

Pharmahemp d.o.o., Koprška ulica 106C, SI-1000 Ljubljana, Slovenia		PharmaHemp®
QUANTITATIVE DETERMINATION OF ERINACINE A BY HPLC		
Issue date	Expiration date	Code: MET-005
		Revision No.: 01
		Appendixes: 2
Author: Marko Dragan	Reviewer: Dr. Boštjan Jančar	Approver: Tine Lavrič

SUMMARY OF AMENDMENTS

Revision Number	Description
01	First issue

DEFINITIONS AND ABBREVIATIONS

ACN	Acetonitrile
HPLC	High Performance Liquid Chromatography
LC	Liquid chromatography
MPA	Mobile phase A
MPB	Mobile phase B
RPC	Reverse Phase Chromatography
SOP	Standard operative procedure
SST	System suitability tests
QA	Quality assurance
QCS	Quality control sample

QUANTITATIVE DETERMINATION OF ERINACINE A BY HPLC**TABLE OF CONTENTS**

1. PURPOSE.....	3
2. RESPONSIBILITIES.....	3
3. THEORETICAL PRINCIPLE	3
4. HANDLING WITH CHEMICALS AND REAGENTS, SAFETY INSTRUCTIONS.....	3
5. CHEMICALS, MATERIALS AND INSTRUMENTS (EQUIPMENT/SOFTWARE).....	4
6. REAGENTS AND SOLUTIONS	4
6.1 Required solutions.....	4
6.2 Reference standards solutions for calibration	5
6.3 Quality control samples.....	6
6.3.1 Blank	6
6.3.2 QCS	6
6.4 Sample preparation.....	6
7. ANALYSIS PROCEDURE AND INSTRUMENT PREPARATION/SETUP.....	7
7.1 HPLC system	7
7.2 Recommended conditions and storage.....	7
7.3 Sequence set- up.....	8
8. EVALUATION, DATA PROCESSING AND RESULT REPORTING.....	8
8.1 Evaluation	8
8.2 Integration.....	8
8.3 Result reporting.....	10
8.4 Data documentation and Control Charts	10
8.5 Data reporting	10
9. REFERENCES.....	11
10. APPENDIX	11

QUANTITATIVE DETERMINATION OF ERINACINE A BY HPLC

1. PURPOSE

This laboratory procedure describes the quantitative determination of Erinacine A on HPLC system. The description includes sample preparation and handling as well as detailed description of experimental and data processing parameters / settings.

The method enables determination of Erinacine A in different formulations. The given result of the concentration of Erinacine A is calculated using calibration curve from the known concentration of Erinacine A in reference solutions. Internal quality control samples are used for the evaluation of validity of each analysis.

2. RESPONSIBILITIES

All the employees within analytical laboratory are obligated to use this laboratory procedure in accordance with the instruction given within for all the samples received.

Table 1: List of responsibilities

Role:	Responsible for:
Analytical Laboratory Technician (ALT)	Using this laboratory procedure.
Analytical Laboratory Manager (MAN/LAB)	The preparation, evaluation and maintenance of this laboratory procedure.
Chief Technology Officer (CTO)	Reviewer of this laboratory procedure.
QA Manager (QA)	Approval of this laboratory procedure.

3. THEORETICAL PRINCIPLE

High performance liquid chromatography (HPLC) has recently become the method of choice for the quantitative determination of small molecules such is Erinacine A. The most common used columns are based on reverse phase (RP) C₁₈ stationary phases - thus the name RPC is used. The great advantage in the use of such columns lies in fast analyses and high separation efficiency.

4. HANDLING WITH CHEMICALS AND REAGENTS, SAFETY INSTRUCTIONS

When handling with chemicals and reagents safety precautions on safety data sheets of materials used have to be followed. The analyst must use the protective equipment described in the table below.

Table 2: Safety precautions when handling with chemicals and reagents

Substance	Danger	Precautions
Acetone	Highly flammable liquid and vapor. Causes severe eye irritation. May cause drowsiness and dizziness.	Wear protective gloves/protective clothing/eye protection. When aerosol formation or mist occurs wear suitable protective mask. In case of contact with eyes, rinse thoroughly with clean running water for at least 10 minutes.
Acetonitrile	Highly flammable liquid and vapor. Harmful if swallowed, in contact with skin or if inhaled. Causes severe eye irritation.	Wear protective gloves/protective clothing/eye protection. When aerosol formation or mist occurs wear suitable protective mask. In case of contact with eyes, rinse thoroughly with clean running water for at least 10 minutes. If exposed or concerned: Get medical advice/attention.
Ethanol	Highly flammable liquid and vapor. Causes severe eye irritation.	Wear protective gloves/protective clothing/eye protection. In case of contact with eyes, rinse thoroughly with clean running water for at least 10

QUANTITATIVE DETERMINATION OF ERINACINE A BY HPLCCode: **MET-005**Revision No.: **01**

Substance	Danger	Precautions
Isopropanol	Highly flammable liquid and vapor. Causes severe eye irritation. May cause drowsiness and dizziness.	minutes. Wear protective gloves/protective clothing/eye protection. When aerosol formation or mist occurs wear suitable protective mask. In case of contact with eyes, rinse thoroughly with clean running water for at least 10 minutes.

5. CHEMICALS, MATERIALS AND INSTRUMENTS (EQUIPMENT/SOFTWARE)

The stated materials, standards and chemicals were tested and proven to be suitable for the current procedure. Other suitable products of the same quality and performance can also be used. If different materials and/or chemicals are used, at least SST criteria have to be fulfilled. If this is not applicable, comparable control chart data and/or chromatograms have to show equivalency and suitability.

Table 3: List of chemicals

Chemicals / reagents	Producer and order number
Acetonitrile	LabExpert; KE00.0174.2500
Ethanol	PharmaChem; Denaturiran tip B, 10 L
Isopropanol	J.T. Baker, 9095
Formic acid	Carlo Erba Reagents, 405824
MQ-Water	Adrona, B30

Table 4: List of instruments, consumables / disposables

Instruments/Materials	Producer/Supplier and order number
HPLC System	Agilent; 1260 infinity
HPLC software	Agilent, OpenLab Chromatography Data System (CDS)
Analytical balance	Mettler Toledo; XPE204
Test weight	Kern & Sohn GmbH; 316-05
Pipets	ErgoOne; 10-1000 µl
Tips	TipOne; S1161-1820 (1250 µl) and S1163-1800 (200 µl)
Pre-column	Agilent, Poroshell 120 EC-C18 4.6 x 5mm 2.7 Micron
Column	Agilent; Poroshell 120 SB-C18 4.6 x 150mm 2.7 Micron
Water bath	Julabo, Pura 14
Chromafil Filters	KEFO, 7658851
Parafilm	KEFO, 9170002
Spatulas	KEFO, 9220204 and 9220208
Vials	Agilent, 5183-2069 or KEFO 11090520
Vial caps	Agilent, 5182-0717 or KEFO 4008218
Cleaning agent for Dishwasher	KEFO; 9192425 and 9192446

6. REAGENTS AND SOLUTIONS

Note: If larger or smaller volumes of the solutions are required the quantities in the instructions may be adapted proportionally.

6.1 Required solutions

MPA (0,1% formic acid)	<u>Preparation:</u> Add 2.5 ml of formic acid to 2,5 l Milli-Q water and mix the solution by shaking. <u>Stability:</u>
----------------------------------	---

QUANTITATIVE DETERMINATION OF ERINACINE A BY HPLC

Code: **MET-005**

Revision No.: **01**

	3 days at RT
MPB (0,1% formic acid in ACN)	<u>Preparation:</u> Add 2,5 ml of formic acid and 25 ml of Milli-Q water to 2,5 L ACN (originally packed) and mix the solution by shaking. It is recommended that the MPB is prepared one day in advance. <u>Stability:</u> 2 weeks at RT
Sample dilution buffer (70 % Ethanol)	<u>Preparation:</u> In a volumetric flask measure 730 ml of 96 % Ethanol and 270 ml of Milli Q water. Mix by shaking. <u>Stability:</u> 1 month at RT
Column storage solution (70 % ACN)	<u>Preparation:</u> In a volumetric flask measure 700 ml of ACN and 300 ml of Milli Q water. Mix by shaking. <u>Stability:</u> 6 months at RT
Solution for (70 % ACN)	<u>Preparation:</u> In a volumetric flask measure 700 ml of ACN and 300 ml of Milli Q water. Mix by shaking. <u>Stability:</u> 6 months at RT
Column storage solution (70 % ACN)	<u>Preparation:</u> In a volumetric flask measure 700 ml of ACN and 300 ml of Milli Q water. Mix by shaking. <u>Stability:</u> 6 months at RT

6.2 Reference standards solutions for calibration

Concentration of Erinacine A in samples is determined using calibration curve from the known concentration of reference standard solutions - dilutions from reference standard.

Preparation:

Prepare calibration points - dilutions from reference standard solution with concentration of 1000 µg/ml.

Table 5: Reference standard dilution and injection volumes for Calibration

Sample name	Start C [µg/ml]	Sample V [ml]	Solvent V (EtOH) [ml]	Final C [µg/ml]
Erinacine A_78	78	500	0	78.0
Erinacine A_40	78.0	256	244	40.0
Erinacine A_20	40.0	250	250	20.0
Erinacine A_10	20.0	250	250	10.0
Erinacine A_5	10.0	250	250	5.0
Erinacine A_1	5.0	100	400	1.0
Erinacine A_0.1	1.0	100	900	0.1
Erinacine A_0.01	0.1	100	900	0.01

Abbreviations: V = volume, C = concentration *Used also for further dilution

QUANTITATIVE DETERMINATION OF ERINACINE A BY HPLC

Calculate the accurate concentration of each Reference standard solution and enter the calculated values into required fields in the OpenLab software - defined in the Levels in Peaks/Group category.

6.3 Quality control samples

Quality control samples must be used in every analysis and the results must be tracked in control charts. Based on quality control samples the validity of each analysis is confirmed.

The quality control samples are used for:

- **Composition of sample dilution buffer**
The blank injections are used to confirm that no Erinacine A is present in the sample dilution buffer.
- **Peak identification**
When the peak identification in the sample is questionable, QCS sample for identification is used. Sample is overlapped with the QCS to assure that the retention time of the unknown sample peak is the same as the retention time of the Erinacine A in QCS.
- **Conformation of uniformity of chromatographic conditions**
Every 15 injections and at least at the end of each sequence the QCS is re-injected to confirm the suitable chromatographic conditions (same RT times of Erinacine A, same peak shapes, same resolution, same measured concentration).

6.3.1 Blank

The blank sample contains sample dilution buffer and it is used to define a baseline and thus enable the analyst to distinguish sample signals from system signals occurring due to the chromatographic conditions and buffer components. It is crucial that the same dilution buffer as blank is used.

The blank samples are used at the beginning of the sequence, where more than one can be injected, and also can be re-injected anywhere in the sequence if necessary.

6.3.2 QCS

QCS is in advance prepared sample which contains Erinacine A. The QCS is used to assess the repeatability of the measurements.

Preparation:

Prepare the sample with at least 0.5 mg/g Erinacine A - follow the sample preparation as described in chapter 6.4. After the extraction filter the solution through 0.45 µm filter and pipet into vials as 1000 µl aliquots. Store the samples at - 20°C.

6.4 Sample preparation

The concentration of Erinacine A in the sample must be in the concentration range of the method. If the concentration of Erinacine A is higher than 7.5 mg/g, the one-step dilution is required. Both the concentrating and the diluting of the sample can be performed.

General sample preparation protocol:

1. Weight 1000 ±100 mg of the sample on analytical balance.
2. Dilute the sample in 100 mL sample dilution buffer using the mark on the volumetric flask (bottom of a meniscus).
3. Close the measuring flask with parafilm and start the extraction process.
4. Extraction process: Fill the water bath with MQ water and pre-heat it to 65°C. Put the samples in the water bath at 65°C for 3h.
5. After the extraction put 1.5 ml of the sample solution into 2 ml tube and centrifuge at 12000 RPM for 20 min.
6. After the centrifugation, filter 1 ml of the supernatant through 0.45 µm filter into the glass vial.

QUANTITATIVE DETERMINATION OF ERINACINE A BY HPLC

Preparation of concentrated sample (initial weight of the sample from 1.0 g to 10.0 g):

The preparation of the concentrated sample is the same as the general sample preparation protocol, only the initial weight of the sample is greater. When more sample is weighted, the lowest reported concentration is lower - as many times as the weighted sample amount is higher. Example: When 10.0 g of sample is prepared instead of 1.0 g, the lowest reported concentration of erinacine A in the sample is 10x lower.

Preparation of concentrates or extracts:

For concentrates and extracts which contain more than 7 mg/g of Erinacine A, the lower amount of the sample can be prepared instead of the further diluting of the sample. As less sample is weighted the lowest reported concentration is higher. Example: When 100 mg of sample is prepared instead of 1000 mg, the lowest reported concentration of erinacine A in the sample is 10x higher.

Sample dilution

If the concentration of Erinacine A in the sample exceeds the analytical range of the method, dilute the sample by factor of 10 gravimetrically. Example: weigh 100 µl sample solution and dilute it with 900 µl of 70 % ethanol. Weigh the final solution. Enter the measured weights in the fields Dilutor and Multiplier in OpenLab software.

7. ANALYSIS PROCEDURE AND INSTRUMENT PREPARATION/SETUP

7.1 HPLC system

HPLC parameters and conditions are described in a table below.

Table 6: HPLC parameters and conditions

Parameter	Condition
Flow rate	1.2 mL/min
Elution type	Isocratic
Column temperature	35°C, OpenLab settings: Temperature delta: 0.8°C
Injection volume	10.0 µL
Run Time	5 minutes
Auto-sampler temperature	4°C, OpenLab settings: Lower limit: 4°C / Upper limit: 7°C
Detection	340 nm
Step for the UV detector	OpenLab settings: > 0.05 min (1 s response time) (5 Hz) (corresponds to a step of 0.1 s)

7.2 Recommended conditions and storage

Before column installation and after column removal

The HPLC system should be flushed for at least 20 minutes with solution for column storage (70 % ACN) at 1.2 mL/min.

New column

Before using a new column first flush the column with at least 5 column volumes of 70 % ACN (at least 12,5 mL, e.g. for a flow rate of 1.2 ml/min it would take at least 10 minutes) to remove potential residual impurities. Follow the vendors recommendation and gradually increase the flow to 1.2 ml/min.

Next equilibrate the column with the initial mobile phase gradient for 30 minutes with a flow rate of 1.2 mL/min.

QUANTITATIVE DETERMINATION OF ERINACINE A BY HPLC

Already used column

Flush an already used column with the initial mobile phase gradient for 30 minutes with a flow rate of 1.2 mL/min. Follow the vendors recommendation and gradually increase the flow to 1.2 ml/min.

Column storage

The column must be stored in ACN/MQ water mixture (without HFO), 70% ACN (v/v) is recommended. After each sequence equilibrate the column with long term column storage solution (30 minutes with a flow rate of 1.2 mL/min). Column is stored at room temperature, installed on the system or in the provided box.

Conditioning of the HPLC system

Use initial mobile phase gradient and gradually increase the flow on the HPLC system from 0.1 to 1.2 mL/min. Monitor the pressure on the system and when is stable wait for another 15 min before starting the sequence.

7.3 Sequence set- up

Table 7: Example of sequence of injections up to 43 samples

Sample name	No. of injections
Blank	1-3
QCS	1
Sample 1	1
Sample...	1
Sample 14	1
QCS	1
Sample 14	1
Sample...	1
Sample 29	1
QCS	1
Sample 29	1
Sample...	1
Sample 44	1
QCS	1
Blank	1-3
Flushing_1*	1
Flushing_2**	1
Shutdown***	1

* 20 min, 90% ACN flushing

** 40 min, 20% ACN flushing

*** 30 min, 70% ACN flushing

8. EVALUATION, DATA PROCESSING AND RESULT REPORTING

8.1 Evaluation

Determine the peak area of each peak in the chromatogram, which are not present in the blank sample. Use correct baseline integration (see Chapter 8.2).

8.2 Integration

Integration of chromatograms is performed automatically by using the OpenLAB software, but can be corrected manually. Automatic peak labelling may not be correct due to the peak RT shifting. In that case it has to be corrected manually according to the retention times of the peaks in the QCS sample.

As a general rule, draw one baseline from the first to the last peak eluting within the group. Set the baseline considering the course of the blank. Within a group of peaks, separate all peaks normally by an orthogonal

QUANTITATIVE DETERMINATION OF ERINACINE A BY HPLC

split. The split should be set if the two peaks are separated by a well-defined valley or at least the maxima of the two peaks are clearly distinguishable. If shoulders of the peak are present, these should also be integrated using orthogonal split at the inflection point. A separate baseline should be set for the baseline separated peaks.

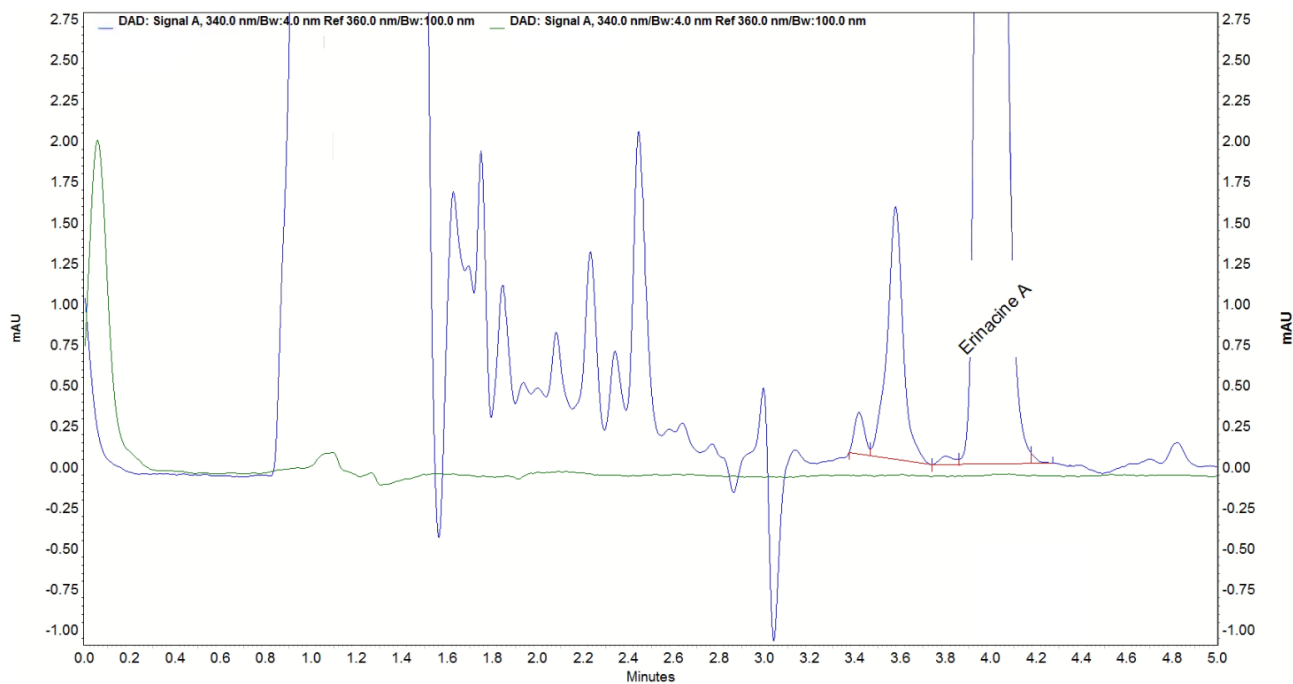


Figure 1: Overlay of the Blank and random sample (Sample - blue, Blank - green); integration of unknown compounds is not mandatory

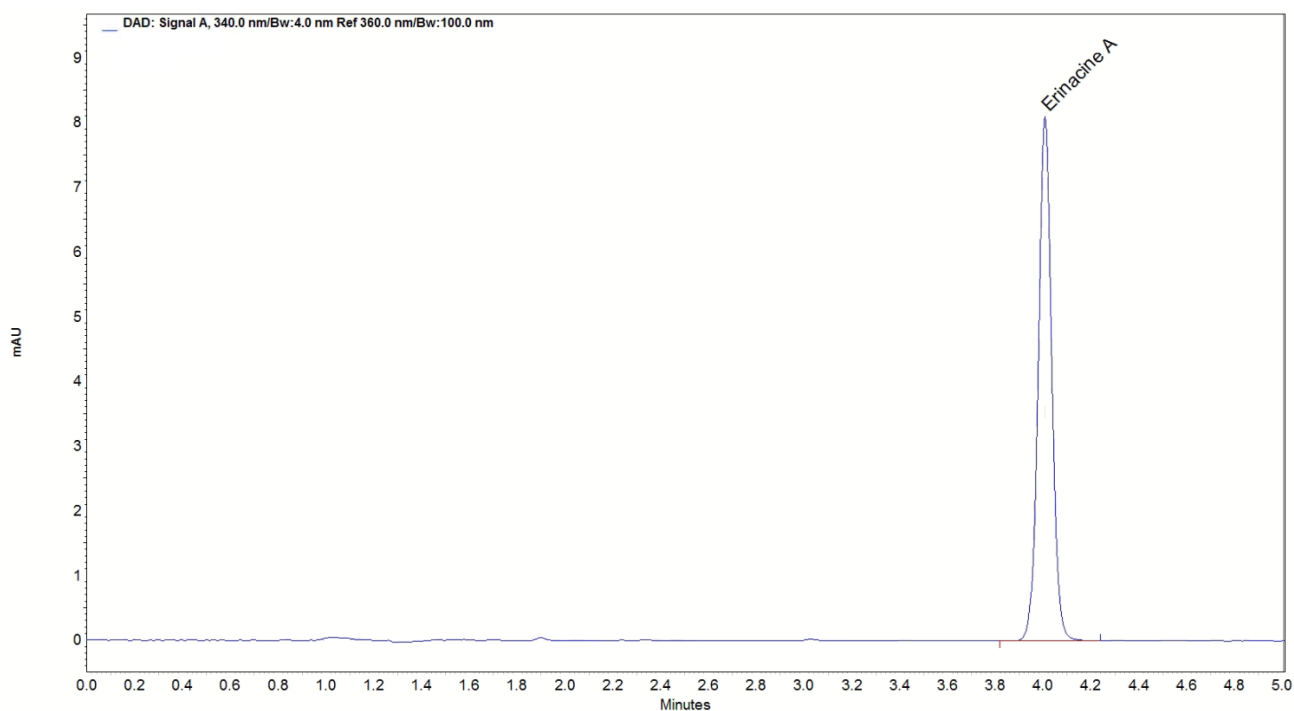


Figure 2: Chromatogram of Erinacine A reference standard

QUANTITATIVE DETERMINATION OF ERINACINE A BY HPLC

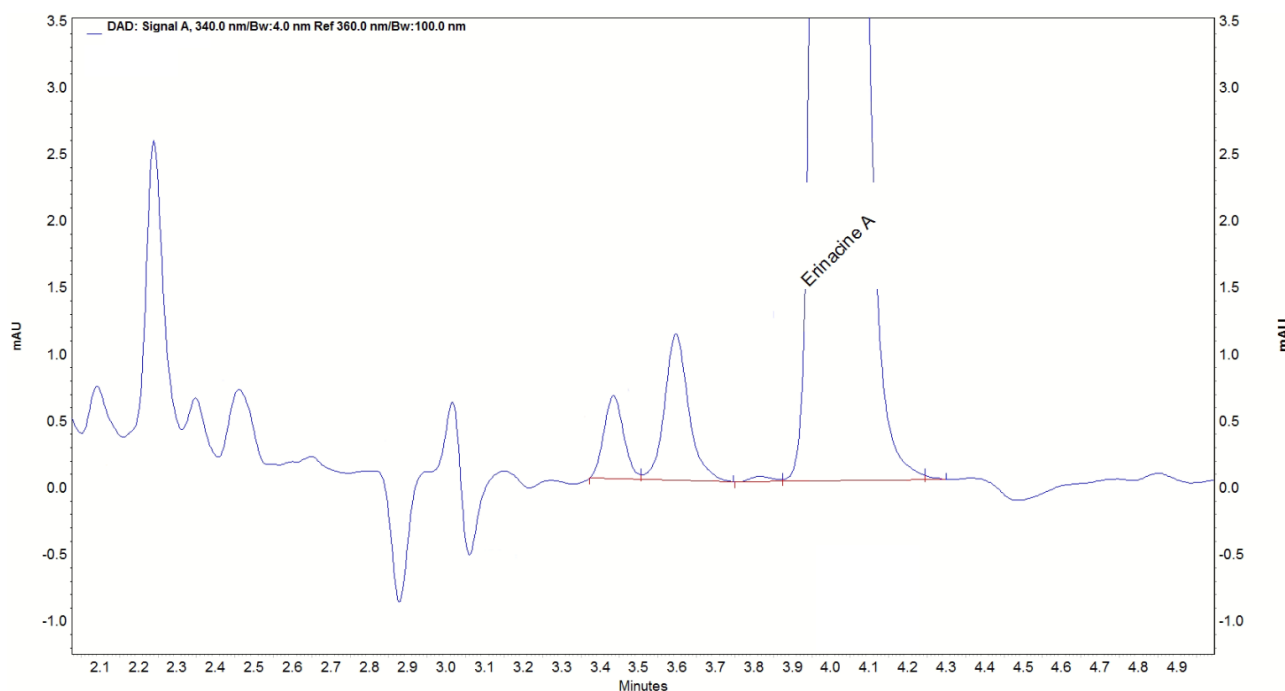


Figure 3: Example for correct baseline determination

8.3 Result reporting

Report the concentration of Erinacine A in the sample as mg per 1 g of sample. Any calculations in OpenLab software have to be checked by LAB/MAN. The reporting results can be different if required by the client.

Reported concentration of erinacine A in the sample solution must be in the concentration range of the method. If the concentration is higher, the sample must be further diluted and the results from the diluted sample are reported.

Concentration of erinacine A in first injection of QCS must be entered in corresponding control chart.

Based on the trends or other observations of the data in control chart re-calibration of the method could be required.

8.4 Data documentation and Control Charts

Analysis has to be documented. All documents have to be e-signed. The results have to be checked and signed.

Control chart (i.e. in the form of an Excel table) relating the method should be stored in the corresponding directory, which is backed up to the server on a daily basis.

8.5 Data reporting

Report the concentration of erinacine A in the samples above the LOQ. LOQ concentration depends on the preparation (less sample weighted = higher LOQ; more sample weighted = lower LOQ).

QUANTITATIVE DETERMINATION OF ERINACINE A BY HPLC	Pharma  Hemp®
	Code: MET-005
	Revision No.: 01

9. REFERENCES

- Kozmetika - Dobra proizvodna praksa (GMP) - Smernice za dobro proizvodno prakso (ISO 22716:2007)
- Prehranski dodatki - Dobra proizvodna praksa (GMP) - Smernice za dobro proizvodno prakso (Food Supplements Europe, 2014)
- Sistemi vodenja varnosti živil - Zahteve za vsako organizacijo v prehranski verigi (ISO 22000:2018)
- Splošne zahteve za usposobljenost preskuševalnih in kalibracijskih laboratorijev (ISO/IEC 17025:2017)
- Sistemi vodenja kakovosti - Zahteve (ISO 9001:2015)

10. APPENDIX

- Appendix 1: Training/Familiarity sheet
- Appendix 2: Logbook
- Appendix 3: History of the document

**QUANTITATIVE DETERMINATION OF CANNABINOIDS BY
REVERSE PHASE CHROMATOGRAPHY**



Code: **MET-005**

APPENDIX 1: Training/Familiarity sheet

Revision No.: **01**

Obseg usposabljanja	LAB, ASS/R&D
Obseg seznanjenosti	

Ime in priimek	Org. enota	Delovno mesto	U - Usposabljanje S - Seznanjenost	Datum	Podpis
Janez Gerdenc	LAB	ALT	☐ U ☐ S		
Janja Ahej	R&D	ASS	☐ U ☐ S		
Aleksander Jefim	LAB	ALT	☐ U ☐ S		
Karmen Korbar	LAB	ALT	☐ U ☐ S		
			☐ U ☐ S		
			☐ U ☐ S		
			☐ U ☐ S		
			☐ U ☐ S		
			☐ U ☐ S		
			☐ U ☐ S		
			☐ U ☐ S		
			☐ U ☐ S		
			☐ U ☐ S		
			☐ U ☐ S		
			☐ U ☐ S		

QUANTITATIVE DETERMINATION OF ERINACINE A BY HPLC

APPENDIX 2: Logbook

Code: **MET-005**Revision No.: **01**

IME ANALIZE: PHL_RPC_ERI

PROJEKT: PHL_RPC_ERI

DATUM ANALIZE:

PODPIS IZVAJALCA:

Stran: ___ od ___

1. MOBILNE FAZE

MFA: _____

MFB: _____

2. RAZTOPINA ZA REDČENJE VZORCEV / Blank: _____

3. PRIPRAVA VZORCEV:

Podrobnejša navodila za določitev koncentracije erinacina A v vzorcih so v SOP: **MET-005**.

#	Ime vzorca	Zatehta [mg]	Dilutor 1	Multiplier 1	Datum in podpis - izvajalec	Datum in podpis - vnos v OpenLAB	Pregled (datum in podpis)
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							
16							
17							
18							
19							
20							

Dodatni komentar pri pripravi vzorcev:

4. QCS: Priprava v: _____; Številka vial: _____ Ustreznost: DA / NE

5. REZULTATI

Obdelava podatkov (datum podpis): _____ Pregled in odobritev rezultatov (datum podpis): _____

Rezultati poslani (datum podpis): _____

6. KOMENTAR:

QUANTITATIVE DETERMINATION OF ERINACINE A BY HPLC**APPENDIX 3: Laboratory notebook**Pharma  Hemp®Code: **MET-005**Revision No.: **01**

Revision Number	Amendments	Issue date	Changed with new version (date, signature)
01	First issue		