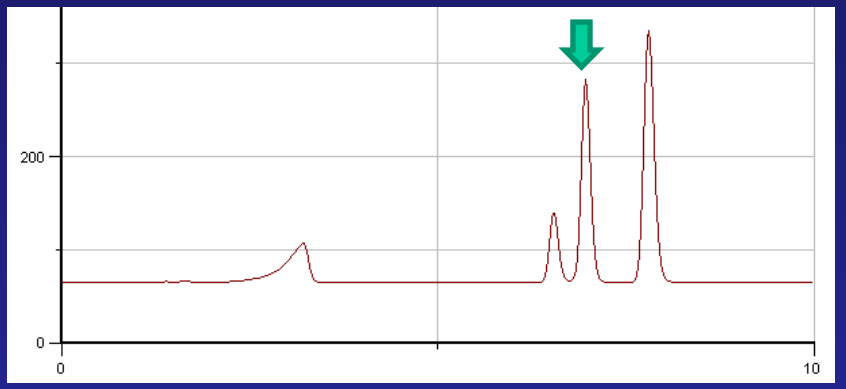
R	Name
– $(CH_2)_{17}CH_3$	C18 reverse phase
– $(CH_2)_6Ph$	Phenylhexyl phase
– $(CH_2)_nCN$	Nitrile phase

- **> 15 columns evaluated**
- **8 different phases tested**

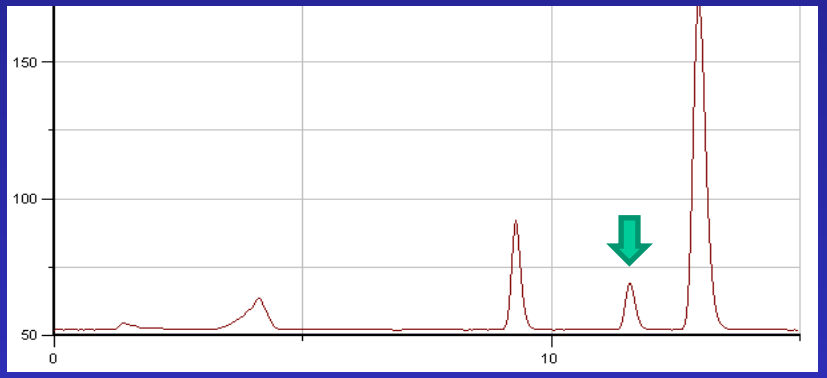
Comparison of C18 and aromatic bonded columns (ELSD detection)



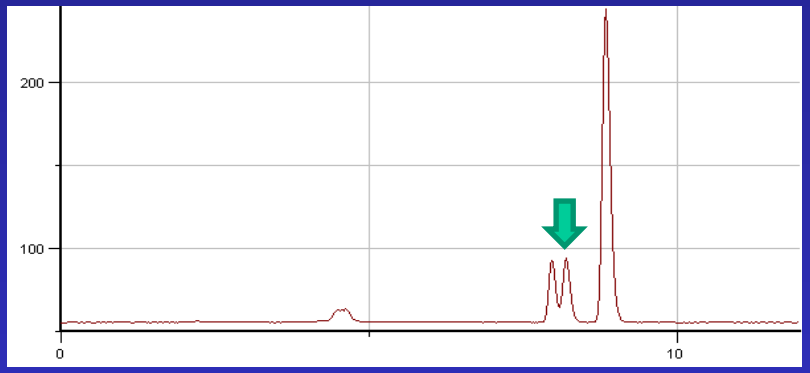
C18 Betasil (250mm) 25%aqMeOH



Polar RP (150mm) 25%aqMeOH



Phenylhexyl (150mm) 30%aqMeOH



Diphenyl (250mm) 20%aqMeOH

- **Clear differences between C18 and aromatic phases**

Monograph HPLC-UV method - LIMITATIONS

- Is UV the best detector for quantifying artemisinin? NO
- Can separation of interfering compounds be improved?

YES:
Stationary phase, solvent and column dimensions can result in improved separations

Monograph HPLC-UV method - LIMITATIONS

HPLC-UV method needs to be updated

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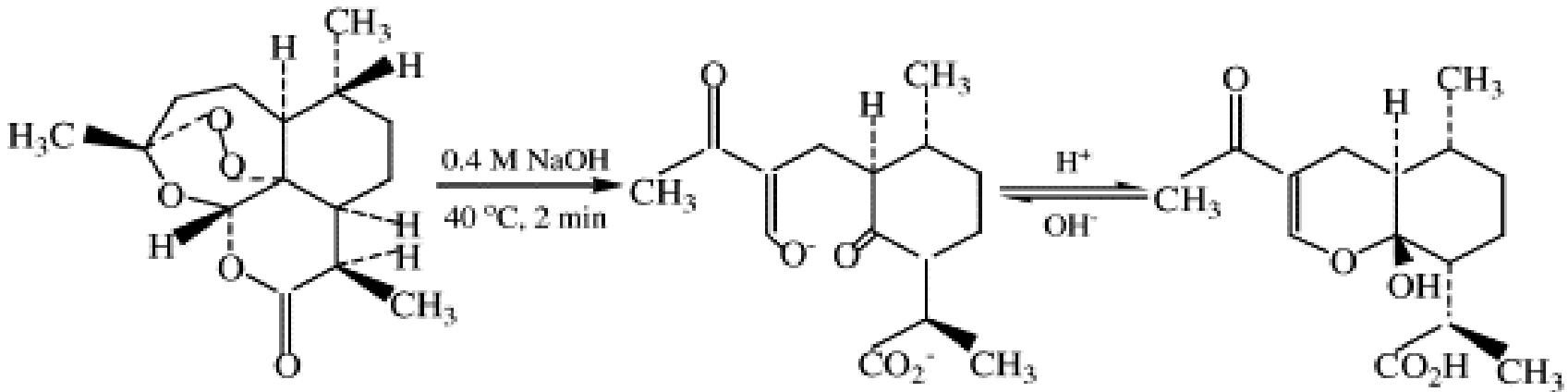
Chemical transformation – UV method



(I) Artemisinin (II) Q292 (III) Q260

UV transparent

UV active than ART

Chemical transformation-UV method

- Method relies on products formed by chemical reaction – complete reaction?
- Very sensitive to reaction conditions
- Dependent upon experimental skill

More reliable methods now available

Monograph protocols for Artemisinin

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TLC-densitometer	??

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TLC-Densitometer by CAMAG

MS interface available - 2009

- Mixture applied to TLC plate and developed
- Plate sprayed with reagent and heated
- ART produces coloured spots and measured with densitometer

TLC-densitometer method

- Simple and economical method
- Amenable to high throughput
- Widely used

However

- Over-estimation of ART reported
- Need for proper validation with reference samples

Comparison of analytical methods

Technique	Precision (RSD)	Additional comments
HPLC-ELSD	<3%	• RSD can be less than ± 2% • Intra-day precision needs to be monitored • Careful control of operating parameters necessary
TLC-Densitometer	?	• Transformation yield <85% • Major advantage of high throughput
HPLC-UV	2%	• Poor sensitivity but good for ART crystals • Not acceptable for extracts
HPLC-MSMS	<2%	• 0.4-2.4ng mL^{-1} has been reported
HPLC-RI	6%	• Low sensitivity - potential impact on HPLC separation
TLC-FID [Iatroscan]	8%	• Separation of compounds questionable

Several factors influence choice of analytical technique

Examples:

- HPLC-UV for analysis of crystalline ART
- ELSD and TLC-densitometer for extracts
- HPLC-MSMS for standardising protocols

Summary

- Separation of minor interfering impurities cannot be guaranteed by current Monograph HPLC-UV method.
- Potential for improving current methods has been demonstrated.
- New methods (e.g LCMSMS) should be considered for inclusion.
- Need to:
  - reconsider choice of internal reference standards
  - identify interfering compounds and their effect on quantification of ART
  - ensure availability of reference standards

Acknowledgments

- MMV/FSC for funding
- Rothamsted Research for facilities and general support

THANK FOR YOUR ATTENTION

MMV/FSC funded study

Title: Validation of the monograph HPLC analytical protocol for artemisinin quantification in biomass and extracts

Alexei Lapkin, Belindha Mlambo and Smain Chemat – University of Bath
Adam Walker – Bioniqs Ltd
Neil Sullivan – SensaPharm Ltd
Bhupinder PS Khambay – Kamtech Technologies Ltd

http://www.mmv.org/IMG/pdf/HPLC_methods_for_Artemisinin_Rep