

WEB302 - SOMT Activity Factorial

Benjamin Weigel

August 4, 2014, Halle

1 Deduced from WEB299

SOMT seems to be active:

- in reducing conditions (1 mM DTT)
- in the presence of divalent cations (2 mM Mg²⁺)
- at a pH of 8.5

SOMT activity:

- is somewhat peculiar (at least over long incubation times)
- peaks appear in the chromatograms, that are not supposed to be there (e.g. very early retention time [Peak 1], early shoulder of Naringenin [Peak 2] Fig.1)

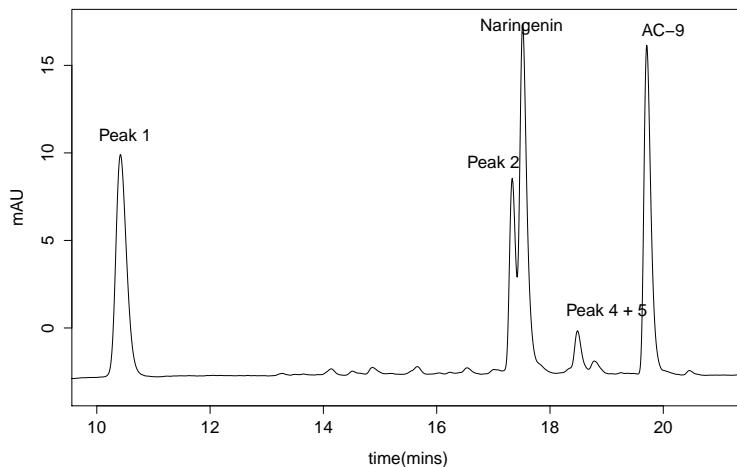


Figure 1: HPLC Trace at 325 nm of WEB299.12 activity test.

2 (Fractional) Factorial to optimize assay conditions

- optimize assay conditions with refolded protein from WEB301.12 (Tab. 1)

variable Factors:

- temperature	- pH	- reaction time
- <i>divalent cations</i>	- <i>redox conditions</i>	- DES
- SAM concentration	- concentration of substrate	- substrate
- enzyme concentration		

	Mg ²⁺ [mM]	Ca ²⁺ [mM]	DTT [mM]	DES [% (V/V)]	e1	e2	e3
1	0.00	5.00	2.00	25.00	-1.00	1.00	-1.00
2	0.00	0.00	0.00	0.00	-1.00	-1.00	-1.00
3	5.00	5.00	0.00	25.00	-1.00	-1.00	1.00
4	0.00	5.00	0.00	0.00	1.00	1.00	1.00
5	5.00	5.00	2.00	0.00	1.00	-1.00	-1.00
6	5.00	0.00	0.00	25.00	1.00	1.00	-1.00
7	0.00	0.00	2.00	25.00	1.00	-1.00	1.00
8	5.00	0.00	2.00	0.00	-1.00	1.00	1.00
9	2.50	2.50	1.00	12.50	0.00	0.00	0.00

Table 1: 4 Factor fractional factorial design with centerpoint for assessing SOMT activity influences. The variable factors are divalent cations (Mg²⁺ and Ca²⁺), DTT and 1,2-propanediol:cholin chloride:water (PCH) DES.e1, e2, e3 are dummy variables important for the factorial design process.

WEB303 - Analytical Gefi with reduced refolded Protein

Benjamin Weigel

August 6, 2014, Halle

1 Description

- from WEB301 experiments with analytical gel filtration it seems as if SOMT elutes in tetrameric, as well as monomeric form
 - however those runs were under **non-reducing** conditions
 - ↪ re-run a sample eluted from the WEB301 GPC run under **reducing** conditions

2 Experimental

2.1 Protein sample

Sample: concentrated eluate from pooled fractions A2 and A3 of **WEB301.Run2**

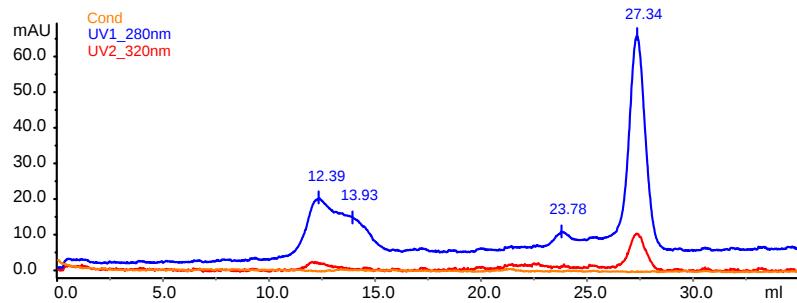
Treatment: added approximately 10 mM DTT to sample → let incubate on ice for approx. 1h

2.2 ÄKTA Run conditions (Fig. 1a)

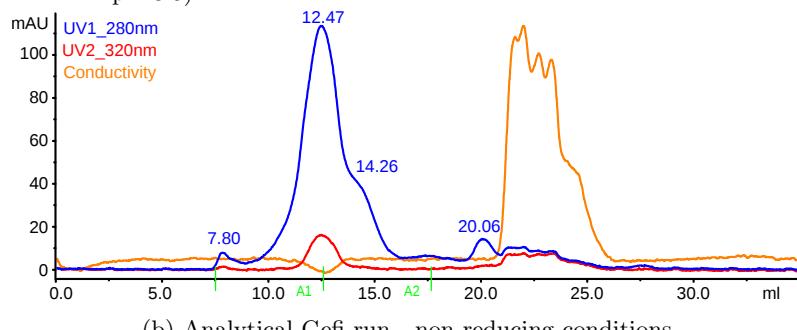
ÄKTA column: Superdex 200 10/300 GL

Running buffer: 0.1 M Tris/HCl, 150 mM NaCl, **1 mM DTT**, pH 8.5

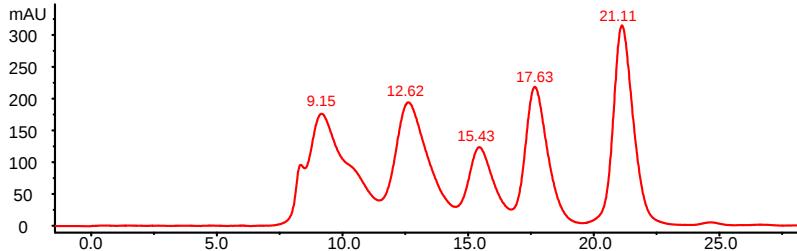
Sample: 100 µl of the sample from **2.1** (xx mg/mL) [100 µl loop]



(a) Analytical gel filtration run with protein that was subjected to gel filtration before (WEB301 Run2, see Fig. 1b). The protein was treated with 10 mM DTT prior to the run. Buffer: 0.1 M Tris/HCl, 150 mM NaCl, 1 mM DTT pH 8.5)



(b) Analytical Gefi run - non-reducing conditions



(c) Analytical standards

Figure 1: ÄKTA runs

WEB305 - Chromabond HR-X Test

Benjamin Weigel

August 27, 2014

1 Introduction

For the analysis of methyl transferase reaction via the LAAO-assay it is necessary to remove flavonoids and phenyl propanoids. Those substances interfere with the detection of H₂O₂ via HRP, because rather than TMB these substances tend to get oxidized.

Trying to remove the flavonoid substrates and products of a MT-reaction by solid phase extraction columns(SPE).

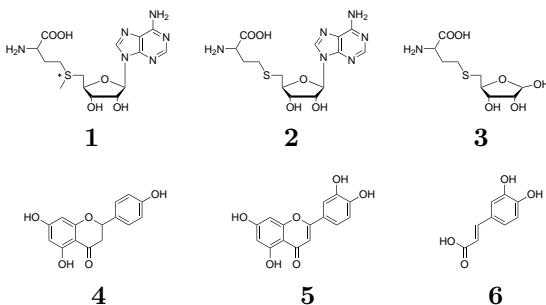


Figure 1: Substances to be separated for analysis by LAAO assay. Polar substances to be eluted for analysis are **1** - SAM, **2** - SAH and **3** - Ribosyl homocysteine. Flavonoids and phenyl propanoids (**4–5**) are problematic during detection with LAAO/HRP.

2 Experimental

2.1 Sample preparation

Sample solutions:

1. 100 μM SAH in ddH₂O
2. 100 μM SAH, 0.2 mM Eriodictyol in ddH₂O

Sample treatment:

A1: 100 μM SAH in ddH₂O passed through HR-X column

A2: 100 μM SAH, 0.2 mM Eriodictyol in ddH₂O passed through HR-X column

B1: 100 μM SAH in ddH₂O

B2: 100 μM SAH, 0.2 mM Eriodictyol in ddH₂O

Column preparation:

Conditioning of HR-X column:

1.5 mL MeOH, then 1.5 mL Water

Sample aspiration:

0.5 mL sample passed through column by pressure (syringe)

Washing/Elution:

eluted polar components by washing with 0.5 mL water

Regeneration:

regenerated by passing through 2 mL of MeOH

2.2 LAAO-Reaction

1. add 75 μ L LAAO-reagent to 25 μ L of sample
2. incubate for 10 min @ RT
3. stop reaction with 50 μ L 12 N H₂SO₄
4. measure absorption at 450 nm

WEB308 - SOMT ammonium sulfate precipitation

Benjamin Weigel

23rd September, 2014

Introduction

- refolded 2.5 mL 1 mg/mL SOMT in solubilization buffer in 50 mL Buffer 12 by fast dilution (in 50 mL tube)
- incubation over night at 4°C
- centrifuged at 16.000 $\times g$ / 4°C to remove precipitate
- took 5 mL aliquots and performed ammonium sulfate precipitation test (see Figure 1)

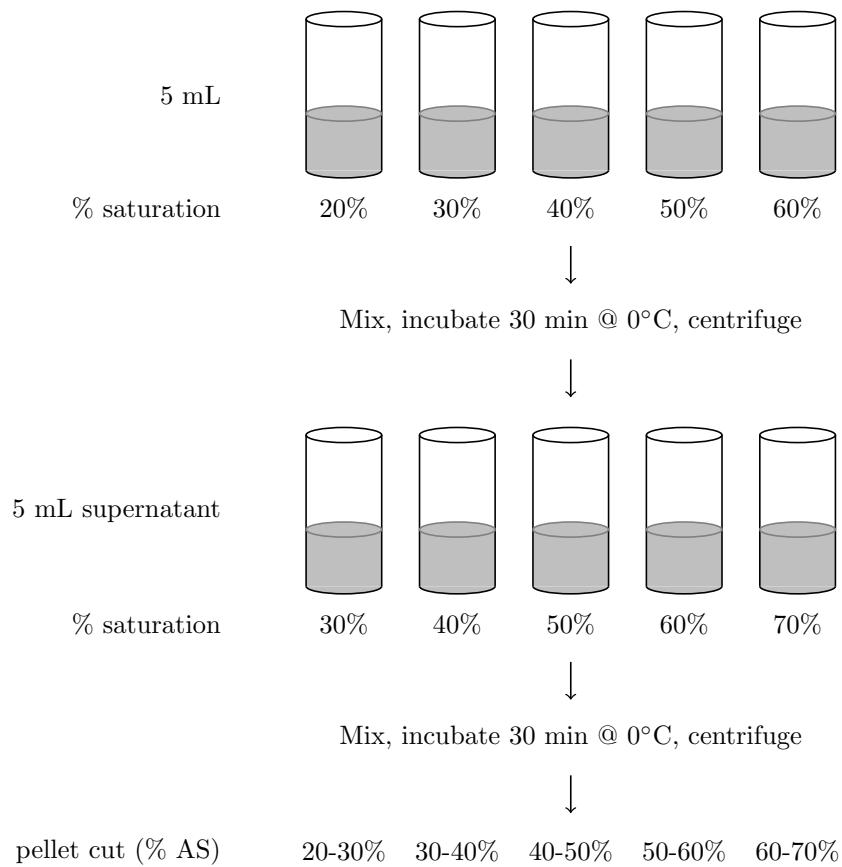


Figure 1: Experimental setup for the ammonium sulfate precipitation.

Table 1: Amount of Ammonium sulfate to add to 5 mL solution at each step.

AS cut	20-30%	30-40%	40-50%	50-60%	60-70%
AS (1 st step)	0.53 g	0.82 g	1.13 g	1.455 g	1.8 g
AS (2 nd step)	0.275 g	0.28 g	0.29 g	0.3 g	0.31 g

1 SDS-PAGE

- ca. 50 μ g/mL protein in refolding reaction
- precipitation via ammonium sulfate
- SDS-PAGE analysis of pellet & supernatant of first step
- SDS-PAGE analysis of pellet & supernatant of second step \rightarrow 16 samples
- 200 uL = 10 ug protein \rightarrow take 200 uL from precipitation in eppi & centrifuge
- resuspend AS pellet (1) in 50 uL PBS \rightarrow 5 uL for Gel
- TCA precipitation with supernatant, take up pellet from TCA-precipitation in 10 uL PBS and analyze via SDS-PAGE
- resuspend protein pellet from AS-precipitation (2) in 0.25 mL PBS \rightarrow 10 uL (ca 10 ug) on gel

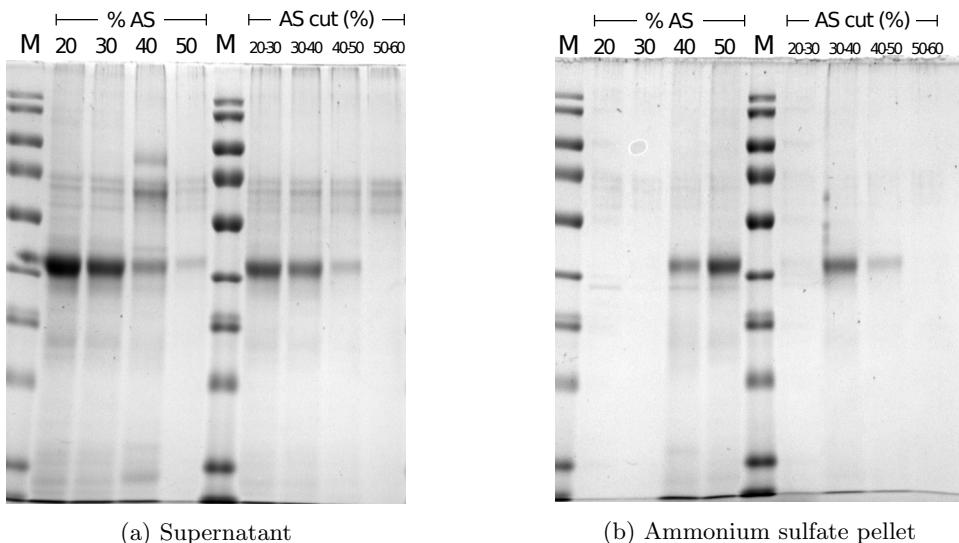


Figure 2: Ammonium sulfate precipitation test straight from refolding SOMT in refolding buffer 12. The majority of SOMT protein is precipitated in the 30–40% cut of ammonium sulfate saturation (Fig. 2b). However, there is some SOMT still in solution even at a 50% AS saturation (Fig. 2a).

Table 2: Setup of the gels for SDS-PAGE analysis

lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
pellets	M	20%	1 st precipitation	30%	40%	50%	M	30%	2 nd precipitation	40%	50%	60%	M			
supernatant	M	20%	1 st precipitation	30%	40%	50%	M	30%	2 nd precipitation	40%	50%	60%	M	MTAN		

WEB309 - SOMT Expression

Benjamin Weigel

23rd September, 2014

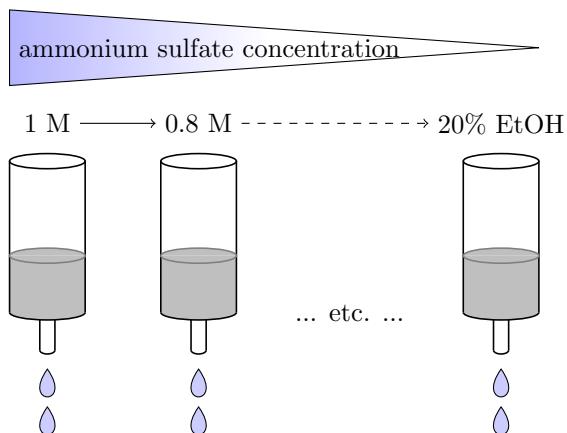


Figure 1: Experimental setup for the HIC.

time	OD ⁶⁰⁰	comment
10.40	0.51	placed at 30°C
11.20	0.592	induced with 1 mM IPTG
17.15	6.98	collected cells (1 mL sample for SDS-PAGE etc.)

1 Cell lysis & Treatment

- cells were resuspended in ca. 40 mL of lysis buffer
- spatula tip of lysozyme was added and incubated at room temp for 15 min on a platform shaker
- three time sonication for 30 s at 70% amplitude
- addition of DNase salts and 1/1000 volume of XX U/mL DNase I
- incubation for 15 min on ice
- centrifugation at 10.000 $\times g$, 4°C for 15 min
- addition of an equal volume (40 mL) of 2 M ammonium sulfate, pH 7.4 to the supernatant (add while stirring on ice) → this takes the supernatant to 1 M ammonium sulfate
→ the solution becomes turbid from precipitating proteins and needs to be centrifuged
- centrifugation at 10.000 $\times g$, 4°C for 30 min
→ **took sample of pellet for SDS-PAGE**

lysis buffer

50 mM Tris/HCl
500 mM NaCl
10 % glycerol
0.1 % Triton X100
pH 7.4

2 Phenyl sepharose column

- Filled self-pack column with 6 mL Phenyl-sepharose suspension in 20 % EtOH → about 4 mL column material
- washed with water (~4 CV)
- equilibrated with binding buffer (~3 CV)
- applied 100 mL of cell lysate (@ 1 M ammonium sulfate) from section 1
→ **sample for SDS-PAGE (applied 2 HIC)**
- **started fractionation** (4 mL fractions in 5 mL eppis)
- washed with 2 CV binding buffer
- eluted stepwise with 2 CV each, 0.8/0.6/0.4/0.2/0 M ammonium sulfate, 50 mM KP_i pH 7
- eluted with 2 CV 50 mM KP_i, 10 % EtOH, pH 7
- **ended fractionation**

Table 1: Fractions collected from stepwise elution of HIC

Fraction	1	2	3	4	5	6	7	8	9	10	11	12	13	14
AS [M]	1		0.8		0.6		0.4		0.2		0		20 % EtOH	

Binding Buffer

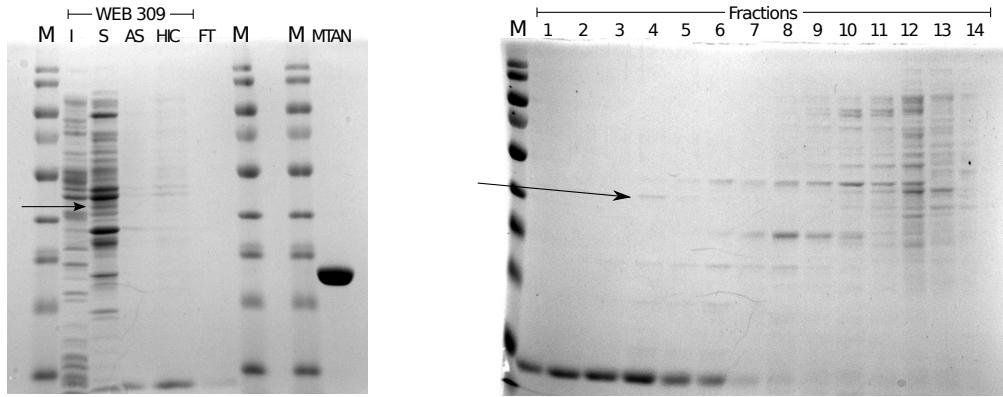
50 mM Phosphate, 1 M (NH₄)₂SO₄

Elution buffer

stepwise lowering of (NH₄)₂SO₄ concentration (0.2 M steps)

3 SDS-PAGE

- samples from fractions were analyzed by UV-VIS (A²⁸⁰) and Bradford for protein estimation
- dilution factor for Bradford was 5
- 10 µl of each sample were applied directly on SDS-PAGE (10% acrylamid)
- 500 µl of each fraction were also precipitated by TCA and resuspended in 100 µl PBS + 20 µl SDS loading dye for further analysis, due to low protein concentration on first SDS-PAGE (see below) → these samples were stored at -20°C for later analysis
- the sample taken from culture prior to harvesting were lysed with B-PER II reagent and subfractioned into soluble and insoluble fraction for SDS-PAGE



(a) Various samples from during workup.

(b) Fractions eluted during HIC.

Figure 2: SDS-PAGE of WEB309. I – insoluble fraction, S – soluble fr., AS – pellet after ammonium sulfate addition, HIC – supernatant after ammonium sulfate addition (this was applied to HIC), FT – flowthrough. M – Marker, MTAN – E.coli methylthioadenosyl nucleosidase.

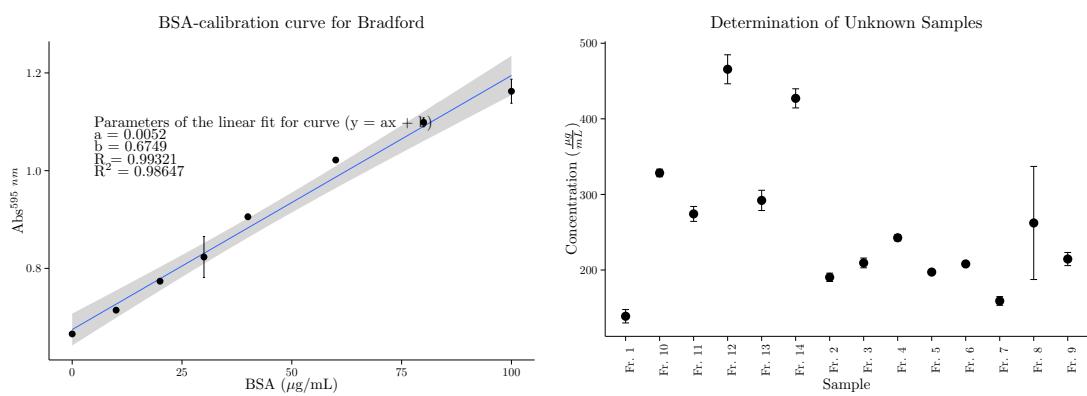


Figure 3: Protein determination by bradford

Table 2: Setup of the gels for SDS-PAGE analysis

	Sample	protein concentration ($\frac{\mu g}{mL}$)	SD
1	Fr. 1	138.82	8.84
2	Fr. 10	328.40	5.03
3	Fr. 11	274.20	9.86
4	Fr. 12	465.56	19.18
5	Fr. 13	292.09	13.40
6	Fr. 14	427.09	12.65
7	Fr. 2	190.33	5.51
8	Fr. 3	209.37	6.46
9	Fr. 4	242.65	4.01
10	Fr. 5	197.25	1.02
11	Fr. 6	208.03	2.11
12	Fr. 7	159.07	5.78
13	Fr. 8	262.23	74.81
14	Fr. 9	214.52	8.71

WEB310 - SOMT Refolding & HIC

Benjamin Weigel

30rd September, 2014

1 Refolding

- refolded 2.5 ml $1 \frac{mg}{mL}$ SOMT in 50 mL buffer 12 over night at 4°C
- added 1 Volume (50 mL) of 2 M $(\text{NH}_4)_2\text{SO}_4$
- adjusted pH to 7 using NaOH

ATTENTION: Added 50 mL of an unknown solution (from DMEs bench) → to compensate 100 mL of 2 M $(\text{NH}_4)_2\text{SO}_4$ were added and then adjusted to pH 7

2 HIC

- equilibrated 4 mL phenyl sepharose matrix with 3 CV water & 3 CV 1 M $\text{NH}_4)_2\text{SO}_4$
- applied 200 mL of sample
- eluted stepwise from 1 M $(\text{NH}_4)_2\text{SO}_4$ to 20 % EtOH (see WEB309) and collected 4 mL fractions
- for SDS-PAGE 1 mL of each fraction was precipitated using TCA and resuspended in 10 μl PBS and 2 μl SDS loading dye (heated to 95 °C and applied to gel)

ATTENTION: after cleaning the HIC column with 4 CV of 1 M acetic acid and 4 CV of 0.1 M NaOH protein precipitated in the washed out solution → took a sample and centrifuged to collect pellet → large pellet of probably protein → do SDS-PAGE

Table 1: Setup of the gels for SDS-PAGE analysis

lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Fraction	Flowthrough	M	1	2	3	4	5	6	7	8	9	10	11	12	13	14

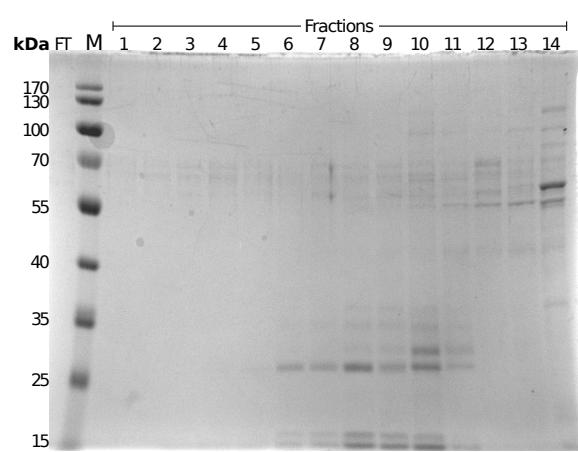


Figure 1: SDS-PAGE of fractions eluted from HIC.

WEB311 - SOMT Refolding & HIC

Benjamin Weigel

1st October, 2014

1 Refolding

- refolded 2.5 ml $1 \frac{mg}{mL}$ SOMT in 50 mL buffer 12 over night at 4°C
- added 1 Volume (50 mL) of 2 M $(\text{NH}_4)_2\text{SO}_4$
- adjusted pH to 7 using 5 M KOH → solution turned slightly turbid
- centrifuged to remove precipitate (20.000 x g, 4°C , 30 min)

2 HIC

- equilibrated 4 mL phenyl sepharose matrix with 3 CV water & 3 CV 1 M $(\text{NH}_4)_2\text{SO}_4$ pH 7
- applied 100 mL of clarified sample
- eluted stepwise from 1 M $(\text{NH}_4)_2\text{SO}_4$ to 20 % EtOH (see WEB309), then 70 % EtOH, 0.1 M NaOH and 0.5 M NaOH, and collected 4 mL fractions
- for SDS-PAGE 1 mL of each fraction was precipitated using TCA and resuspended in 10 μl PBS and 2 μl SDS loading dye (heated to 95 °C and applied to gel)

ATTENTION: after increasing EtOH to 70% there is visible precipitate at the top of the column

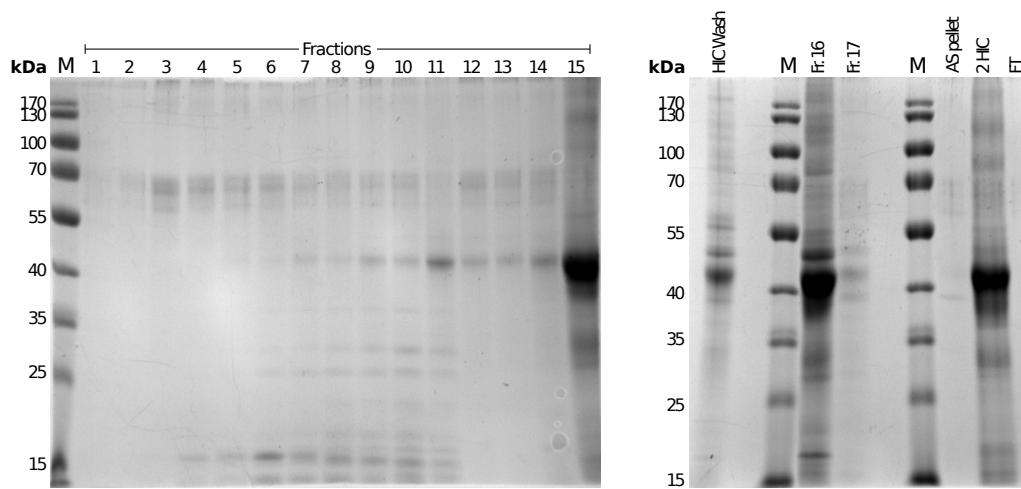


Figure 1: SDS-PAGE of fractions eluted from HIC.

Table 1: Setup of the gels for SDS-PAGE analysis

gel	lane	sample	comment
Gel 1 / Fractions	1	Marker	
	2	Fr. 1	1 M AS
	3	Fr. 2	1 M AS
	4	Fr. 3	0.8 M AS
	5	Fr. 4	0.8 M AS
	6	Fr. 5	0.6 M AS
	7	Fr. 6	0.6 M AS
	8	Fr. 7	0.4 M AS
	9	Fr. 8	0.4 M AS
	10	Fr. 9	0.2 M AS
	11	Fr. 10	0.2 M AS
	12	Fr. 11	0 M AS
	13	Fr. 12	0 M AS
	14	Fr. 13	20 % EtOH
	15	Fr. 14	20 % EtOH
	16	Fr. 15	70 % EtOH
Gel 2	1		
	2	HIC Wash	applied precipitate that appeared after stringent wash (0.1 M acetic acid [4 CV], 0.1 M NaOH [4 CV]) - WEB310
	3		
	4	Marker	
	5	Fr. 16	
	6	Fr. 17	
	7		
	8	Marker	
	9	AS pellet	precipitate from after AS addition
	10	2HIC	sample applied to HIC (after AS addition & centrifugation)
	11	FT	flowthrough from HIC column
	12		
	13		
	14		
	15		
	16		

WEB312 - SOMT Refolding & HIC

Benjamin Weigel

2nd October, 2014

1 Refolding

- refolded 1.25 ml $1\frac{mg}{mL}$ SOMT in 25 mL buffer 12 over night at 4°C
- adjusted pH to 7 using 5 N KOH
- took four 2.5 mL aliquot
- added 2.5 mL of each of the following to obtain the half of these concentrations to apply samples A-D to HIC
 - a) 2 M $(NH_4)_2SO_4$
 - b) 1.2 M $(NH_4)_2SO_4$
 - c) 0.6 M $(NH_4)_2SO_4$
 - d) 2 M NaCl
- for SDS PAGE 500 μL of each sample (5 mL total volume) was precipitated using TCA

ATTENTION: only had about 100 μL of C for precipitation with TCA and subsequently SDS-PAGE

2 HIC

- prepared four phenyl sepharose columns (1 mL matrix each)
- equilibrated matrix with 3 CV water & 3 CV of each for columns (A-D):
 - a) 1 M $(NH_4)_2SO_4$
 - b) 0.6 M $(NH_4)_2SO_4$
 - c) 0.3 M $(NH_4)_2SO_4$
 - d) 1 M NaCl
- applied 4.5 mL of each sample to column
- washed with 4 mL of solutions (a-d, 1 M, 0.6 & 0.3 M AS and 1 M NaCl, see above)
- eluted with 4 mL of 50 mM KPi pH 7
- for SDS-PAGE 2 mL of each fraction was precipitated using TCA and resuspended in 10 μL PBS and 2 μL SDS loading dye (heated to 95 °C and applied to gel)

Table 1: Sample numbering.

No.	Sample	description
1		flow through
2		wash
3		elution
A	1 M $(\text{NH}_4)_2\text{SO}_4$	applied 2 HIC
4		flow through
5		wash
6		elution
B	0.6 M $(\text{NH}_4)_2\text{SO}_4$	applied 2 HIC
7		flow through
8		wash
9		elution
C	0.3 M $(\text{NH}_4)_2\text{SO}_4$	applied 2 HIC
10		flow through
11		wash
12		elution
D	1 M NaCl	applied 2 HIC

WEB313 - SOMT Golden Gate Cloning

cloning to pICH41308 and subcloning, for SOMT2 expression in *Nicotiana benthamiana* (with

Sylvestre Marillonet)

Benjamin Weigel

2nd October, 2014

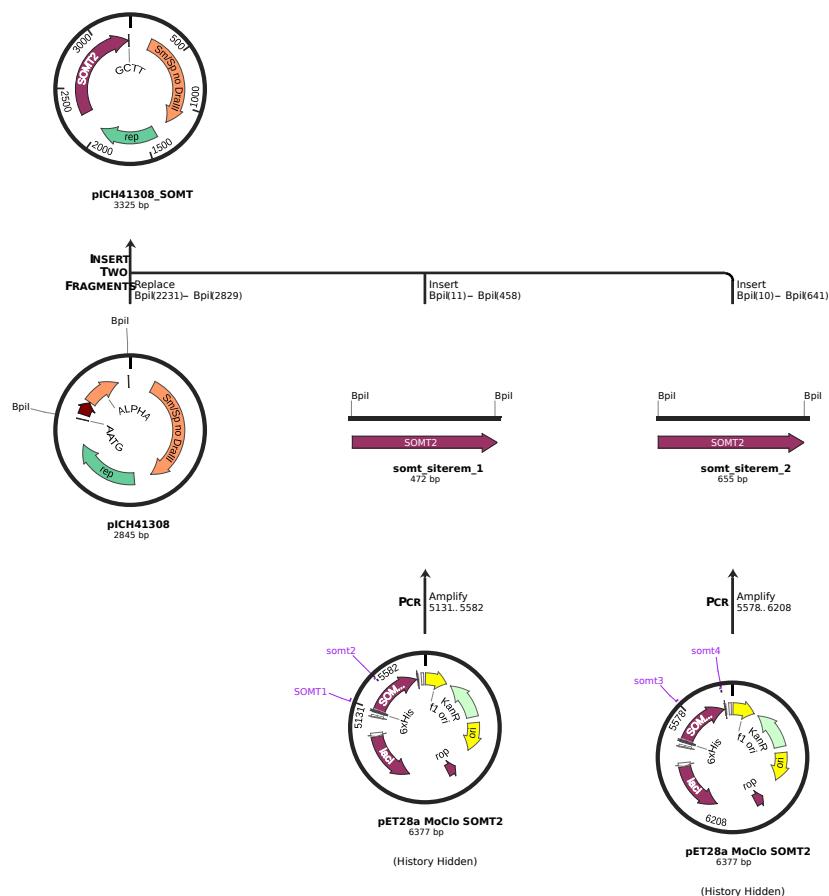


Figure 1: Schematic drawing of the **Level 0** cloning of SOMT2 into pICH41308

1 Introduction

- according to literature SOMT can methylate 4'-hydroxy group of isoflavones and flavones, namely naringenin
- no activity could be shown *in vitro*, neither from refolded, nor from solubly expressed (*E. coli*, periplasmatic expression) protein
- to verify the activity described in literature → eucaryotic expression *in planta* (*N. benthamiana*)
- cooperation with Sylvestre Marillonet (SZB)
- SZB already has the pathway cloned up to naringenin (Figure 2)
- **Aim:**
 - A. clone SOMT in vector for expression in *N. benthamiana*
 - B. infiltrate plant using *Agrobacterium tumefaciens*
 - C. (hopefully) detect product, 4'-O-methyl naringenin

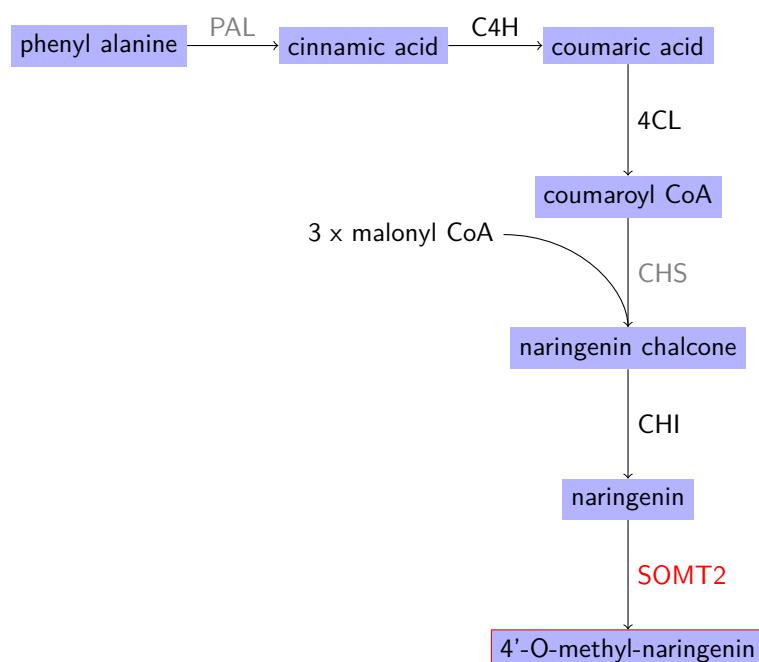


Figure 2: Semi-synthetic pathway to naringenin and 4'-O-methyl naringenin in *N. benthamiana*. Enzymes not endogenous to *N. benthamiana* are in gray. PAL - phenylalanine ammonia lyase, C4H - cinnamic acid 4-hydroxylase, 4CL - 4-coumaric acid:CoA ligase, CHS - chalcone synthase, CHI - chalcone isomerase, SOMT2 - soy O-methyl transferase 2

2 Cloning to pICH41308

ATTENTION: SOMT gene has an endogenous Bsal cleavage site → site needs to be removed

- four primers used for cloning (due to endogenous Bsal site) (see Table 1 for sequences)
 - a) *somt1* - forward primer for first fragment

- b) *somt2* - reverse primer for first fragment (also primer with **C>G** mutation to remove endogenous Bsal site)
- c) *somt3* - forward primer for second fragment
- d) *somt4* - reverse primer for second fragment

Table 1: The primers used for cloning.

name	sequence [5'→3']
somt1	tt gaagac aa aatggcttcattaaacaatggccg
somt2	tt gaagac aa ggacaccccaaatactgtgagatcttcc
somt3	tt gaagac aa gtccttaggaacaccttctggac
somt4	tt gaagac aa aagctcaaggatagatctcaataagagac

2.1 PCR SOMT2

- cloning from pET28a MoClo SOMT2 (2) [51 $\frac{\mu\text{g}}{\mu\text{l}}$]

Reaction mix:

substance	volume	per 50 μl
10x KOD Buffer	10 μl	1x
25 mM MgSO ₄	6 μl	
2 mM dNTPs	10 μl	0.2 mM
51 ng/ μl template	0.5 μl	12.5 ng
KOD HS Polymerase	2 μl	1 μl
water	65.5 μl	

Reactions:

Reaction 1	Reaction 2
47 μl Reaction mix	47 μl Reaction mix
1.5 μl somt1	1.5 μl somt3
1.5 μl somt2	1.5 μl somt4

Theoretical size of fragment

472 bp 655 bp

PCR Program:

Step	Description	Temperature	Time	
1	Denaturation	95°C	2 min	
2	Denaturation	95°C	20 sec	
3	Annealing	55°C	10 sec	
4	Extension	70°C	12 sec	repeat 2-3 24x
5	Final Extension	70°C	2 min	
	Pause	4°C	∞	

3 Level 0: Digestion-Ligation

Reaction:

- 1:10 diluted DNA is used

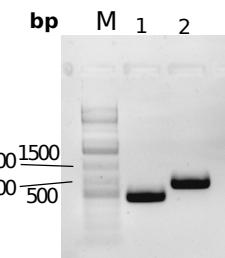


Figure 3: Agarose gel electrophoresis results of PCR. Bands correspond to the theoretical size of the fragments (472 and 655 bp).

DNA	concentration $\frac{ng}{\mu L}$	dilute (1:10)	$\left(\frac{ng}{\mu L}\right)$
pCH41308	420		42
SOMT.f1	305		30.5
SOMT.f2	275		27.5

component	amount	volume
pICH41308	20 fmol	0.89 μL
SOMT.f1	20 fmol	0.20 μL
SOMT.f2	20 fmol	0.31 μL
T4 Ligase (Fermentas)	5 U	1 μL
Bpil (Fermentas)	5 U	0.5 μL
Ligase buffer (Fermentas)	1x	1.5 μL
ad to 15 μL MQ-H ₂ O (10.6 μL)		

Cycler Program:

step	temperature	time
	37°C	4 h
	50°C	5 min
	80°C	5 min
Pause	4°C	∞

4 Level 1: Digestion-Ligation

DNA	concentration $\frac{ng}{\mu L}$	dilute (1:10)	$\left(\frac{ng}{\mu L}\right)$
pCH51266	428		42
pCH41421	155		15
pICH75044	241		24
pICH41308_SOMT	285		28

Reaction:

- 1:10 diluted DNA is used

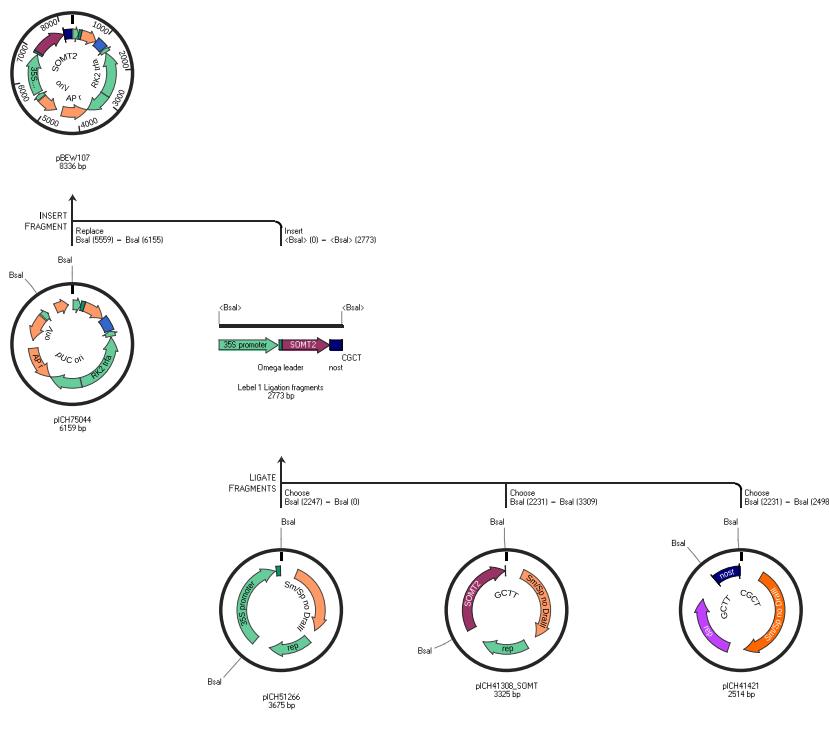


Figure 4: Level 1 Cloning into expression vector for *Agrobacterium tumefaciens* transformation.

component	amount	volume
pICH75044	20 fmol	3.4 μ l
pICH51266	20 fmol	1.2 μ l
pICH41421	20 fmol	2.9 μ l
pICH41308_SOMT	20 fmol	1.2 μ l
T4 Ligase (Fermentas)	5 U	1 μ l
Bsal (Fermentas)	5 U	0.5 μ l
Ligase buffer (Fermentas)	1x	1.5 μ l
ad to 15 μ l MQ-H ₂ O (3.3 μ l)		

Cycler Program:

step	temperature	time	
	37°C	2 min	↳ 50 x
	16°C	5 min	
	50°C	5 min	
	80°C	10 min	
Pause	4°C	∞	

- transform 5 μ l ligation reaction into 50 μ l DH5 α cells

- add 500 μ l SOC
- plate 100 μ l onto LB + S-Gal + Ampicillin Agar
- incubate at 37°C → ~ 50 colonies, no blacks (blue)
- 3 mL cultures of two colonies → plasmid prep and digestion with Bpil

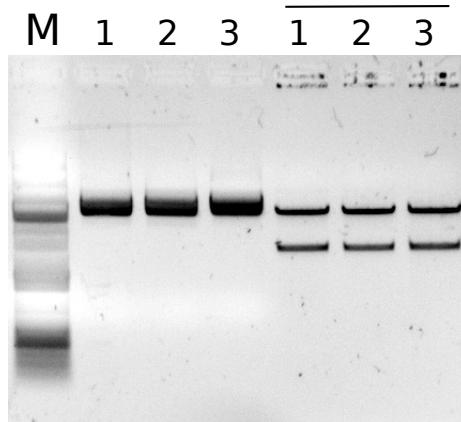


Figure 5: Bpil digestion of pBEW107 (overlined, right) and undigested vector controls (left).

5 Infiltration of *Nicotiana benthamiana*

5.1 Transformation into *Agrobacterium tumefaciens*

- ! cool cuvettes and DNA on ice, work quickly
- thaw electrocompetent agrobacteria on ice
- add 0.5 μ l plasmid to 50 μ l electrocompetent agrobacterium suspension and mix by tapping
- place into electroporation cuvette and pulse
- add 1 mL LB and transfer culture to 1.5 mL tube
- shake for 3-4 hours at 28°C
- centrifuge and remove 900 μ l of supernatant
- plate remaining suspension onto LB + 40 μ g/mL rifampicin + 50 μ g/mL carbencillin plates
- grow at 28°C for 2-3 days
- store plate in fridge

5.2 General infiltration of *N. benthamiana*

- inoculate 5 mL LB + 40 $\frac{\mu\text{g}}{\text{mL}}$ rifampicin and 50 $\frac{\mu\text{g}}{\text{mL}}$ carbencillin with *agrobacterium*
- grow over night at 28°C and 220 rpm
- measure OD⁶⁰⁰ in 1:10 dilution in water (use LB + rif in water as blank)
- for infiltration make dilutions of cell culture with OD⁶⁰⁰ = 0.2 (10^{-1} dilution) in **infiltration buffer**)
- infiltrate reduced (cut back leaves, except for the ones that will be infiltrated) *N. benthamiana* plants using 2 mL syringe

5.3 Special infiltration for this experiment

- everything as in general protocol (5.2)
- ! two infiltration solutions were prepared, both containing 10406 and 10733 (PAL and CHS genes) and either pBEW107, or the control plasmid

Table 2: OD⁶⁰⁰ values measured for the *agrobacterium* cultures.

Sample	OD ⁶⁰⁰	comment
pBEW107	1.72	measured against water
control (75044)	1.32	"
pBEW107	1.15	measured against LB + rif in water (Ramona)
control (75044)	0.88	"
PAL? (10406)	1.86	"
CHS? (10733)	2.08	"

Table 3: Recipes for infiltration solution. Two infiltrations solutions were prepared. One with the SOMT2 gene (pBEW107) and one with the vector control (75044). Both infiltration solutions also contained the PAL and CHS genes (10406, 10733).

Sample	component	volume (mL)
pBEW107	pBEW107	3.478
	10406	2.15
	10733	1.923
	inf. buffer	12.449
control	75044	4.545
	10406	2.15
	10733	1.923
	inf. buffer	111.382

Infiltration pattern (from leaf top)

- left side of leaf → SOMT2
- right side of leaf → control

6 Preparation and extraction of plant material

6.1 Preparation of leaves

The infiltrated areas of the plants were cut out. Separate samples for each plant, top and bottom leaves and infiltration sides (left/right) were prepared. Twelve (12) samples were prepared in total (Table 4). The cut out areas were weighed (wet weight), frozen in liquid nitrogen, ground into powder and stored at -80°C .

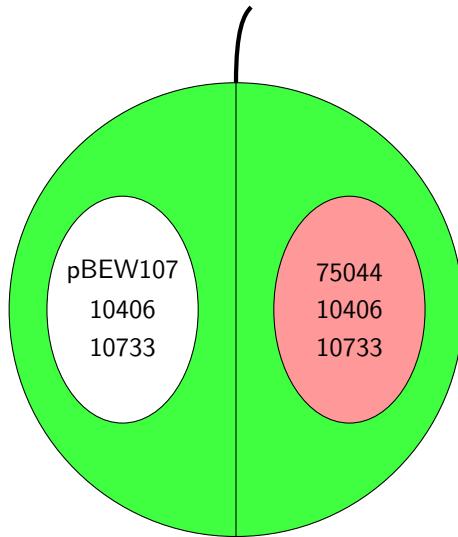


Figure 6: Schematic drawing if the infiltrated leaves (view from top). On the right side the SOMT2 gene is infiltrated, left is the control.

Table 4: Leaf samples from agrobacterium infiltration. The plants were grown for 7 days at (???) after infiltration.

sample	plant	leaf position	leaf side	wet weight	dry weight	tube tara
1tl	1	top	left	0.4204		3.1688
1tr	1	top	right	0.2986		3.1456
1bl	1	bottom	left	1.6333		3.1480
1br	1	bottom	right	1.2322		3.1567
2tl	2	top	left	0.3349		3.1499
2tr	2	top	right	0.4363		3.1523
2bl	2	bottom	left	1.1756		3.1621
2br	2	bottom	right	1.3726		3.1412
3tl	3	top	left	0.7079		3.1464
3tr	3	top	right	0.6861		3.1539
3bl	3	bottom	left	1.0370		3.1380
3br	3	bottom	right	1.1720		3.1539

6.2 Extraction of flavonoids

2-mL tubes were weighed. Two tips of a small spatula of plant material (approx. 6 mg) was added and the tube weighed again. The plant material was extracted with 500 μ l of 1 mM ascorbic acid, 0.2% formic acid, 0.1 mM flavone in 75% MeOH. For extraction the suspension was vortexed for 30 s, rotated in an orbital shaker for 10 min and vortexed again for 30 s. The suspension was centrifuged at 10.000 x g and 4°C for 10 min. The supernatant was centrifuged again, and the resulting supernatant solution was applied to HPLC.

6.3 Hydrolysis of glycosylated compounds

To analyse the aglycone flavonoid, the glycosylated flavonoids were hydrolyzed. Therefore 500 μ l of 2 N 75% methanolic HCl were added to the solid residual plant material obtained from the extraction. The suspension was heated to 90°C for 1 hour.

The result was centrifuged for 10 minutes at 10.000 x g and 4°C , and the supernatant was evaporated in a *SpeedVac* set to 60°C . The residue was resuspended in methanol containing 1 mM ascorbic acid and 0.2% formic acid. The resulting solution was centrifuged at 10.000 x g and 4°C for 10 minutes. Consequently, the supernatant was analyzed by HPLC.

Attention: Some of the tubes sprung open during heating.

7 Buffers and Recipes

7.1 Antibiotics

Rifampicin (40 mg/mL): dissolve 200 mg rifampicin in 5 mL MeOH

Carbencillin (50 mg/mL): dissolve 250 mg carbencillin in 5 mL H₂O

7.2 Buffers

Infiltration buffer: 10 mM Mes/NaOH, 10 mM MgSO₄ pH 5.5

WEB314 - MTAN Activity measurement for DIM

Benjamin Weigel

Oct. 6th, 2014

1 Experimental

- Calibration curve Adenine (HPLC)
- measurements (4 time points, 0, 5, 10, 20, 30 min)

1.1 Assay

Assay buffer: 0.1 M Tris/HCl, 0.2 M KCl, 20 mM MgCl₂, pH 7.5

Substrate Solution: 10 mM SAH in 50 mM HCl

Reaction Mix (1000 µl):

2.5 mM SAH	316.2 µl 7.9 mM SAH
2 µM ~ 53 $\frac{\mu g}{mL}$ MTAN in assay buffer	38 µl 1.4 $\frac{mg}{mL}$ MTAN (omit until start of reaction) 100 µl 1 M Tris/HCl 50 µl 4 M KCl 50 µl 0.4 M MgCl ₂ 445.8 µl ddH ₂ O

Reaction:

1. 192.4 µl Reaction Mix
2. add 7.6 µl 1.4 $\frac{mg}{mL}$ MTAN
3. measure UV absorption at 276 nm (JASCO) in 1 mm cuvette R abline dashedfor 300 seconds at room temp (25°C)

2 Fitting the data

sample	lower fit limit (s)	upper fit limit (s)
WEB314.buffer	0	300
WEB314.denat.1	0	300
WEB314.S.1	0	30
WEB314.S.2	0	30
WEB314.S.3	0	30

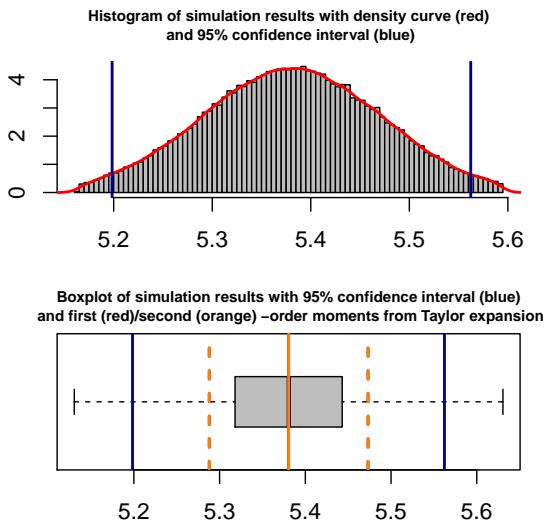


Figure 1: The plot of the object obtained from the error propagation

Table 1: The specific activity of MTAN in $\frac{U}{mg}$ as calculated from the slopes of the progress curves. Calculations from triplicate measurements were made using the *propagate* package for R.

	Mean.1	Mean.2	sd.1	sd.2	2.5%	97.5%
$\frac{U}{mg}$	5.3805248	5.3805248	0.0926499	0.0926499	5.1989500	5.5617620

WEB315 - SOMT2 expression from pET20b in *E.coli* C43(DE3)

Benjamin Weigel

Oct. 14th, 2014

1 Expression

- 3 mL pre-culture of *E.coli* C43(DE3) pET20b SOMT2 from glycerol stock in LB-medium with 200 $\frac{\mu\text{g}}{\text{mL}}$ ampicillin
- incubation at 37°C and 220 rpm over night
- next morning → measured OD⁶⁰⁰
- inoculated 100 mL culture in TB medium to an OD of ~0.06 (1.7 mL)
- incubated at 37°C/220rpm for 1.5 h
- incubated at 30°C for 1 hour
- induced with 1 mM IPTG and incubated at 30°C for 5 hours
- collected cells by centrifugation at 4°C and 10.000 x g for 10 min
- drained and weighed cell pellet

time	OD ⁶⁰⁰	comment	SDS-PAGE sample
0935	3.578	inoculated main culture with 1.7 mL	
1113	0.307	set at 30°C	
1217	0.72	induce with 1 mM IPTG	X (VI, 1 ml)
1700	5.341	1.22 g cell pellet	X (NI, 900 μl)

2 Periplasmic Subfractionationing

- resuspended pellet in 100 mL (80 mL per gram wet weight) buffer A
- stirred suspension slowly at room temp for 10 min
- centrifuged at 10.000 x g, 4°C, 10 min
- drained pellet

- resuspended pellet in 100 mL ice cold 5 mM MgSO₄
- stirred suspension slowly on ice for 10 min
- centrifuged at 10.000 x g, 4°C, 10 min
- collected supernatant (100 mL) and adjusted to 1 M (NH₄)₂SO₄ using 100 mL 2 M (NH₄)₂SO₄
- adjusted pH to 7 using 3.1 mL K₂HPO₄ and 1.9 mL KH₂PO₄

3 Phenyl Sepharose HIC Column

- 1 mL phenyl sepharose matrix
- washed with water and equilibrated with 50 mM KPi, 1 M (NH₄)₂SO₄ pH 7
- applied sample (200 mL) to column at room temp
- washed/eluted stepwise with (3 mL each):
 - 1) 1 M (NH₄)₂SO₄, 50 mM KPi pH 7
 - 2) 0.6 M (NH₄)₂SO₄, 50 mM KPi pH 7
 - 3) 0.3 M (NH₄)₂SO₄, 50 mM KPi pH 7
 - 4) 50 mM KPi pH 7
 - 5) 20 % EtOH
 - 6) **5 mL 0.1 N NaOH**
- collected 3 mL fractions
- for SDS-PAGE 1 mL of each fraction was precipitated with TCA

sample	comment	SDS-PAGE sample	resusp. in PBS [μl]
1	Periplasma Prep	PP	10
2	PP after AS addition	AS	10
3	applied to HIC	2HIC	10
4	HIC flowthrough	FT	10
5	HIC Wash	1 M	100
6	HIC Elution 0.6 M	0.6	100
7	HIC Elution 0.3 M	0.3	100
8	HIC Elution 0 M	0	100
9	HIC Elution 20% EtOH	EtOH	100

3.1 Observations

- after about 50 mL of sample applied to HIC flow slowed down notably (maybe aggregated protein, DNA etc. on column)
- washing with 0.1 N NaOH speeds up flow again

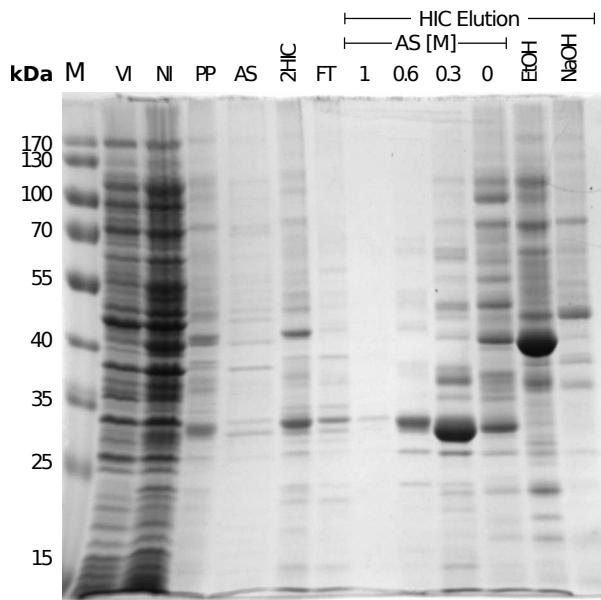


Figure 1: SDS-PAGE of Expression and fractions from HIC column. **VI** - pre-induction, **NI** - post-induction, **PP** - periplasm fraction, **AS** - pellet after addition of 1 M $(\text{NH}_4)_2\text{SO}_4$ to the periplasm fraction, **2HIC** - supernatant (applied to HIC), **FT** - flow through, 1 through 0 correspond to the AS concentrations during the elution, **EtOH** - washed with 20% EtOH, **NaOH** - washed with 0.1 N NaOH

4 Activity Test

- using the 20% ethanol fraction
- control experiment with 20% EtOH instead of elution fractin

Reaction Mix

0.1 M HEPES pH 7
 0.2 mM Naringenin
 0.2 mM Daidzein
 0.2 mM Eriodictyol
 0.25 mM SAM
 in eluate

Reaction:

10 μl 1 M HEPES pH 7
 2 μl 10 mM Naringenin
 2 μl 10 mM Daidzein
 2 μl 10 mM Eriodictyol
 6.8 μl 5 mM SAM (73.5%)
 77.2 μl eluate (EtOH fraction)

sample	substrates	description
1+2	Naringenin, Daidzein, ED	
3+4	Quercetin, Genistein	
0/1+2	Naringenin, Daidzein, ED	control
0/3+4	Quercetin, Genistein	control

5 Activity Test - Day 2

- using the 20% ethanol fraction
- control experiment with 20% EtOH instead of elution fractin

Reaction Mix

0.1 M HEPES pH 7
 0.2 mM Quercetin 0.25 mM SAM
 in eluate

Reaction:

10 μ l 1 M HEPES pH 7
 2 μ l 10 mM Quercetin 6.8 μ l 5 mM SAM
 (73.5%)
 77.2 μ l eluate (EtOH fraction)

sample	\sim Enzym (mg/mL)	SAM (uM)	Quercetin (uM)	EtOH (%)
A	0.2	200	200	20
B	0.1	200	200	10
C	0.02	200	200	2
D	0.2	1000	200	20
E	0.2	200	0	20
F	0	200	200	20
G	0.2	0	200	20

sample	Fraction	SAM	Quercetin (10 mM)	1 M Hepes pH 7	H ₂ O
A	81.2	6.8	2	10	-
B	40.6	6.8	2	10	40.6
C	8.12	6.8	2	10	73.08
D	40.6	27.2	2	10	20.2
E	81.2	6.8	0	10	2
F	-	6.8	2	10	81.2
G	81.2	0	2	10	6.8

WEB316 - SOMT Refolding & ÄKTA HIC

Benjamin Weigel

22nd October, 2014

1 Refolding

- refolded 2.5 ml $1 \frac{mg}{mL}$ SOMT in 50 mL buffer 12 over night at 4°C
- added 1 Volume (50 mL) of 2 M $(\text{NH}_4)_2\text{SO}_4$
- adjusted pH to 7 using 5 M KOH → solution turned slightly turbid
- centrifuged to remove precipitate (20.000 x g, 4°C , 30 min)

2 HIC

- equilibrated 1 mL phenyl sepharose column (HiTrap Phenyl FF (lows sub)) with 5 CV water & 5 CV 1 M $(\text{NH}_4)_2\text{SO}_4$, 50 mM HEPES pH 7
- applied 50 mL of clarified sample
- eluted stepwise from 1 M $(\text{NH}_4)_2\text{SO}_4$ to 20 % EtOH (see WEB309), then 70 % EtOH, 0.1 M NaOH and 0.5 M NaOH, and collected 4 mL fractions
- for SDS-PAGE 0.5 mL of selected fractions were precipitated using TCA and resuspended in 10 μl PBS and 2 μl SDS loading dye (heated to 95 °C and applied to gel)

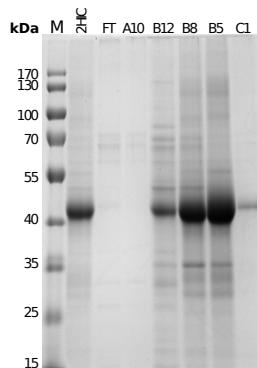


Figure 1: Refolding of SOMT and subsequent HIC (ÄKTA). SDS-PAGE of selected fractions.

Table 1: Setup of the gels for SDS-PAGE analysis

gel	lane	sample	comment
Gel 1 / Fractions	1		
	2		
	3		
	4	Marker	
	5	2HIC	applied to column
	6	FT	flowthrough
	7	A10	0.6 M AS
	8	B12	0.4 M AS
	9	B8	0.4 M AS
	10	B5	0.2 M AS
	11	C1	
	12		
	13		
	14		
	15		
	16		

3 Activity Test

- using fraction B12, B8 and B5 of Run 1 and A8 of Run 2
- control experiment with 20 mM Hepes pH 7 instead of elution fraction
- 6 x MM for each substrate group
 - **Group 1:** Naringenin, Daidzein, ED
 - **Group 2:** Genistein, Quercetin, HED
 - **Group 3:** Apigenin, Hesperetin

Reaction Mix

0.1 M HEPES pH 7

0.2 mM substrate

0.25 mM SAM

in eluate

Mastermix Group 1 (6x)

60 μ l 1 M HEPES pH 7

12 μ l 10 mM Naringenin, Daidzein, ED

40.8 μ l 0.25 mM SAM

43.2 μ l H₂O

Mastermix Group 2 (6x)

60 μ l 1 M HEPES pH 7

12 μ l 10 mM Genistein, Quercetin, HED

40.8 μ l 0.25 mM SAM

43.2 μ l H₂O

Universal-Werkzeugkoffer von Famex

Mastermix Group 3 (6x)

60 μ l 1 M HEPES pH 7

12 μ l 10 mM Apigenin, Hesperetin

40.8 μ l 0.25 mM SAM

55.2 μ l H₂O

Reaction:

30 μ l mastermix

70 μ l eluate fraction

sample	\sim Run/Fraction	Substrate group
A	1/B12	1
B	1/B12	2
C	1/B12	3
D	1/B8	1
E	1/B8	2
F	1/B8	3
G	1/B5	1
H	1/B5	2
I	1/B5	3
J	2/A8	1
K	2/A8	2
L	2/A8	3
M	buffer	1
N	buffer	2
O	buffer	3

WEB317 - SOMT Refolding & ÄKTA HIC

Benjamin Weigel

23nd October, 2014

1 Refolding

- refolded 5 ml $2\frac{mg}{mL}$ SOMT in 100 mL buffer 12 over night at 4°C
- added 1 Volume (100 mL) of 2 M $(NH_4)_2SO_4$
- adjusted pH to 7 using 5 M KOH → solution turned slightly turbid
- centrifuged to remove precipitate (20.000 x g, 20°C , 30 min)

2 HIC

! all steps (equilibration, sample injection and elution) were conducted at room temperature !

- equilibrated 1 mL phenyl sepharose column (HiTrap Phenyl FF (lows sub)) with 5 CV water & 5 CV 1 M $(NH_4)_2SO_4$, 50 mM HEPES pH 7
- applied 50 mL of clarified sample
- eluted stepwise from 1 M $(NH_4)_2SO_4$ to 20 % EtOH (see WEB309), then 70 % EtOH, 0.1 M NaOH and 0.5 M NaOH, and collected 4 mL fractions

3 Activity Test

- using fraction B5 (WEB316), A4 and A6
- control experiment with 20 mM Hepes pH 7

Reaction Mix

0.1 M HEPES pH 7
±0.2 mM Quercetin
0.25 mM SAM
70 μ L of corresponding fraction

Table 1: Activity Test setups

sample	~ Experiment/Run/Fraction	Quercetin (uM)	SAM (uM)
A	WEB316/1/B5	200	250
B	WEB316/1/B5	200	0
C	WEB317/1/A4	200	250
D	WEB317/1/A4	200	0
E	WEB317/1/A6	200	250
F	WEB317/1/A6	200	0
G	buffercontrol	200	250

Table 2: Activity test pipetting scheme

sample	Fraction (70 uL)	Quercetin (10 mM)	SAM (5 mM, 73.5%)	Buffer	H ₂ O
A	WEB316/1/B5	2	6.8	10	11.2
B	WEB316/1/B5	2	0	10	18
C	WEB317/1/A4	2	6.8	10	11.2
D	WEB317/1/A4	2	0	10	18
E	WEB317/1/A6	2	6.8	10	11.2
F	WEB317/1/A6	2	0	10	18
G	buffercontrol	2	6.8	10	11.2

Table 3: Layout Bradford Plate

Well	sample	dilution factor
A3	WEB316 Fr. B12	1
B3	WEB316 Fr. B8	1
C3	WEB316 Fr. B5	1
D3	WEB317 Fr. A4	1
E3	WEB317 Fr. A5	1
F3	WEB317 Fr. A6	1
G3	WEB317 Fr. A7	1
H3	WEB317 Fr. A8	1
A4	WEB317 Fr. A9	1
A5	WEB316 Fr. B12	5
B5	WEB316 Fr. B8	5
C5	WEB316 Fr. B5	5
D5	WEB317 Fr. A4	5
E5	WEB317 Fr. A5	5
F5	WEB317 Fr. A6	5
G5	WEB317 Fr. A7	5
H5	WEB317 Fr. A8	5
A6	WEB317 Fr. A9	5

WEB318 - COMT von Thomas Vogt

Benjamin Weigel

6th November, 2014

1 Introduction

- obtained 4 cultures of COMT expression *E.coli* host from the group of Thomas Vogt
- cultures possess **Ampicillin resistance**
- Culture **COMT 1** was used for plasmid mini prep → sequenced with *pQE_for* and *pQE_rev* primers
- COMT1 gene from *Arabidopsis thaliana* (NCBI Accession Number: NM_124796.3)
- codes for caffeic acid/5-hydroxyferulic acid O-methyltransferase (363 amino acid protein, Accession: NP_200227)

2 Expression

- COMT cultures were incubated over night at 37°C and 200 rpm
- inoculated two 250 mL ZYP-5052 (+ 200 $\frac{\mu g}{mL}$ ampicillin) cultures with 1 mL of COMT 2 each
- incubated at 37°C and 220 rpm from 13.00
- 2030 → $OD^{600} = 3.8$ → cell collection & 1 mL sample
- resuspended cells in 60 mL **lysis buffer** (50 mM Tris/HCl, 0.5 M NaCl, 10% glycerol, 10 mM Imidazole, 1% Tween-20, pH 7.4)
- added a spatula tip of lysozyme and incubated on a shaker platform at room temp for 15 min
- lysed cells by sonication (3x @ 70% amplitude, 30 s, 1 s on-off cycle)
- centrifuged at 10.000 x g, 4°C for 30 min to remove debris → **Prepped IBs from debris**
- filtered the cleared lysate and subjected to metal affinity chromatography (ÄKTA, HiTrap Talon 1 mL FF)
Wash Buffer: 50 mM Tris/HCl, 0.5 M NaCl, 10% glycerol, 10 mM Imidazole, pH 7.4
Elution Buffer: 50 mM Tris/HCl, 0.5 M NaCl, 10% glycerol, 250 mM Imidazole, pH 7.4
- no protein eluted :(→ redo with more time and LB, TB, ZY medium

WEB319 - SOMT Refolding & ÄKTA HIC

Benjamin Weigel

5th November, 2014

1 Refolding

- refolded 2.5 ml 1 $\frac{mg}{mL}$ SOMT in 50 mL reducing buffer 12 (0.1 M Mes, 10% glycerol, 0.5 M Arginine*HCl, 2 mM MgCl₂, 2 mM CaCl₂, 0.5 mM Tween-80, 10 mM NaCl, 0.5 mM KCl, 5 mM DTT pH 5.5) over night at 4°C
- added 1 Volume (50 mL) of 2 M (NH₄)₂SO₄
- adjusted pH to 7 using 5 M KOH → solution turned slightly turbid
- centrifuged to remove precipitate (20.000 x g, 20°C , 30 min)

2 HIC

- ! all steps (equilibration, sample injection and elution) were conducted at room temperature !
- equilibrated 1 mL phenyl sepharose column (HiTrap Phenyl FF (lows sub)) with 5 CV water & 5 CV 1 M (NH₄)₂SO₄, 50 mM HEPES pH 7
 - applied 50 mL of clarified sample
 - eluted stepwise from 1 M (NH₄)₂SO₄ to 20 % EtOH (see WEB309), then 70 % EtOH, 0.1 M NaOH and 0.5 M NaOH, and collected 4 mL fractions

3 Activity Test

- using fraction A4, A6 and A9
- control experiment with 20 mM Hepes pH 7
- 6 x MM for each substrate group
 - **Group 1:** Naringenin, Daidzein, ED
 - **Group 2:** Genistein, Quercetin, HED
 - **Group 3:** Apigenin, Hesperetin

Reaction Mix

0.1 M HEPES pH 7
0.2 mM substrate
0.25 mM SAM
in eluate

Mastermix Group 1 (5x)

50 μ l 1 M HEPES pH 7
 10 μ l 10 mM Naringenin, Daidzein, ED
 34 μ l 5 mM SAM
 36 μ l H₂O

Mastermix Group 2 (5x)

50 μ l 1 M HEPES pH 7
 10 μ l 10 mM Genistein, Quercetin, HED
 34 μ l 5 mM SAM
 36 μ l H₂O

Mastermix Group 3 (6x)

50 μ l 1 M HEPES pH 7
 10 μ l 10 mM Apigenin, Hesperetin
 34 μ l 5 mM SAM
 46 μ l H₂O

Reaction:

30 μ l mastermix
 70 μ l eluate fraction

Reaction for WEB316/B5:

2 μ l Quercetin in DMSO or MeOH
 10 μ l 1 M Hepes pH 7
 18 μ l ddH₂O
 70 μ l eluate fraction

Reaction Conditions: incubate 2 h at 30 °C

sample	Fraction	Substrate group
A	A4	1
B	A4	2
C	A4	3
D	A6	1
E	A6	2
F	A6	3
G	A9	1
H	A9	2
I	A9	3
J	buffer	1
K	buffer	2
L	buffer	3
O	WEB316/B5	Quercetin from MeOH stock
P	WEB316/B5	Quercetin from DMSO stock

WEB320 - Docking experiments for PFOMT Paper

Benjamin Weigel

10th November, 2014

1 Docking using AutoDockTools and autodock4 (v. 4.2.5.1)

2 141125

2.1 1112

Receptor: 3c3y from PDB, waters deleted, hydrogens added

Ligand: S-Eriodictyol

Software: Yasara using dock_runensemble.mcr Makro

good Poses (orientation):

7, 13, (15), (20), 27, 33, 41 (4'), 51, 58, 73

cluster 5 (receptor ensemble 1/5), cluster 16 (ensemble 5/5)

2.2 1455

Receptor: 3c3y from PDB, waters deleted, hydrogens added;

mutations: Y51R, N202W (chainA)

Ligand: S-Eriodictyol

Software: Yasara using dock_runensemble.mcr Makro

good Poses (orientation):

7, 13, (15), (20), 27, 33, 41 (4'), 51, 58, 73

cluster 5 (receptor ensemble 1/5), cluster 16 (ensemble 5/5)

3 Docking using Vina - 20140105

3.1 Setup

- loaded 3c3y into pymol
- removed waters from structure and saved :

Table 1: Parameters of docking

Name	Ligand	Macromolecule	Grid center (xyz)	grid dimensions (xyz)	grid spacing (Å)
taxifolin.dlg	taxifolin	PFOMT + Ca^{2+}	1.581, 5.196, 25.718	58, 54, 68	0.375
taxifolin.b.dlg	taxifolin	only PFOMT	1.581, 5.196, 25.718	58, 54, 68	0.375
taxifolin.c.dlg	taxifolin	PFOMT + SAH + Ca^{2+}	1.581, 5.196, 25.718	58, 54, 68	0.375
taxifolin.c2.dlg	taxifolin	PFOMT + SAH + Ca^{2+}	1.581, 3.196, 27.718	58, 54, 68	0.375
R-ED.a.dlg	R-eriodictyol	PFOMT + SAH + Ca^{2+}	1.581, 5.196, 25.718	58, 54, 68	0.375
S-ED.a.dlg	S-eriodictyol	PFOMT + SAH + Ca^{2+}	1.581, 5.196, 25.718	58, 54, 68	0.375

Table 2: Files associated with docking

Name (Docking-Log-File)	Ligand-File	Macromolecule-File
taxifolin.dlg	taxifolin	PFOMTChainA_noWater_addedHydrogens

```
fetch 3c3y
select H20, ////HOH
remove H20
save 20140105_3c3y_clean.pdb, 3c3y
```

- loaded clean structure into ADT
- added polar hydrogens: Edit -> Hydrogens -> Add - (polar only)
- to save: Grid -> Macromolecule -> Choose then select a filename to save

3.2 Vina 1.1.2 (May 11, 2011)

- grid center (xyz): 1.581, 5.196, 25.718
- box size (xyz, Å): 22, 20, 25
- typical configuration file for vina:

```
receptor = 20140105_3c3y_clean
ligand = S_ED

out = dock1_all
log = dock1_log

center_x = 1.581
center_y = 5.196
center_z = 25.718

size_x = 22
size_y = 20
size_z = 25

exhaustiveness = 25
```

- start calculation with: vina --config <configfile.cfg>

3.3 Mutating Y51R and N202W using Pymol Mutagenesis

Used Pymol to mutate the residues Tyr51 and Asn202 to Arg and Trp, respectively. Unfortunately, every rotamer of **Tryptophane** clashes with other residues in the active site. Used rotamer number 7, which has the least clashes to mutate.

Table 3: Parameters of *vina* docking

Output (*.pdbsqt)	Log	Config (*.cfg)	Ligand	Macromolecule	minimization FF	comment
dock1_all	dock1_log	dock1	S-ED	PFOMT	-	rigid receptor
dock1_flex1_all	dock1_flex1_log	dock_flex1	S-ED	PFOMT	-	flexible Y51 and N202
dock_flex2_all	dock_flex2_log	dock_flex2	S-ED	PFOMT	amber03	flexible resis: 51, 157, 184, 202
dock_flex3_all	dock_flex3_log	dock_flex3	S-ED	PFOMT	yasara	flexible resis: 51, 157, 202
dock_flex4_all	dock_flex4_log	dock_flex4	S-ED	PFOMT	yasara	yasara for mutation; flexible resis: 51, 157, 184, 202
dock_flex5_all	dock_flex5_log	dock_flex5	S-ED	PFOMT	-	no minimization; flexible resis: 51, 157
dock_flex6_all	dock_flex6_log	dock_flex6	R-ED	PFOMT	-	flexible resis: 51, 202
dock_flex7_all	dock_flex7_log	dock_flex7	S-ED	PFOMT	-	flexible resis: 51, 157, 184, 202
dock8_all	dock8_log	dock8	S-ED	R51W202	amber03	rigid
dock9_all	dock9_log	dock9	S-ED	R51W202	yasara	rigid
dock10_all	dock10_log	dock10	S-ED	R51W202	yasara	yasara for mutation, rigid
dock11_all	dock11_log	dock11	S-ED	PFOMT	-	rigid receptor

View that was used below (Figure 1):

```
set_view (\n    0.387520641,    0.813636184,   -0.433348358,\n    -0.865751505,    0.482709885,   0.132122964,\n    0.316683739,    0.323972791,   0.891478777,\n    0.000000000,    0.000000000,  -84.650573730,\n    4.299249649,    -5.553125381,   28.580125809,\n    66.064750671,   103.236396790,  -20.000000000 )
```

Minimized the energy of the structure using amber03 and yasara forcefields to prevent clashing. The minimized structures (Figure 2) align equally well. The most movement is in the residue W202. Most notably, the tryptophane residue adopts another conformation, when using Yasara to mutate the N202.

- used Yasara to do an energy minimization.
- the forcefields used for minimization were *amber03* and *yasara*

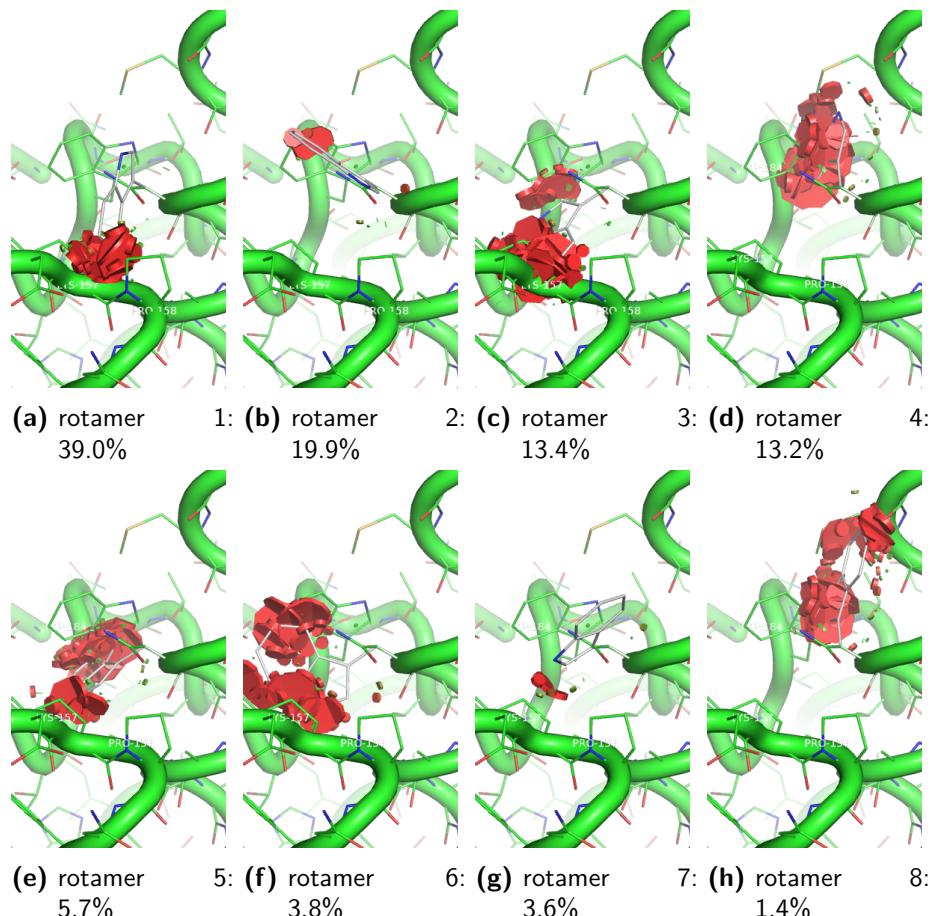
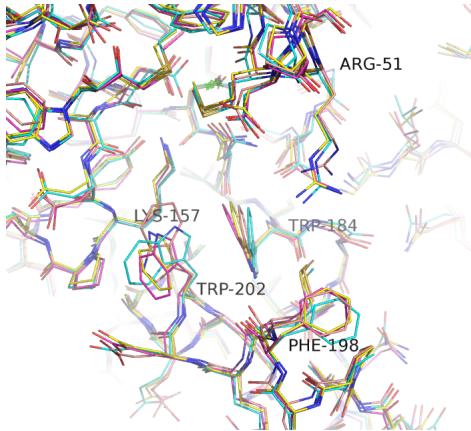


Figure 1: Mutagenesis of Asn202 to tryptophane using Pymol. Clashes with Trp184, Pro158 and Lys157 are common.



```

set_view (\n
    0.817390680, -0.485194504, 0.310517728,\n
    0.283943981, 0.808350325, 0.515652537,\n
    -0.501217663, -0.333326846, 0.798540056,\n
    0.000000000, 0.000000000, -109.694801331,\n
    4.772169590, -4.331441402, 31.016996384,\n
    95.474258423, 123.915359497, -20.000000000 )

```

Figure 2: Alignment of differently energy minimized structures of the mutated Y51R N202W PFOMT. **yellow** - unminimized (mutated using PyMol), **magenta** - minimized using yasara FF, **cyan** - minimized using amber03 FF, **orangeish** - mutated using yasara, minimized using yasara FF.

All docking poses were examined by eye using pymol. Poses, that were chemically sensible, were flagged with a `chemical_sense` tag in a table for later analysis using R (`chemical_sense = TRUE`). The distances of the 3' and 4' oxygen atoms to the sulfur atom in SAH were calculated for all poses using a Pymol script using the formula for the *euclidean distance*:

$$d(p, q) = d(q, p) = \sqrt{\sum_{i=1}^n (q_i - p_i)^2} \quad (1)$$

```

# — StateGetDistances.py
#
# from pymol import cmd, stored
import math
import numpy

def state_get_dist( sel1 , sel2 , writeout ):
    """
DESCRIPTION
Brief description what this function does goes here
@sel1 — selector for the receptor
@sel2 — selector for the ligand
@writeout — filename to write the data to
"""

# to store coordinates
stored.sel1 = []
stored.sel2 = []
# for the distances
dist = []

cmd.iterate_state(0, selector.process(sel1), 'stored.sel1.append((x,y,z))')
cmd.iterate_state(0, selector.process(sel2), 'stored.sel2.append((x,y,z))')

```

```

# calculate the distances between sel1 and sel2
if (len(stored.sel1)) == 1:
    L = len(stored.sel2)
    assert( L > 0 )
    for x in range(L):
        dist.append( math.sqrt ( (stored.sel1[0][0] - stored.sel2[x][0])**2 +
(stored.sel1[0][1] - stored.sel2[x][1])**2 +
(stored.sel1[0][2] - stored.sel2[x][2])**2 ) )
    else:
        assert( len(stored.sel1) == len(stored.sel2) )
        L = len(stored.sel2)
        assert( L > 0 )
        for x in range(L):
            dist.append( math.sqrt ( (stored.sel1[x][0] - stored.sel2[x][0])**2 +
(stored.sel1[x][1] - stored.sel2[x][1])**2 +
(stored.sel1[x][2] - stored.sel2[x][2])**2 ) )

id1 = cmd.identify(sel1, mode = 1)
id2 = cmd.identify(sel2, mode = 1)

print "Distances between %s and %s:" % (id1, id2)
print dist

with open(writeout, 'w') as f:
    f.write('Distance between ID1 = ' + str(id1) + ' and ID2 = ' + str(id2) + ':\n')
    f.write('\n'.join( map(str, dist) ) )

cmd.extend( "state_get_dist", state_get_dist );

```

If a pose had a greater distance between 3'O-S than between 4'O-S and was chemically sensible, it was also flagged with a switched = TRUE.

3.4 Results of Vina Docking

The following shows the summary of all dockings. Generally, a pose was chemically sensible if the distance **O-S** was below 4.5 Å (Figure 3a). The type of oxygen atom (3' or 4') did not matter for this distinction. The unproductive poses (Fig. 4d) showed high distances **O-S**. The conformation **C** (Fig. 4c) almost exclusively showed a 4' productive conformation.

The docking itself performed well for the wildtype PFOMT, where about 40% of the poses were chemically sound (Table 5, Figure 5). Only 12.5% of these poses showed 4' specificity. However, in the variant only 16% of the poses made sense chemically, but 78% of these showed 4' specificity (Table 6).

3.5 Poses selected for PFOMT Paper

- selected for wildtype poses: rigid docking 1 using state 1 for pose B and state 2 for pose A
- selected for variant poses: rigid docking 9 using state 2 for pose A and state 6 for pose C
- made pictures for each pose with SAH from xtal structure and SAM from previous docking
- **viewX_wt_SAM_poseA.png** selected as **Fig. 4 for paper**

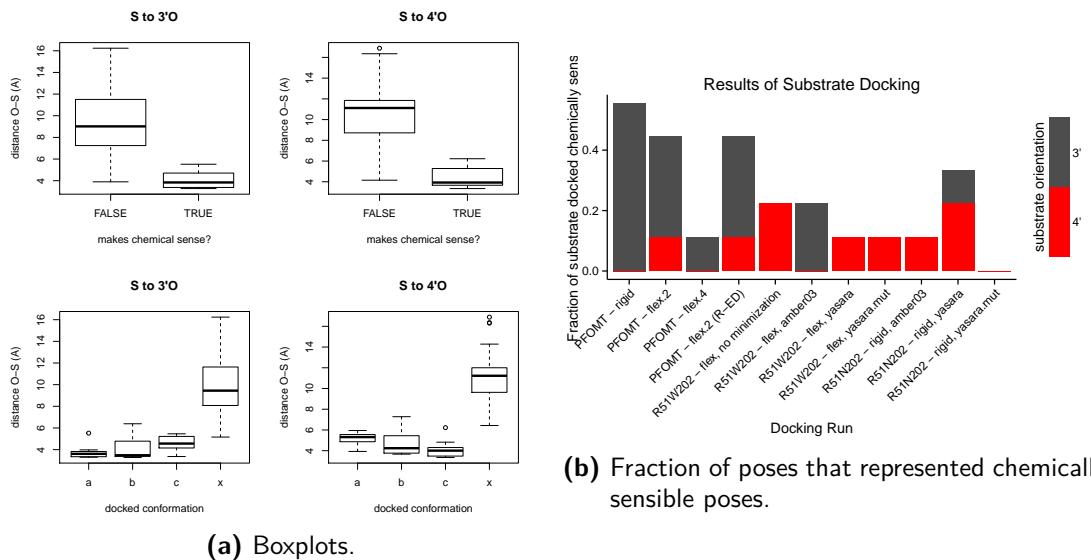


Figure 3: Graphical Summary of Dockings.

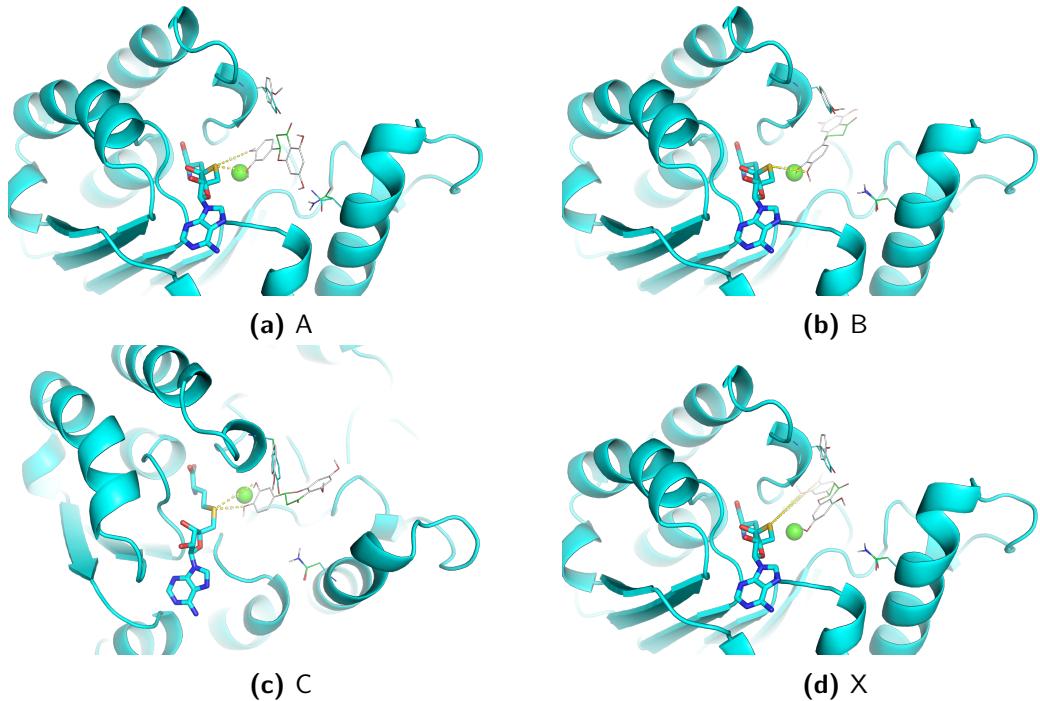


Figure 4: Different docking poses taken from flexible docking number 1. There were four main docking poses in all dockings. **a** - the pose that was also discussed in ???, **b** - another different pose, that should also lead to product, **c** - this pose usually adapted a pro-4' conformation, **d** - all other non chemically sensible poses

Table 4: Fraction of poses that were switched in specificity and that were chemically sound.

	dockrun	wt	switched	chemical_sense
1	1r	TRUE	0.00	0.56
2	1	TRUE	0.25	0.44
3	7	TRUE	0.00	0.11
4	6	TRUE	0.25	0.44
5	5	FALSE	1.00	0.22
6	2	FALSE	0.00	0.22
7	3	FALSE	1.00	0.11
8	4	FALSE	1.00	0.11
9	8	FALSE	1.00	0.11
10	9	FALSE	0.67	0.33
11	10	FALSE		0.00

Table 5: *summary* table for wildtype PFOMT (ref. Table 4).

```
##      dockrun      wt          switched      chemical_sense
## 1r      :1 Mode:logical Min.   :0.000 Min.   :0.1111
## 1       :1 TRUE:4        1st Qu.:0.000 1st Qu.:0.3611
## 7       :1 NA's:0        Median :0.125 Median :0.4444
## 6       :1             Mean   :0.125 Mean   :0.3889
## 5       :0             3rd Qu.:0.250 3rd Qu.:0.4722
## 2       :0             Max.   :0.250 Max.   :0.5556
## (Other):0
```

Table 6: *summary* table for variants (ref. Table 4).

```
##      dockrun      wt          switched      chemical_sense
## 5       :1 Mode :logical Min.   :0.0000 Min.   :0.0000
## 2       :1 FALSE:7        1st Qu.:0.7500 1st Qu.:0.1111
## 3       :1 NA's :0        Median :1.0000 Median :0.1111
## 4       :1             Mean   :0.7778 Mean   :0.1587
## 8       :1             3rd Qu.:1.0000 3rd Qu.:0.2222
## 9       :1             Max.   :1.0000 Max.   :0.3333
## (Other):1             NA's   :1
```

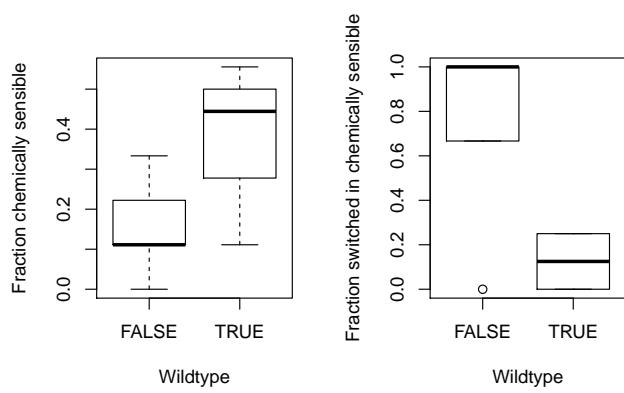


Figure 5: Graphical summary of the fraction of switched specificity (3' to 4') and the fraction that was chemically sensible.

WEB321 - COMT expression test

Benjamin Weigel

11th November, 2014

1 Day 1

- 3 mL over night culture (+ 200 $\frac{\mu g}{ml}$ Amp) inoculated with toothpick of COMT (3) from Th. Vogt

2 Day 2

- three different media for expression test (**LB**, **TB** and **ZYP-5052**)
- 20 mL cultures (+ 200 $\frac{\mu g}{ml}$ Amp) inoculated to $OD^{600}=0.075$ from overnight culture ($OD^{600}=5.59$)
- started incubation at 10.00

LB:

incubated at 37°C & 220 rpm until $OD^{600}=0.6$ - 1
added 1 mM after 2 hours ($OD^{600}=0.8$) & took **pre-induction sample**
set on ice briefly
incubate at 30°C & 220 rpm for 5 hours (17.00)
at harvesting $OD^{600}=4.9$ → **post-induction sample** & fractionated into soluble and insoluble

TB:

incubated at 37°C & 220 rpm until $OD^{600}=0.6$ - 1
added 1 mM after 2 hours ($OD^{600}=0.9$) & took **pre-induction sample**
set on ice briefly
incubate at 30°C & 220 rpm for 5 hours (17.00)
at harvesting $OD^{600}=8.0$ → **post-induction sample** & fractionated into soluble and insoluble

ZYP-5052:

incubated at 30°C and 220 rpm for 7 hours (17.00)
at harvesting $OD^{600}=5.7$ → **post-induction sample** & fractionated into soluble and insoluble

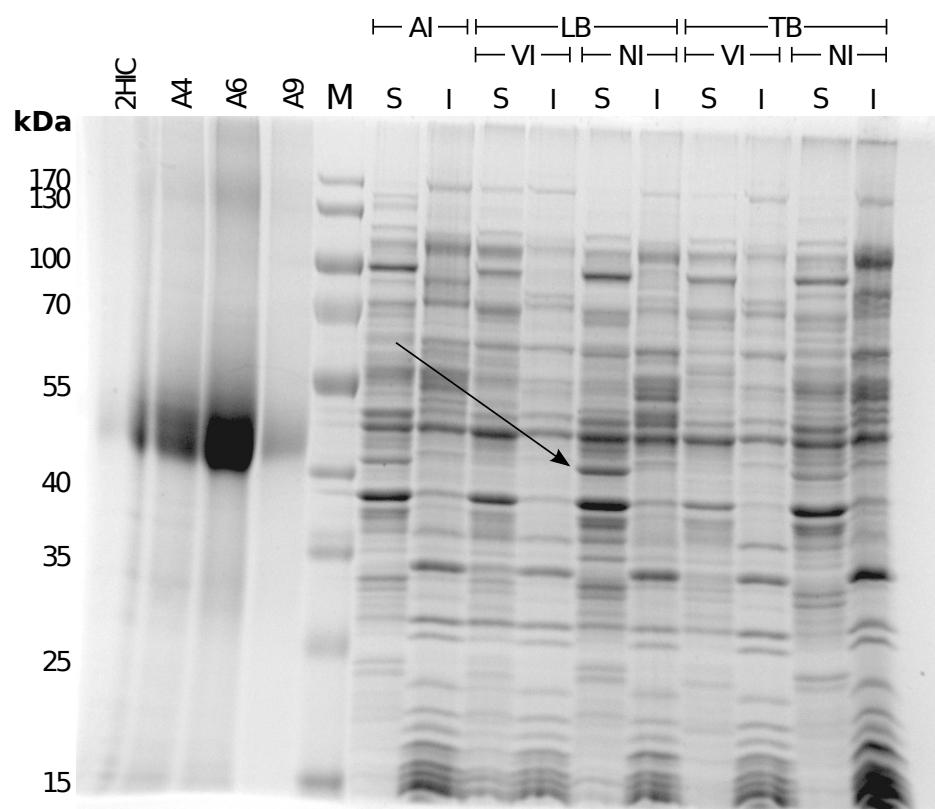


Figure 1: SDS-PAGE of Expressiontest. M - Marker, S - soluble fraction, I - insoluble fraction, AI - autoinduction medium, LB - LB-medium, TB - TB -medium, VI - pre-induction, NI - post-induction. The arrow indicates the suspected COMT protein.

WEB322 - COMT expression

Benjamin Weigel

17th November, 2014

1 Day 1

- 500 mL over night culture (+ 200 $\frac{\mu\text{g}}{\text{ml}}$ Amp) of **ZYP-5052** (autoinduction medium) inoculated with toothpick of COMT (3) from Th. Vogt

2 Day 2

- measured $\text{OD}^{600} = 10.56 \rightarrow 500 \mu\text{l SDS-PAGE sample}$
- harvested cells by centrifugation (10.000 x g, 4°C , 5 min)
- inoculated 250 mL culture of **LB** (+ 200 $\frac{\mu\text{g}}{\text{ml}}$ Amp) with 1.8 mL of overnight AI culture
- started incubation at 10.50

time	sample	OD ⁶⁰⁰	comments
10.50	AI	10.56	
13.00	LB culture	0.852	
18.00	LB culture	3.6	SDS-PAGE sample NI

3 Purification from AI-medium expression

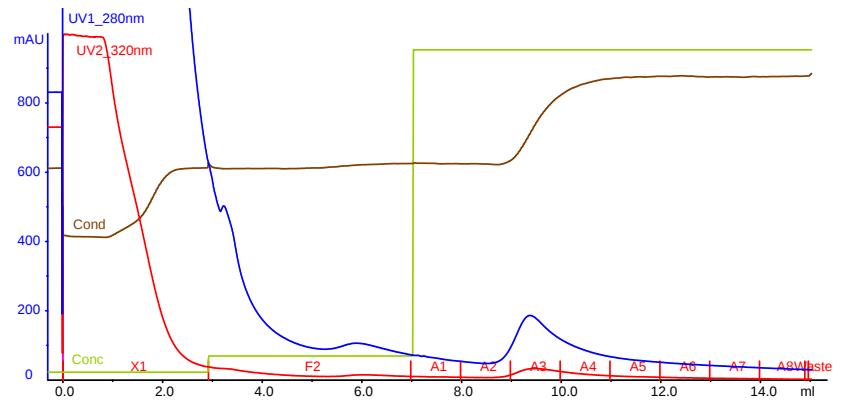
- resuspended pellet (~3 g) in 60 mL **Lysis Buffer** (50 mM Tris/HCl, 0.5 M NaCl, 10% (v/v) glycerol, 10 mM Imidazole, 1% Tween 20)
- added a spatula tip of HEWL and incubated for 15 min at RT while inverting
- lysed cells by sonication (3 times for 30 s at 70% amplitude, 1s-1s on-off-cylce)
- added DNase buffer and 200 μl DNase I and incubated on ice for 15 min
- removed debris by centrifugation (20 min, 10.000 x g, 4°C)

3.1 ÄKTA

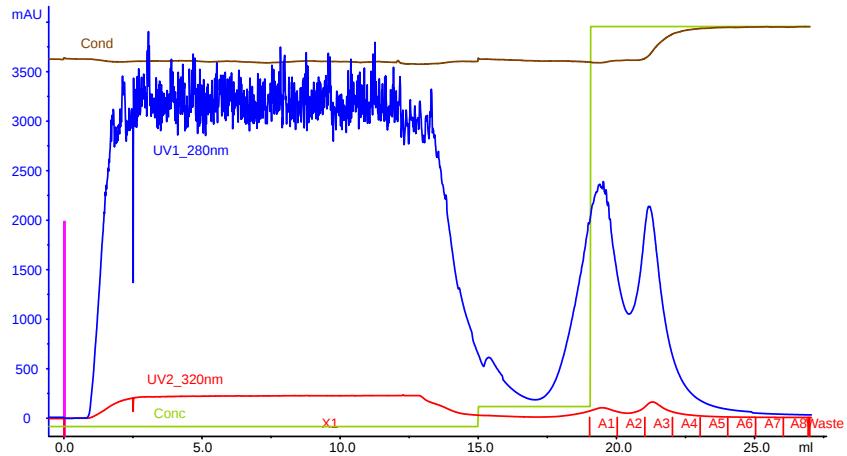
- filtered through a 0.45 μm filter and injected onto 1 mL HiTrap Talon FF column on ÄKTA
 - ! buffers, lysate and also the collection tubes were cooled
 - column was equilibrated with **buffer A** (50 mM Tris/HCl, 500 mM NaCl, 10% (v/v) glycerol, 2.5 mM imidazole, pH 7.4)
 - after sample injection the column was washed with 4 CV of buffer A and 4 CV of 5% buffer B
 - eluted with 8 CV **buffer B** (50 mM Tris/HCl, 500 mM NaCl, 10% (v/v) glycerol, 300 mM imidazole, pH 7.4)
- !!! only a very small protein peak elutes at 100% B (Fig. 1a) → SDS-Gel to clarify what went wrong in purification (Fig. 2a)
- precipitated 100 μl of fractions X1 and A3 with TCA and used for SDS-PAGE

4 Purification from LB-medium expression

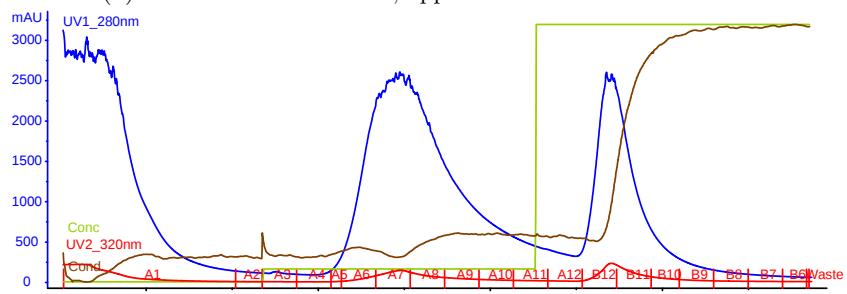
- harvested cells by centrifugation (10.000 x g, 4°C , 5 min) and stored at -20°C



(a) ÄKTA-Run 1. Co-NTA, application @ 10 mM imidazole



(b) ÄKTA-Run 2. Ni-NTA, application @ 2.5 mM imidazole



(c) ÄKTA-Run 3. Ni-NTA, application @ 2.5 mM imidazole, prolonged steps

Figure 1: ÄKTA Runs. **1a:** Raw lysate was injected at 2 mL/min on a HiTrap Talon FF 1mL (Co-NTA). **1b:** The flow-through of 1a was diluted with 3 volumes of buffer A.2 and 30 mL were injected at 0.5 mL/min on a HisTrap FF 1mL (Ni-NTA). **1c:** The same protocol as used before (Fig. 1b), however the wash and elution steps were prolonged.

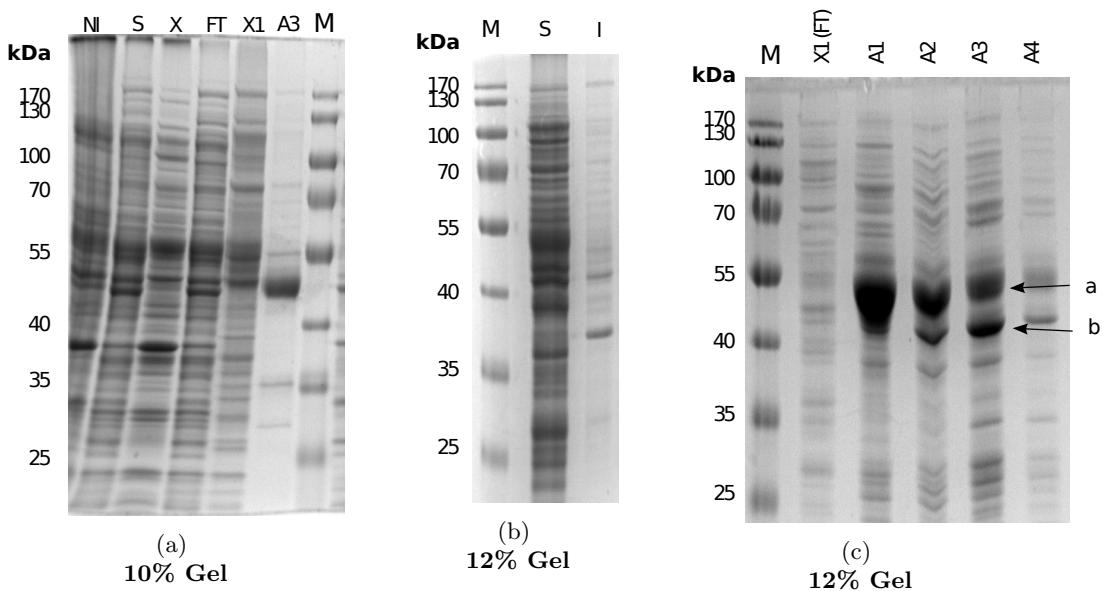


Figure 2: COMT purification.

2a: COMT purification using 1 mL HiTrap Talon FF (left side). NI - after induction (total), S - after induction (soluble), X - not sure (discard), FT - ÄKTA flowthrough, X1 - fraction X1, A3 - elution fraction A3.

2b: Flowthrough, that was collected during COMT purification using 1 mL HiTrap Talon FF. The flowthrough stood at room temp for ~3 hours. The turbid solution was centrifuged to separate soluble and aggregated protein. S - soluble, I - insoluble.

2c: Flowthrough, that was collected during first COMT purification trial (2a) was diluted with 3 volumes of **buffer A.2** (50 mM Tris/HCl, 500 mM NaCl, 10% (v/v) glycerol, pH 7.4). 30 mL of the diluted flowthrough (2.5 mM imidazol concentration) were applied to a HisTrap (Ni-NTA) 1 mL FF column at 0.5 mL/min flow. X1 - flowthrough, A1:4 - fractions A1 to A4.

WEB323 - SOMT2 expression from pET20b in *E.coli* C43(DE3)

Benjamin Weigel

Nov. 17th, 2014

1 Expression

- 3 mL pre-culture of *E.coli* C43(DE3) pET20b SOMT2 from glycerol stock in LB-medium with 200 $\frac{\mu g}{mL}$ ampicillin
- incubation at 37°C and 220 rpm over night
- next morning → measured OD⁶⁰⁰
- inoculated 100 mL culture in TB medium to an OD of ~0.06 (1.7 mL)
- incubated at 37°C /220rpm for 1.5 h
- incubated at 30°C for 1 hour
- induced with 1 mM IPTG and incubated at 30°C for 5 hours
- collected cells by centrifugation at 4°C and 10.000 x g for 10 min
- drained and weighed cell pellet

time	OD ⁶⁰⁰	comment	SDS-PAGE sample
1100	6.0	inoculated main culture with 1 mL	
1230	0.44	set at 30°C	
1330	0.846	induce with 1 mM IPTG	X (VI, 1 ml)
1830	5.55	~1 g cell pellet	forgot to take sample

2 Periplasmic Subfractionationing

- resuspended pellet in 100 mL (80 mL per gram wet weight) buffer A (30 mM Tris/HCl, 20% (w/v) D-Saccharose, 1 mM EDTA pH 8)
- stirred suspension slowly at room temp for 10 min
- centrifuged at 10.000 x g, 4°C , 10 min

- drained pellet
- resuspended pellet in 100 mL ice cold 5 mM MgSO₄
- stirred suspension slowly on ice for 10 min
- centrifuged at 10.000 x g, 4°C , 10 min
- collected supernatant (100 mL) and adjusted to 1 M (NH₄)₂SO₄ using 100 mL 2 M (NH₄)₂SO₄
- adjusted pH to 7 using 3.1 mL K₂HPO₄ and 1.9 mL KH₂PO₄

3 Phenyl Sepharose HIC Column (Fig. 1a)

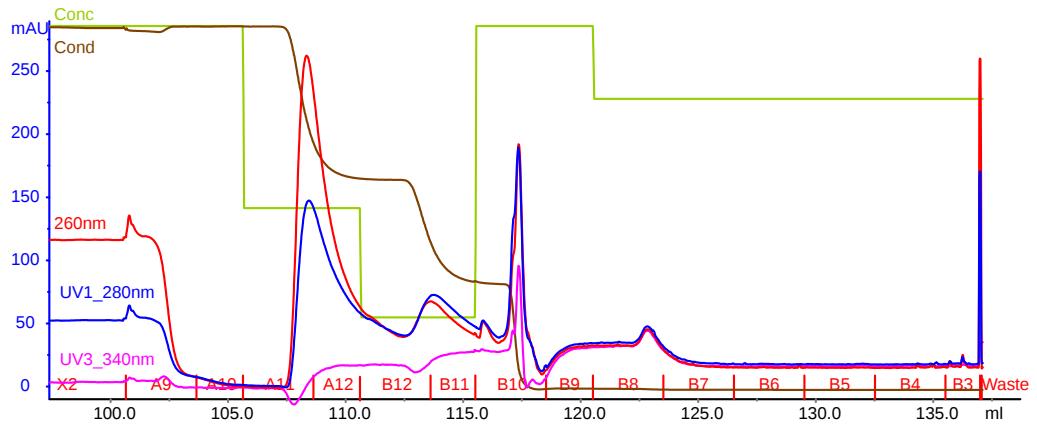
- HiTrap phenyl sepharose FF low-sub 1 mL
- washed with water and equilibrated with 50 mM Hepes/NaOH, 1 M (NH₄)₂SO₄ pH 7
- applied sample (100 mL) to column at room temp
- washed/eluted stepwise with (5 mL each):
 - 1) 1 M (NH₄)₂SO₄, 50 mM Hepes/NaOH pH 7
 - 2) 0.5 M (NH₄)₂SO₄, 50 mM KPi pH 7
 - 3) 0.2 M (NH₄)₂SO₄, 50 mM KPi pH 7
 - 4) 50 mM Hepes/NaOH pH 7
 - 5) 20 % EtOH, 50 mM Hepes/NaOH pH 7
- collected 3 mL fractions
- for SDS-PAGE 1 mL of each fraction was precipitated with TCA

3.1 Buffers:

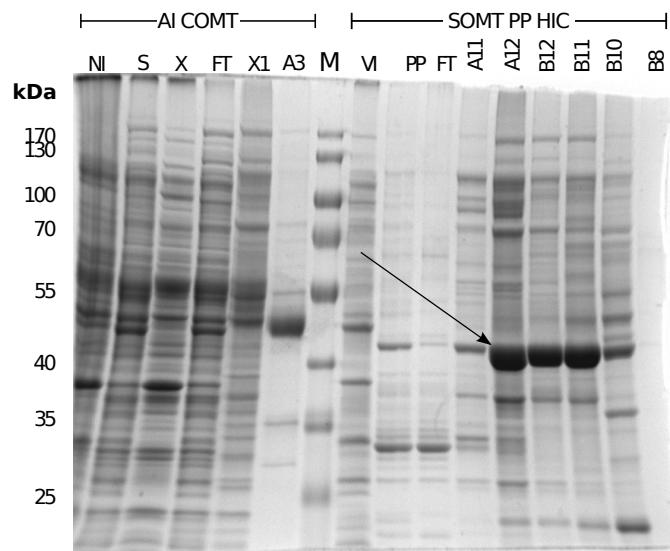
- **inlet A1** – Buffer A.1: 50 mM Hepes, pH 7.0
- **inlet A2** – Buffer A.2: 96% EtOH
- **inlet B1** – Buffer B.1: 1 M ammonium sulfate, 50 mM Hepes, pH 7.0
- **inlet B2** – Buffer B.2: 50 mM Hepes, pH 7.0

4 Activity Test

- using fraction A12, B12 and B11
- control experiment with 20 mM Hepes pH 7
- 4 x MM for each substrate group
→ **Group 1:** Naringenin, Daidzein, Quercetin



(a) HIC Run of periplasma preparation. Gradient (green line): 100% Buffer B (10 CV), 50% B (5 CV), 20% B (5 CV) – change inlets to A2 (96% EtOH) and B2 (50 mM Hepes pH 7) – 100% B (5 CV), 80% B (15 CV).



(b) SDS-PAGE of purification (only to the right of the marker). The arrow indicates the SOMT protein.

Figure 1: Hydrophobic interaction chromatography (HIC) using HiTrap Phenylsepharose 1 mL (lowsub) and SDS-Gel of collected fractions.

Reaction Mix	Mastermix Group 1 (4x)
0.1 M HEPES pH 7	40 μ l 1 M HEPES pH 7
0.2 mM substrate	8 μ l 10 mM Naringenin, Daidzein, Quercetin
0.25 mM SAM	27.2 μ l 5 mM SAM
in eluate	28.8 μ l H ₂ O
Mastermix Group 2 (4x)	
40 μ l 1 M HEPES pH 7	
8 μ l 10 mM Naringenin, Daidzein, Quercetin	
0.8 μ l 1M DTT	
27.2 μ l 5 mM SAM	
28 μ l H ₂ O	

Reaction:

30 μ l mastermix
70 μ l eluate fraction

Reaction Conditions: incubate 2 h at 30 °C

sample	Fraction	Reducing	comment
A	A12	no	decoloration
B	B12	no	decoloration
C	B11	no	decoloration
D	A12	yes	decoloration
E	B12	yes	decoloration
F	B11	yes	decoloration
G	buffer	no	visibly more yellow than reactions with enzyme
H	buffer	yes	visibly more yellow than reactions with enzyme

WEB324 - Cloning SOMT into GST-tag containing pET41a(+)

Benjamin Weigel

18th November, 2014

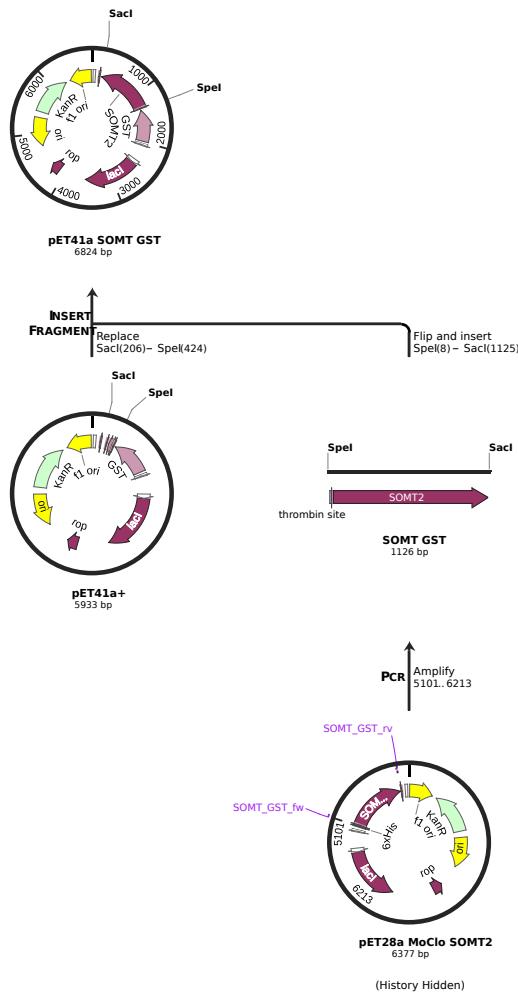


Figure 1: Cloning strategy towards GST-tagged SOMT2. pET41a(+)SOMT.

1 Cloning to pET41a(+)

ATTENTION: SOMT gene has an endogenous Bsal cleavage site → site needs to be removed

Table 1: The primers used for cloning.

name	sequence [5'→3']
somt_gst-fw	cat cat act agt AGC AGC GGC CTG GTG
somt_gst-rv	tt gaagac aa gag ctc TCA AGG ATA GAT CTC AAT

1.1 PCR SOMT2

- cloning from pET28a MoClo SOMT2 (2) [51 $\frac{ng}{\mu l}$]

Reaction mix:

substance	volume	final concentration
10x KOD Buffer	5 μl	1x
25 mM MgSO ₄	3 μl	1.5 mM
2 mM dNTPs	5 μl	0.2 mM
51 ng/ μl template	0.25 μl	12.5 ng
10 pmol/ μl SOMT_GST_fw	1.5 μl	
10 pmol/ μl SOMT_GST_rv	1.5 μl	
KOD HS Polymerase	1 μl	1 μl
water	32.75 μl	

PCR Program: theoretical size = 1100 bp

Step	Description	Temperature	Time
1	Denaturation	95°C	2 min
2	Denaturation	95°C	20 sec
3	Annealing	55°C	10 sec
4	Extension	70°C	21 sec
5	Final Extension	70°C	2 min
	Pause	4°C	∞



Figure 2: Agarose gel of PCR to amplify SOMT fragment

2 Digestion of pET41 and fragment by SpeI (BcUL) and SacI

Plasmid digestion:

component	amount	volume
FD Buffer Green		1 μ l
pET41a(+)	1 μ g	2 μ l
SacI FD		0.5 μ l
BcUL FD		0.5 μ l
ad to 10 μ l MQ-H ₂ O (6 μ l)		

fragment digestion:

component	amount	volume
FD Buffer Green		1 μ l
SOMT fragment	1 μ g	5 μ l
SacI FD		0.5 μ l
BcUL FD		0.5 μ l
ad to 10 μ l MQ-H ₂ O (3 μ l)		

- digested plasmid was cleaned by agarose gel > cut band > MN gel cleanup (eluted with H₂O: 40 ng/ μ l)
- digested fragment was only cleaned by MN PCR cleanup (eluted with H₂O: 46 ng/ μ l)

2.1 Second Ligation

Ligation:

component	amount	volume
T4 ligase buffer	1x	1 μ l
cut pET41a(+)	~20 ng	0.5 μ l
cut fragment	?	0.5 μ l
T4 ligase		0.25 μ l
ad to 10 μ l MQ-H ₂ O (7.5 μ l)		

- ligated at 22°C for 1 h and 4°C over the weekend

Colony PCR: Several small colonies → colony PCR (SOMT1, T7-term as primers) → mini-prep of clone 3 and sequencing → Sequencing results were screwed up → Ligation didn't work. → **ligate again**

Colony PCR:

component	amount	volume (* samples)
DreamTaq Buffer	1x	2 μ l
SOMT1 primer		0.4 μ l
T7-term primer		0.4 μ l
dNTP mix		0.4 μ l
DreamTaq Polymerase	0.5 U	0.1 μ l
ad to 20 μ l MQ-H ₂ O (16.7 μ l)		

For more samples the volumes need to be multiplied by the number of samples.

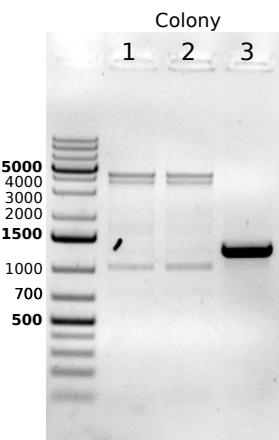


Figure 3: Agarose gel of colony-PCR. The theoretical size of the fragment, if the ligation was successful is 1216 bp. Only clone 3 gave a fragment with a comparable size. This clone was sequenced.

2.2 Second Ligation

Ligation:

component	amount	volume
T4 ligase buffer	1x	1 μ l
cut pET41a(+)	~20 ng	0.5 μ l
cut fragment	18.98 ng	0.41 μ l
T4 ligase		0.25 μ l
ad to 10 μ l MQ-H ₂ O (7.84 μ l)		

- ligated at 22°C for 1 h and 4°C over night

No colonies grew on the plate.

WEB325 - Flavonoids from *N.benthamiana*

Benjamin Weigel

9th December, 2014 - December 11, 2014

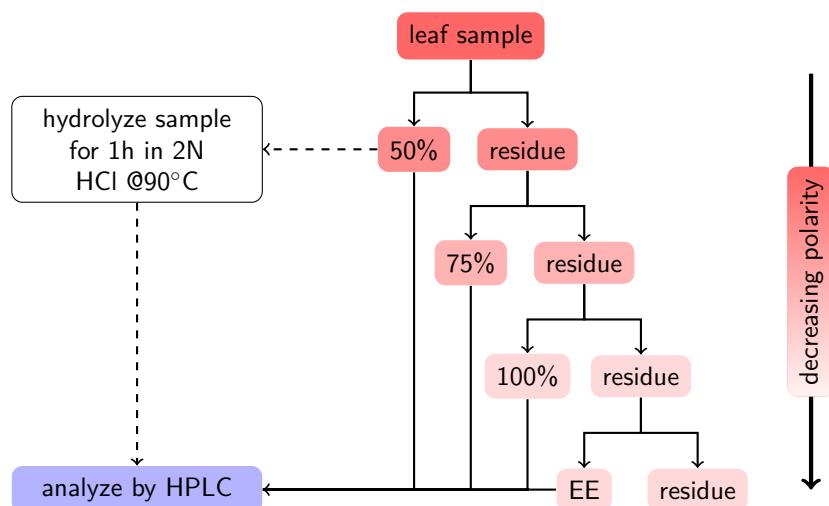


Figure 1: Stepwise extraction of *N. benthamiana* leaves by decreasing polarity. Supernatant fractions:
X% – X% methanol, EE – ethyl acetate.

1 Extraction test of *N.benthamiana* leaves

- froze, grinded and lyophilized residual leaves from WEB313
 - average weight loss of WEB313 samples was 87.5% → use 6.25 mg (12.5% of 50 mg, which is double the amount used in the master thesis of Kristin König) dried plant material for extraction with 500 μ l solvent
1. extract with 500 μ l 1 mM ascorbic acid, 0.2 % formic acid in 50 % (v/v) MeOH
 - 30 s vortex, 10 min rotary shaker, 30 s vortex
 - centrifuge at 4°C 10.000g for 10 min
 - collect supernatant
 - centrifuge at 4°C 10.000g for 10 min
 - collect 400 μ l supernatant

2. extract with 500 μ l 1 mM ascorbic acid, 0.2 % formic acid in 75 % (v/v) MeOH
→ like **1**.
3. extract with 500 μ l 1 mM ascorbic acid, 0.2 % formic acid in MeOH
→ like **1**.
4. extract with 500 μ l 2 % formic acid in ethyl acetate
→ like **1**.
→ dry in speedvac & resuspend in 400 μ l 1 mM ascorbic acid, 0.2 % formic acid in MeOH

1.1 Hydrolysis of glycosylated compounds

To analyse the aglycone flavonoid content in the 50% methanolic sample, the glycosylated flavonoids needed to be hydrolyzed. Therefore 300 μ l of each of the 50% MeOH fractions were hydrolyzed, by adding 300 μ l of 4 N methanolic HCl and heating to 90°C for 1 hour.

The resulting solution was evaporated in a *SpeedVac* set to 60°C and the residue was resuspended in methanol containing 1 mM ascorbic acid and 0.2% formic acid. The resulting solution was centrifuged at 10.000 x g and 4°C for 10 minutes. The supernatant wa analyzed by HPLC.

Attention: Some of the tubes sprung open during heating.

WEB326 - COMT expression

Benjamin Weigel

25th November, 2014

1 Day 1

- 500 mL over night culture (+ 200 $\frac{\mu\text{g}}{\text{ml}}$ Amp) of **ZYP-5052** (autoinduction medium) inoculated with toothpick of COMT (culture that stood at 4°C for 2 weeks)

2 Day 2

- measured OD⁶⁰⁰ = ~ 2.6 at 10.00
 - measured OD⁶⁰⁰ = 6.88 → at 1300
 - harvested cells by centrifugation (10.000 x g, 4°C , 3 min)
- !! cells grew slowly → probably due to old pre-culture....

3 Purification from AI-medium expression

- resuspended pellet (~3 g) in 60 mL **Lysis Buffer** (50 mM Tris/HCl, 0.5 M NaCl, 10% (v/v) glycerol, 10 mM Imidazole, 1% Tween 20)
- added a spatula tip of HEWL and incubated for 15 min at RT while inverting
- lysed cells by sonication (3 times for 30 s at 70% amplitude, 1s-1s on-off-cylce)
- added DNase buffer and 200 μl DNase I and incubated on ice for 15 min
- removed debris by centrifugation (20 min, 10.000 x g, 4°C)

3.1 ÄKTA

- filtered through a 0.45 μm filter and injected onto 1 mL HiTrap Talon FF column on ÄKTA
- ! buffers, lysate and also the collection tubes were cooled
- column was equilibrated with **buffer A** (50 mM Tris/HCl, 500 mM NaCl, 10% (v/v) glycerol, pH 7.4)
- after sample injection the column was washed with 8 CV of buffer A, 8 CV of 5% buffer B and 8 CV of 10% buffer B

- eluted with 8 CV **buffer B** (50 mM Tris/HCl, 500 mM NaCl, 10% (v/v) glycerol, 300 mM imidazole, pH 7.4)

!!! only a very small protein peak elutes at 100% B (Fig. ??) → SDS-Gel to clarify what went wrong in purification

WEB327 - SOMT Xtallization

Benjamin Weigel

9th December, 2014

Table 1: 50 μl total volume

component	concentration	volume	stock
SAE	0.25 mM SAE	0.5 μl	5 mM SAE
MgCl ₂	0.25 mM	0.1 μl	25 mM MgCl ₂
eriodictyol	0.25 mM	0.1 μl	25 mM ED
PFOMT	0.262 mM (7.53 mg/mL)	3.2 μl	24 mg/mL PFOMT
ad to 10 μl 10 mM Hepes pH 7.5		6.1 μl	

WEB328 - PFOMT Xtallization

Benjamin Weigel

12th December 2014

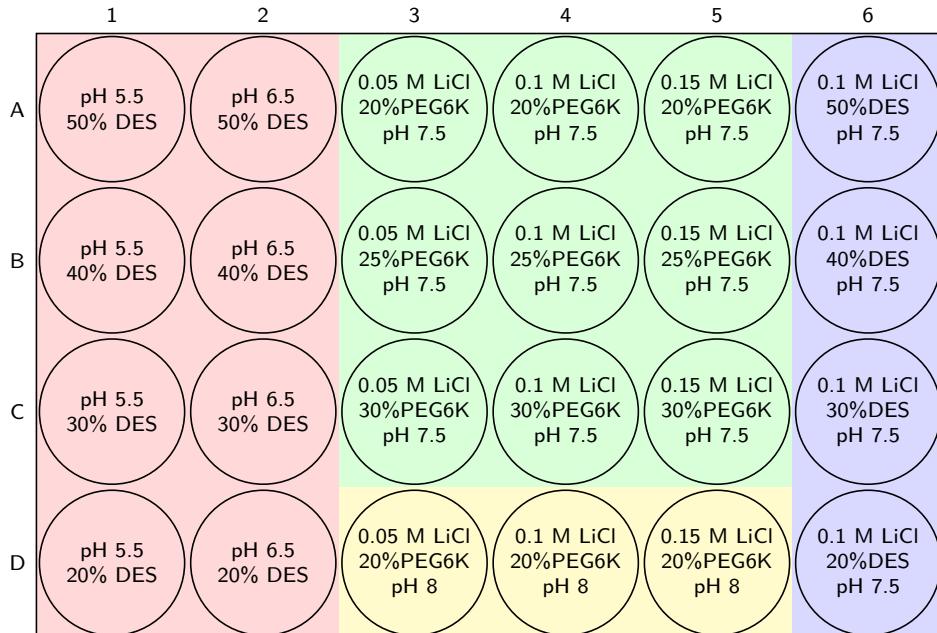


Figure 1: The plate layout for crystallization of proteinase K, lipase B and PFOMT.

component	concentration	volume	stock
SAE	0.25 mM SAE	1 μ l	5 mM SAE
MgCl ₂	0.25 mM	0.2 μ l	25 mM MgCl ₂
eriodictyol	0.25 mM	0.2 μ l	25 mM ED
PFOMT	0.262 mM (7.53 mg/mL)	6.4 μ l	24 mg/mL PFOMT
	ad to 10 μ l water	12.2 μ l	

Table 1: freshly prepared PFOMT xtallization solution.

0.1 Buffers used:

A1:D1 → buffer is 0.1 M NaOAc
A2:D2 → buffer is 0.1 M Na-citrate
A3:D5 → buffer is 0.1 M Hepes
A6:D6 → buffer is 0.1 M Hepes

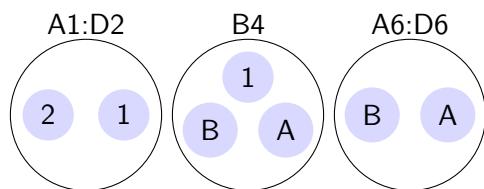


Figure 2: Layout of glass plates in special rows. View from the top. In wells A1 to D1, drop 2 contains proteinase K, drop 1 contains *C. cylindrica* lipase B. In wells A2 to D2, the order of drops is switched. Drop 1 contains Proteinase K and drop 2 contains lipase B. In well B5 drop 1 contains freshly prepared PFOMT (see WEB327) with ED and SAE. Drops B and A contain previously prepared PFOMT xtallization recipes (from WEB293). Wells A6:D6 also contain these preparations. A – 261 uM SAH, MgCl₂, ferulic acid and 7.5 mg/mL PFOMT in 10 mM Tris pH 7.5. B – 0.5 mM SAE, 1 mM MgCl₂, 10 mg/mL PFOMT.

Well	Drop	Protein
A1:D1	1 (right)	lipase B
	2 (left)	proteinase K
A2:D2	1 (right)	proteinase K
	2 (left)	lipase B

Table 2: Protein in wells.

0.2 Method

500 μ l of crystallization buffer was used inside a well. On the glass slide used for crystallization, 1 μ l of protein solution was mixed with 1 μ l of buffer solution. The small end of a cat whisker was used for seeding. The whisker was dragged along the edges of a previous crystal and then through the crystallization drops. Afterwards the glass slide was put onto the well and placed at 4°C for crystallization.

0.3 Observations

Day 0 - directly after seeding: The drop 1 in wells B1 and C1 already contained crystals shortly after seeding. The drop 2 in wells D2 and C2 also already contained crystals along the seeding line.

Day 4 after seeding: In each well of row 1 and 2 the drops 1 and 2 contain crystals respectively.

WEB330 - COMT expression

Benjamin Weigel

20th January, 2015

1 Expression in autoinduction medium

1.1 Day 1

- 500 mL over night culture (+ 100 $\frac{\mu g}{ml}$ Amp, + 50 $\frac{\mu g}{ml}$ Kan) of **ZYP-5052** (autoinduction medium) inoculated with toothpick of COMT plate (E.coli M15[pREP4]) (around 15.00)
- incubated at 220 rmp and 37°C

1.2 Day 2

time	OD ⁶⁰⁰	comment
1015	0,7	
1415	7,8	
1715	6,5	cell harvest

Table 1: OD⁶⁰⁰ of autoinduction sample **A1**

2 Expression in LB medium

2.1 Day 1

- 25 mL over night culture in LB-medium with **A**: 100 $\frac{\mu g}{ml}$ Amp and 50 $\frac{\mu g}{ml}$ Kan, or **B**: 100 $\frac{\mu g}{ml}$ Amp inoculated with toothpick of COMT plate (E.coli M15[pREP4]) (around 18.00)
- incubated at 220 rmp and 37°C

2.2 Day 2

- no growth in sample A (Amp, Kan), growth in sample B (only Amp) → *pREP4 plasmid probably missing in cells*

- **B:** OD⁶⁰⁰ ~ 4.2 → inoculated LB + 100 µg/ul Amp to OD⁶⁰⁰ = 0.1 → 11.4 mL
(11.20)

time	OD ⁶⁰⁰	comment
1120	0.1	
1215	0.191	
1315	0.494	
1415	1.55	induced with 1 mM IPTG, 37°C , 220 rpm
1715	3.5	cell harvest

Table 2: OD⁶⁰⁰ of sample **B**

WEB330 - COMT expression

Benjamin Weigel

January 28th 2015

1 Expression in LB medium

1.1 Day 1

- 25 mL over night culture in LB-medium with 100 $\frac{\mu\text{g}}{\text{ml}}$ Amp inoculated with toothpick of COMT plate (E.coli M15[pREP4]) (around 18.00)
- incubated at 220 rpm and 37°C

1.2 Day 2

- inoculated 500 mL (+ Amp) main culture with 1:10 (5 mL) of ON-culture
- measure OD⁶⁰⁰
- induce with 1 mM IPTG, when $OD^{600} \sim 0.6$
- incubated at 37°C and 220 rpm for 3 hours
- harvested via centrifugation (4°C, 10.000×g, 10 min) and stored pellets at -20°C

time	OD ⁶⁰⁰	comment
0850	0.05	
0950	0.087	
1105	0.508	
1115	0.663	induced with 1 mM IPTG, 37°C, 220 rpm
1415	2.81	cell harvest

Table 1: OD⁶⁰⁰ of sample LB culture COMT

2 ÄKTA purification

column: 1 mL HisTrap FF crude

buffer A: 50 mM KPi, 10% glycerin pH 7.4

buffer B: 50 mM KPi, 10% glycerin, 300 mM imidazole pH 7.4

program: apply sample (1 mL/min) → 0% B (5CV) → 10% B (10CV) → 80% B (10CV)
→ 100% B (5CV)

- pooled fractions A12, B12, B11 and B10 of Run 1 (WEB330) and Run 2 (WEB331), as well as D12 to D10 of Run 3
- used centrifugal filter with 10 kDa MWCO for rebuffering into 25 mM HEPES, 150 mM NaCl, 5% Glycerin pH 7
- concentrated to about $1.13 \frac{\text{mg}}{\text{mL}}$ ($OD^{280} = 1$ is 1 mg/mL)

3 Activity test

- COMT activity test of lysate and fractions (see tab. 2)

Assay	10x Mastermix (800 µl)
0.25 mM SAM	68 µl 5 mM SAM (73.5%) SAM
0.2 mM caffeic acid	20 µl 10 mM caffeic acid
20 µl protein in 0.1 M HEPES pH 7	100 µl 1 M Hepes pH 7
100 µl total volume (80 µl MM + 20 µl protein)	612 µl ddH ₂ O
	(b) Mastermix

(a) Assay conditions for COMT activity assay

Table 2: Activity assay for COMT without MgCl₂

4 Expression in autoinduction (from 150121)

- lysed cells with 50 mM KPi, 10% glycerol, 0.2% Tween-20 pH 7.4

No.	sample	comment
A	lysate	unfiltered
B	lysate	filtered
C	flowthrough	
D	Fr. A4	wash
E	Fr. A12	eluate
F	Fr. B12	eluate
G	Fr. B11	eluate
H	Fr. B10	eluate
I	Fr. B12	eluate (WEB330)
J	Fr. B10	eluate (WEB330)

Table 3: Sample list for activity test.

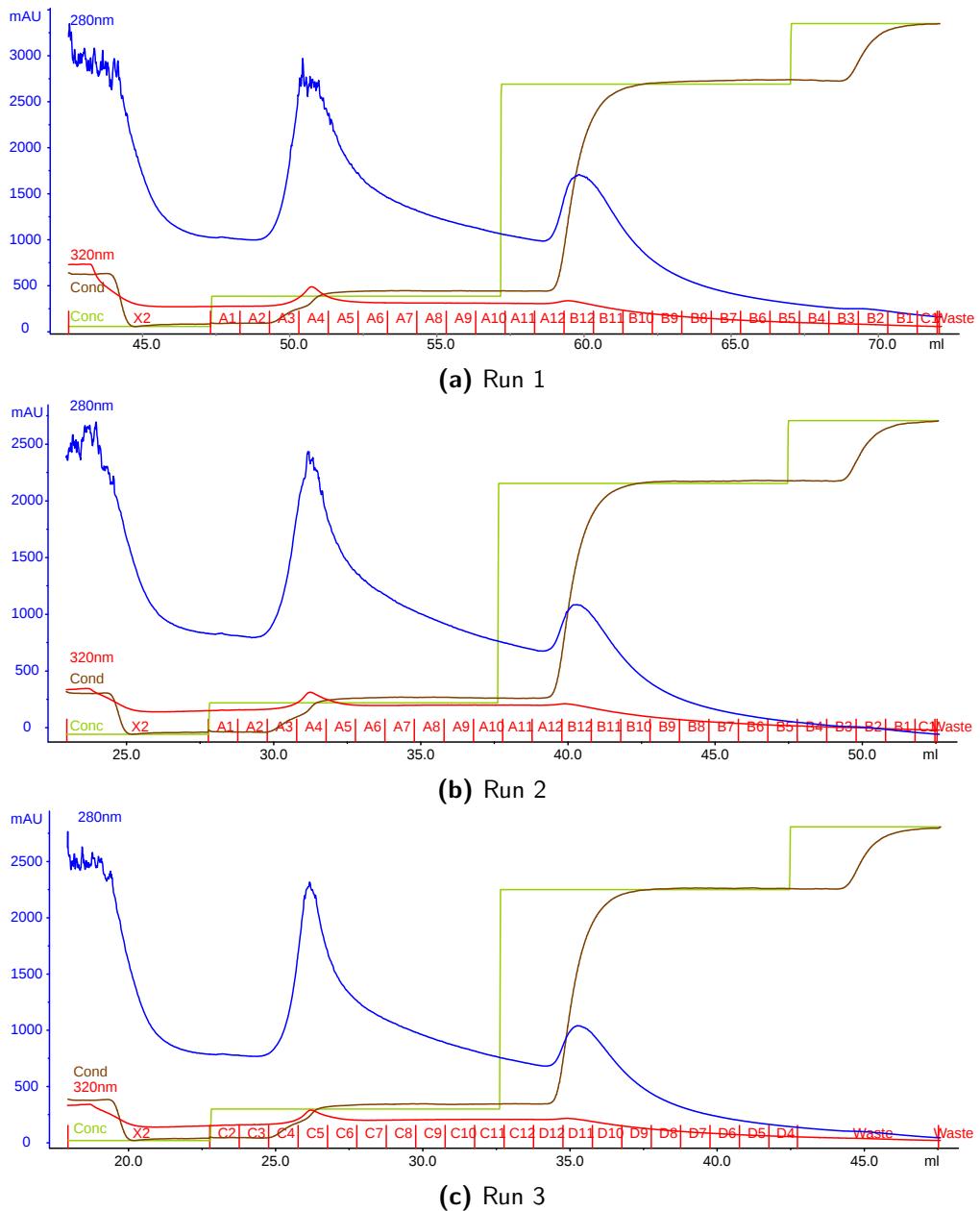


Figure 1: ÅKTA chromatograms.

Column: 1 mL HisTrap FF crude

Buffer A: 50 mM KPi, 10% glycerin, pH 7.4

Buffer B: as A, supplemented with 300 mM imidazole

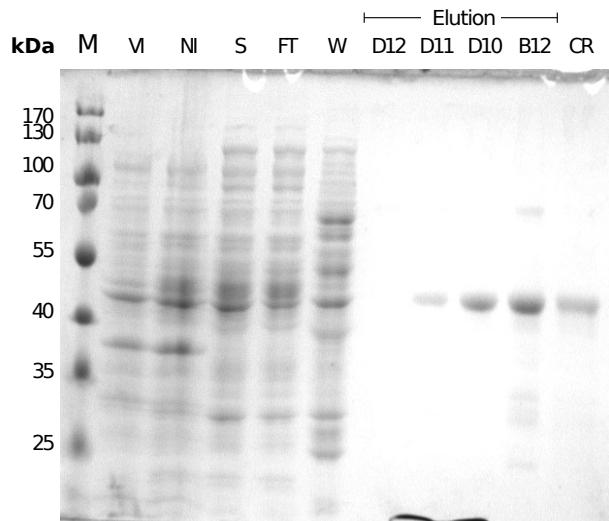


Figure 2: SDS-PAGE of COMT purification.

M – marker, VI – pre-induction, NI – post-induction, S – cleared lysate, FT – flow through (fraction X2), W – wash (fraction A4), D12:D10 – elution fractions D12 to D10 (see fig. 1c), B12 – fraction B12 (fig. 1a), CR – concentrated and rebuffered COMT ($\sim 5 \mu\text{g}$)

- AKTA (like above)
- concentrated and rebuffered into HEPES $\rightarrow \sim 3.5 \text{ mL}$ with an $\text{OD}^{280} = 0.75$

5 Hydrophobic Interaction Chromatography (HIC), see Fig. ??

To concentrate the COMT protein, the pooled and rebuffered eluates were supplemented with 1 M ammoniumsulfate (1 volume 2 M AS) and applied to a 1 mL HiTrap phenyl sepharose FF (low-sub.) column. The program was 1 M AS to 0.5 M AS, to 0.2 M AS to 0 M AS. The Buffer used was 50 mM Hepes pH 7.

Following fractions were pooled: A2–A6, A9–A11, B11–B9, B6–B3. 200 μl of each fraction were precipitated using 1/10 V 100% TCA for SDS-PAGE analysis. The pellets were redissolved in 20 μl PBS and 4 μl SDS sample buffer.

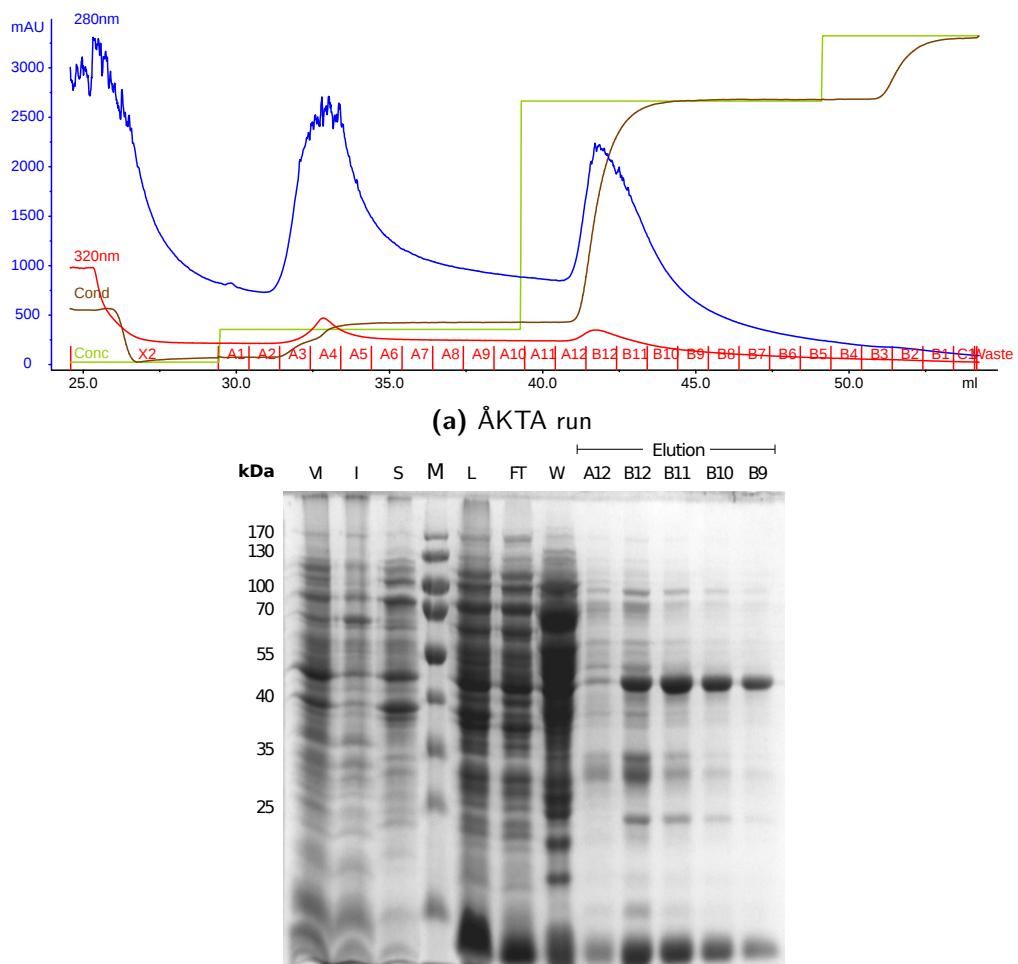
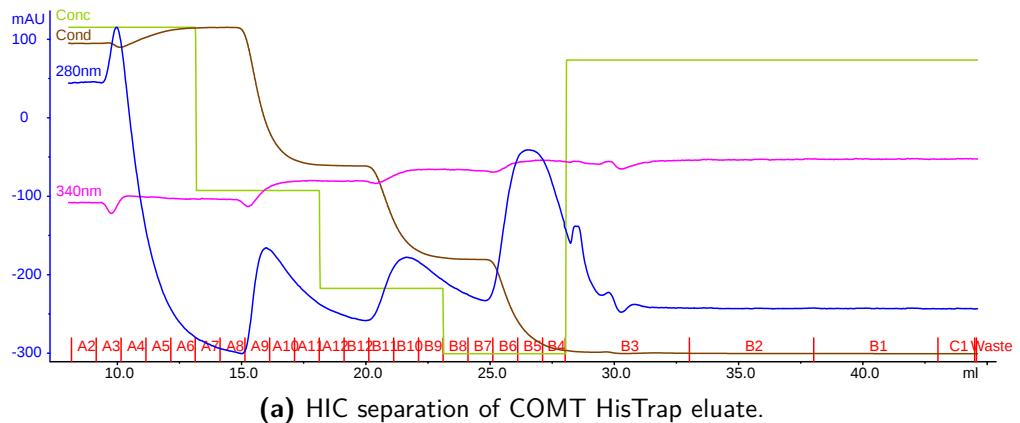
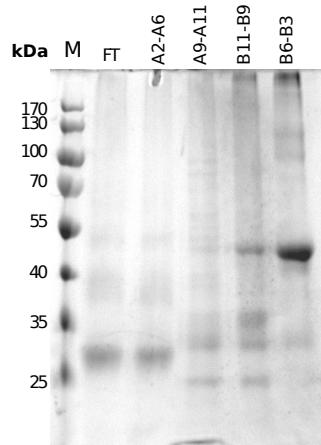


Figure 3: Purification of COMT from expression in autoinduction medium. VI – pre-induction, I – insoluble WEB332, S – soluble WEB332, M – marker, L – lysate, FT – flow-trough, W – wash, A12 to B9 – fractions of ÅKTA eluate (see 3a).



(a) HIC separation of COMT HisTrap eluate.



(b) SDS-PAGE

Figure 4: Polishing of COMT using phenyl sepharose HIC

WEB332 - Expression of GST-tagged SOMT

Benjamin Weigel

29th January 2015

1 Expression (Tab. 1)

- 20 mL overnight culture of LB + 100 µg/mL kanamycin inoculated with 1 colony of *E.coli* BL21(DE3) containing pET41a(+) GST-SOMT → incubate o.n. at 37 °C, 220 rpm
- use 0.1 volumes of ON-culture to inoculate 250 mL LB + kan ($OD^{600} \sim 0.05$)
- incubate at 37 °C, 220 rpm until $0.6 \leq OD^{600} \leq 0.8$
- induce with 0.1 mM IPTG and incubate at 25 °C for 4 hours
- harvest 1 mL sample and check soluble and insoluble fractions for protein via SDS-PAGE
- activity test (Tab. 2) using the soluble protein (B-PER II)

time	OD^{600}	comment
1105	0.056	
1220	0.089	
1335	0.413	
1405	0.7	induced with 0.1 mM IPTG, 25 °C, 220 rpm
1805	$2 \leq OD \leq 3$ (est.)	cell harvest; $OD^{600} = 2.4$ the next day at 1200

Table 1: OD^{600} of sample LB culture COMT

Assay	10x Mastermix (800 µl)
0.25 mM SAM	68 µl 5 mM SAM (73.5%) SAM
0.2 mM Naringenin	20 µl 10 mM Naringenin
20 µl protein	100 µl 1 M Hepes pH 7
in 0.1 M HEPES pH 7	612 µl ddH ₂ O
100 µl total volume	
(80 µl MM + 20 µl protein)	
	(b) Mastermix

(a) Assay conditions for SOMT-2 activity assay

Table 2: Activity assay for SOMT-2 without MgCl₂

2 Activity test

3 *In vivo* biotransformation

- 20 mL overnight culture of LB + 100 µg/mL kanamycin inoculated with 1 colony of *E.coli* BL21(DE3) containing pET41a(+) GST-SOMT → incubate o.n. at 37 °C, 220 rpm
- use 0.1 volumes of ON-culture to inoculate LB + kan ($OD^{600} \sim 0.05$)
- incubate at 37 °C, 220 rpm until $OD^{600} \sim 0.6$
- induce with 0.1 mM IPTG and incubate at 25 °C for 4 hours
- harvest 2 × two 20 mL cell samples (centrifuge at $4000 \times g$, 4 °C for 5 min)
- resuspend pellets in 20 mL LB + kan (**A**), or PBS (no kan!, **B**) and add 0.1 mM flavonoid (*Naringenin*, *Quercetin*) from 10 mM MeOH stock (**duplicates!**)
- incubate at 25 °C, 220 rpm for 18 h

- measure OD
- take 200 µl samples, add 1 µl 10 mM flavone (ISTD) and extract **twice** with 500 µl ethyl acetate + 1% formic acid
- evaporate to dryness and redissolve in 100 µl MeOH → gives in theory 0.2 mM flavonoid in HPLC-sample

sample	OD ⁶⁰⁰
LB - N	6.74
LB - N	6.51
LB - Q	6.34
LB - Q	6.47
PBS - N	2.45
PBS - N	2.31

Table 3: OD⁶⁰⁰ of sample after biotransformation

WEB334

In vivo biotransformation of flavonoids by SOMT-2

Benjamin Weigel

2nd February 2015

1 Plan

- *in vivo* biotransformation using *E.coli* BL21(DE3) harboring the pET41a(+) GST-SOMT construct
- record growth (OD^{600}), protein levels (SDS-PAGE), expression status (glucose assay) and flavonoid content (HPLC)
- use two different flavonoids (*Naringenin* and *Quercetin*)
- same protocol as WEB204 (Notebook No. 3, pp. 115)

2 Protocol

2.1 seed culture

- 1) ~10 mL pre-culture in LB supplemented with proper AB (100 ug/mL kanamycin)
- 2) grow over night at 30 °C and 200 rpm

2.2 main culture

- 1) pellet cells (5 min @ $5000 \times g$, 4 °C) and wash with 2×15 mL PBS
- 2) resuspend pellet ~3 mL of autoinduction medium (ZYP5052) supplemented with 100 ug/mL kanamycin
!! measure and record OD^{600}
- 3) inoculate $2 \times$ (per sample) 20 mL autoinduction medium (+ 100 ug/mL kan) to an $OD^{600} = 0.1$ (use 100 mL flasks)
- 4) add 0.1 mM of flavonoid from 10 mM stock in MeOH (Naringenin [*!from DMSO stock!*] and Quercetin) to the cultures at 3 hours after inoculation ($OD^{600} \sim 0.8$)
- 5) take a 1 mL sample every hour and divide as follows: (**on ice!**)
 - a) measure OD^{600} (~100 μ l)
 - b) 200 μ l for glucose measurement and SDS-PAGE (see 2.3 and 2.4)
 - c) 500 μ l for HPLC (see 2.5)

2.3 SDS-PAGE

1. centrifuge 200 μL aliquot for 3 minutes at $14.000 \times g$ and 4°C to collect the cells
2. transfer the supernatant to a new tube (use for glucose determination → 2.4)
3. resuspend the pellet in the appropriate volume of B-PER II, which is calculated by:

$$V(\mu\text{L}) = OD^{600} \times 50\mu\text{L} \times V_{sample}(\text{mL}) = OD^{600} \times 50\mu\text{L} \times 0.2 \quad (1)$$

4. vortex for 30 s and incubate at RT for 30 min
5. use 10 μl sample + 2 μl SDS sample buffer for SDS-PAGE analysis
6. *optional:*
 - a) centrifuge sample for 10 min at $14.000 \times g$ to pellet the insoluble fraction
 - b) use 10 μl of supernatant + 2 μl SDS sample buffer for SDS-PAGE of soluble fraction
 - c) resuspend (by pipetting and vortexing) pellet in the same volume of B-PER II as above (→ 3)
 - d) use 10 μl of sample + 2 μl SDS sample buffer for SDS-PAGE of insoluble fraction

2.4 glucose measurement

1. mix 2.5 μl of the supernatant (→ 2.3) with 22.5 μl of ddH₂O in a microtiter plate well
2. add 50 μl *glucose determination reagent* (→ 2.4.2)
3. incubate for 30 minutes at 37°C , 200 rpm (in an incubation shaker)
4. add 50 μl *stopp reagent*
5. measure absorption at OD^{540nm}

2.4.1 glucose calibration curve

1. prepare 1 $\frac{\text{mg}}{\text{mL}}$ D-glucose in 0.1% benzoic acid in ddH₂O
2. 100 $\frac{\text{ug}}{\text{mL}}$ D-glucose dilution in ddH₂O
3. prepare serial dilution (5 – 100 $\frac{\text{ug}}{\text{mL}}$) of glucose (→ Tab. 1)
4. use 25 μl of dilution and 50 μl GDR

2.4.2 Reagents

glucose determination reagent (GDR):

1. dissolve 100 U of HRP and 500 U of glucose oxidase (GOD) in 39.2 mL of 50 mM NaOAc pH 5.1 (solution A)
2. dissolve 5 mg of O-dianisidin hydrochloride in 1 mL ddH₂O (solution B)
3. add 0.8 mL of B to 39.2 mL of A

stopp reagent (SR): 6 M H₂SO₄ in ddH₂O

Table 1: Pipetting scheme for glucose determination calibration curve.

c(glucose) [$\frac{\mu\text{g}}{\text{mL}}$]	V(100 $\frac{\mu\text{g}}{\text{mL}}$ glucose) [μl]	water [μl]
100	1000	0
80	800	200
60	600	400
40	400	600
20	200	800
10	100	900
5	50	950
0	0	1000

2.5 HPLC

1. extract 500 μl of culture **twice** with 500 μl ethyl acetate + 1% formic acid
2. vortex for 30 s to extract, centrifuge for 10 min @ $10.000 \times g$, 4°C to separate phases
3. pool organic phases and evaporate in SpeedVac (45°C)
4. solve remainder in 200 μl MeOH
5. analyze via HPLC

Column:

Injection volume: 10 μl

Solvent A: H₂O + 0.2% formic acid

Solvent B: MeCN + 0.2% formic acid

Program: 5% B (hold 4 min) → 21 min ramp → 100% B (hold 5 min)

3 Results

3.1 OD⁶⁰⁰ and glucose

The OD⁶⁰⁰ was measured every hour to control growth. Furthermore the glucose concentration was measured to assess, when expression started. As the OD⁶⁰⁰ increases, the glucose concentration decreases (see Fig. 1). The glucose is used up at around 5 hours. This is also the time, when the expression of SOMT-2 should start. Maximum OD⁶⁰⁰ is reached approximately around 15 hours after inoculation.

The glucose was measured in MTPs columns 1 to 3 contained the glucose calibration standard. Columns 4 and 5 contained the Naringenin samples (duplicates). Columns 6 and 7 contained the Quercetin samples. The sampling time increased with rows (row A: 0h, B: 1h, ..., G: 6h).

3.2 SDS-PAGE

Cell pellets of each sampling were lysed with B-PER II reagent and applied to an SDS-PAGE (see Fig. 2). The putative weight of GST-tagged SOMT-2 is 67 kDa. A band just below the 70 kDa Marker becomes more prominent with time, indicating production of SOMT-2.

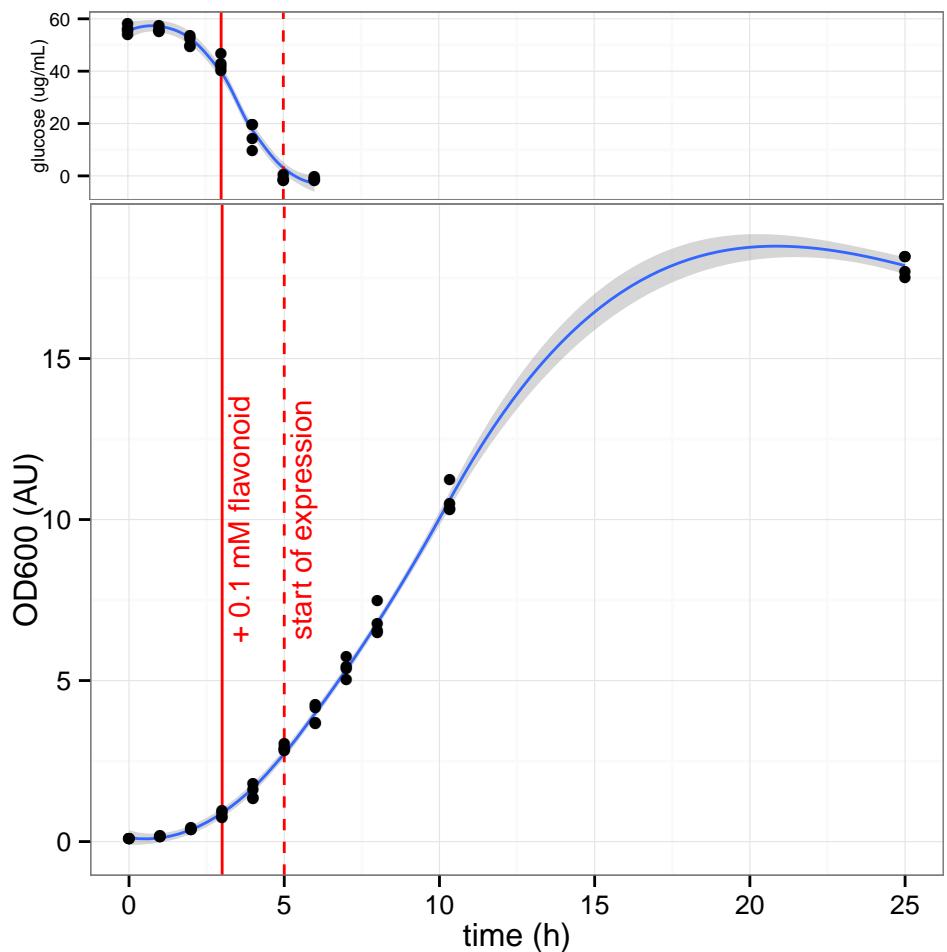


Figure 1: OD⁶⁰⁰ and glucose concentration of samples.

Table 2: OD⁶⁰⁰ of cultures.

time (h)	OD ⁶⁰⁰			
	N1	N2	Q1	Q2
0	0.087	0.088	0.087	0.099
1	0.144	0.143	0.154	0.175
2	0.383	0.384	0.408	0.449
3	0.782	0.756	0.913	0.952
4	1.342	1.362	1.621	1.801
5	2.816	2.857	2.907	3.045

Table 3: Comments on sampling.

time (h)	sample volumes (μl)			Comments
	total	HPLC	PAGE & Glucose	
0	200	-	200	
1	200	-	200	
2	200	-	200	
3	500	500	..	pelleted 500 μl, pellet for PAGE, supernatant for HPLC and Glucose
4	750	500	200	pelleted 200 μl
4	750	500	200	pelleted 200 μl

3 h: HPLC sample cleared before extraction

3.3 GCMS

Analysis of HPLC samples by GCMS. The samples were dried, derivatized with MSTFA and analyzed by GCMS.

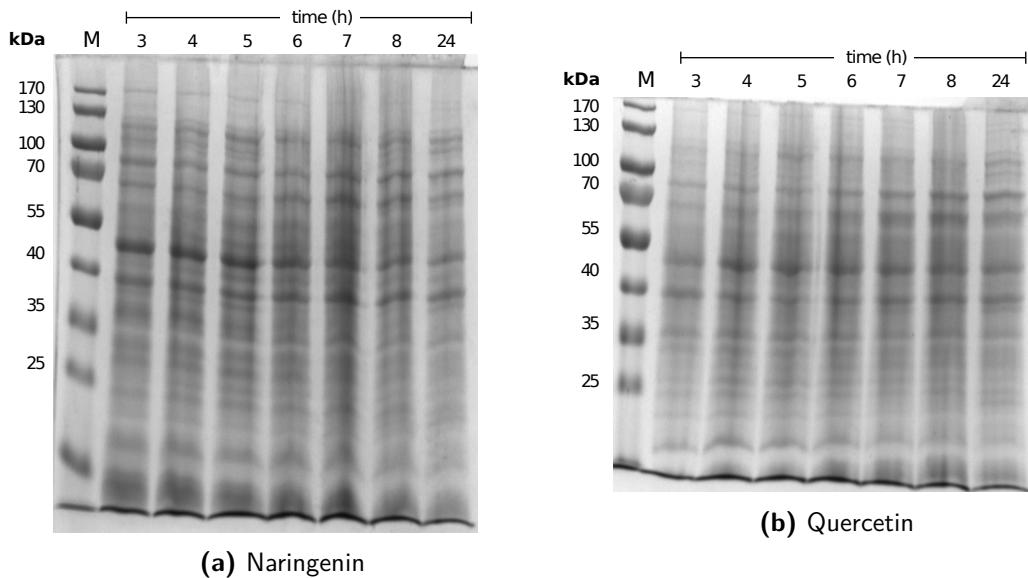


Figure 2: SDS-PAGE of in vivo biotransformation to check for SOMT expression. The size of the GST-tagged SOMT-2 should be approximately 67 kDa.

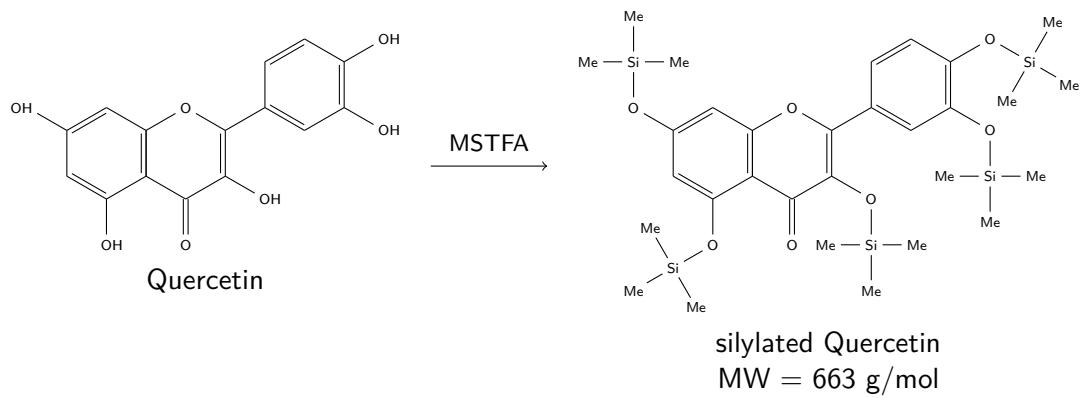


Figure 3: Derivatization of quercetin by MSTFA.

WEB336 - COMT expression

Benjamin Weigel

February 4th 2015

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1 Expression in AI medium

- 4×500 mL ZYP5052 autoinduction-medium with 100 $\frac{\mu\text{g}}{\text{ml}}$ Ampicillin (2 L shaker flasks)
- inoculated each with 20 μl of COMT culture (E.coli M15[pREP4]) from fridge (around 16.00)
- incubated at 220 rpm and 37 °C over night
- measured OD⁶⁰⁰ at 10.00
 - took 500 μl sample for SDS-PAGE (pelleted and resuspended in 250 μl P-BER II)
- harvested via centrifugation (4 °C, 10.000×g, 10 min) and stored pellets at -20 °C

sample	OD ⁶⁰⁰
1	9.887
2	10.03
3	9.857
4	9.499

Table 1: OD⁶⁰⁰ of growth cultures after over night incubation.

- the cell pellets were resuspended into Buffer A (50 mM Tris/HCl, 500 mM NaCl, 10% glycerol pH 7.5)

2 Polishing of rebuffered COMT (RB) by HisTrap column (150209)

- 6 mL of the rebuffered protein (RB) were applied to each, a 1 mL HisTrap FF crude, as well as a HiTrap Talon FF column via FPLC to *polish* it and compare binding between *Co*²⁺ and *Ni*²⁺ columns
- the elution fractions A1, A3 to A6 were precipitated using TCA and analyzed via SDS-PAGE
-

3 Activity of Fractions (see Fig. 3)

Activity test according to *OMT-catechol assay* using Buffer without MgCl₂ (0.1 M Hepes pH 7.5). The reactions, as well as the measurement were performed in microtiter plates.

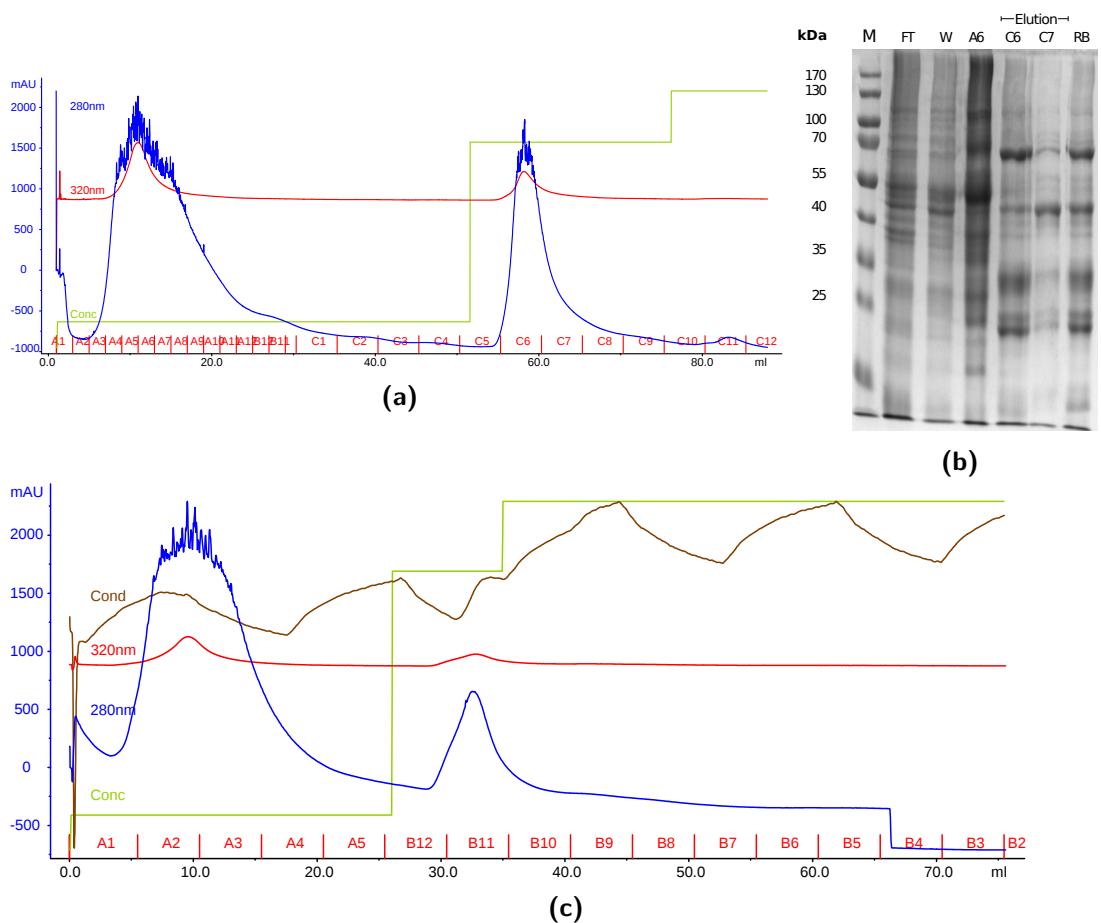
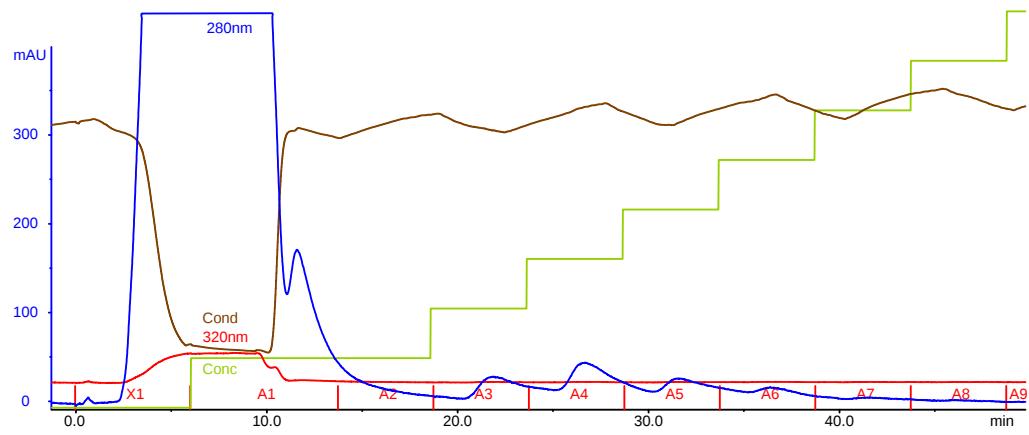
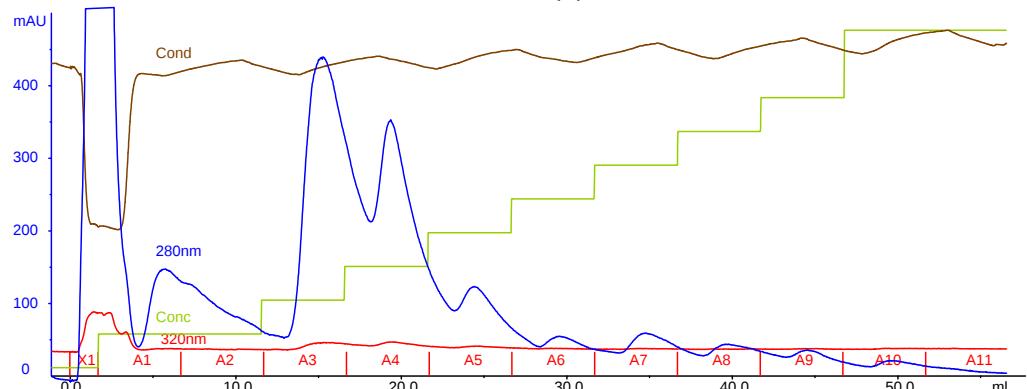


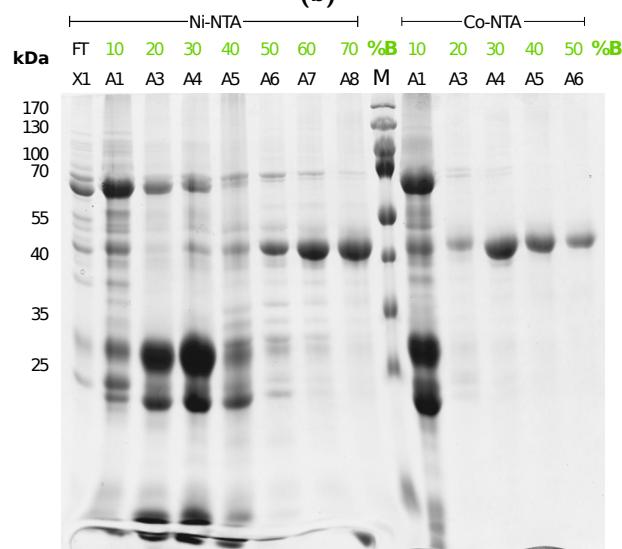
Figure 1: **1a** – Elution of loaded 5 mL HisTrap FF crude column using the ÅKTA FPLC system. The sample was loaded onto the column and also washed using a peristaltic pump. **1b** – SDS-PAGE of the purification. **1c** – the flowthrough of **1a** was loaded onto a 5 mL HisTrap column again and eluted.



(a)



(b)



(c) SDS-PAGE of fractions.

Figure 2: Polishing the COMT protein using Co-NTA (2a) and Ni-NTA (2b).

Mastermix (22x):

volume	compound	final concentration
220 μl	OMT-Buffer	1x
44 μl	10 mM caffeic acid	0.2 mM
55 μl	0.1 M GSH	2.5 μM
149.6 μl	73.5% 5 mM SAM	0.25 mM
ad to 1760 μl H ₂ O (1291 μl)		

Reaction:

1. start reaction by addition of 20 μl protein sample to 80 μl of mastermix
2. incubate at 30 °C for 30 min
3. stop reaction by addition of 50 μl *catechol reagent* (2 mM FeCl₃, 1 mM Triton X-100 in 10 mM HCl)

Table 2: Samplelist for the activity test using the catechol assay.

No	sample	comment	No	sample
1	A1		11	A6
2	A3		12	A7
3	A4	Co-NTA column	13	A8
4	A5		14	FT
5	A6		15	Wash
6	X1		16	A6
7	A1		17	C6
8	A3	Ni-NTA column	18	C7
9	A4		19	Dialyzed
10	A5		20	blank no enzyme

After measuring the absorption, A^{595} , the fraction of caffeic acid, that is still left in the sample is calculated by (1). Consequently the fraction of methylated caffeic acid (ferulic acid) is calculated by $1 - F_{CA}$. The volumetric activity A_V is the concentration change over time, divided by the volume of the sample $\frac{\delta c}{\delta t} \cdot \frac{1}{V}$, which can be calculated by (2). Here, the time $t = 30 \text{ min}$ and the volume of enzyme $V_E = 20 \mu\text{L}$. The total volume V is 100 μl .

$$F_{CA} = \frac{A^{595} - A_{FA}^{595}}{A_{CA}^{595} - A_{FA}^{595}} : 0 \leq F_{CA} \leq 1 \quad (1)$$

$$A_V = \frac{F_{FA} \cdot c_0 \cdot V}{t} \cdot \frac{1}{V_E} = \frac{(1 - F_{CA}) \cdot c_0 \cdot V}{t} \cdot \frac{1}{V_E} \quad (2)$$

4 Activity of COMT of SAE

Activity test according to 3.

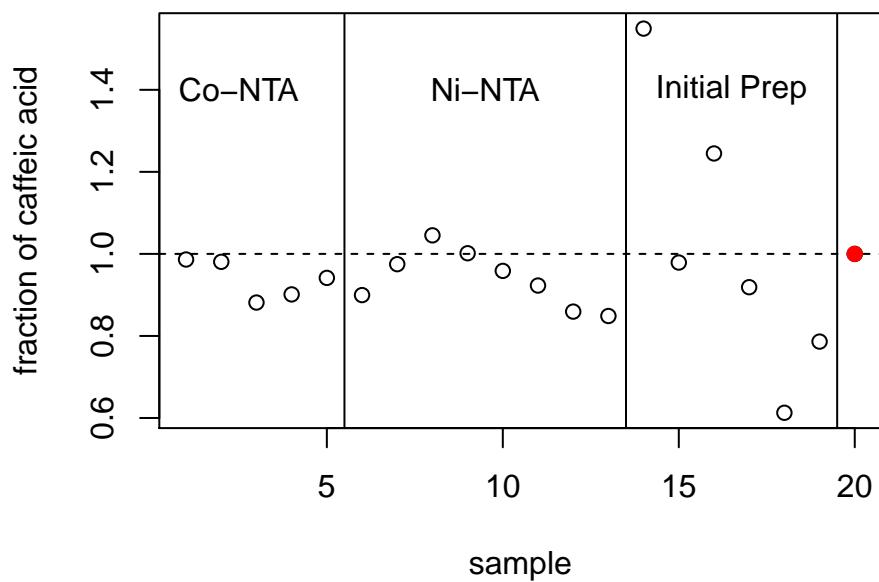


Figure 3: Residual caffeic acid (of starting concentration) of COMT samples as measured by catechol assay. The blank run (20, filled red circle) was taken to be 100% (dashed vertical line) and 0% was assumed to have an absorption of 0, see Tab. 2.

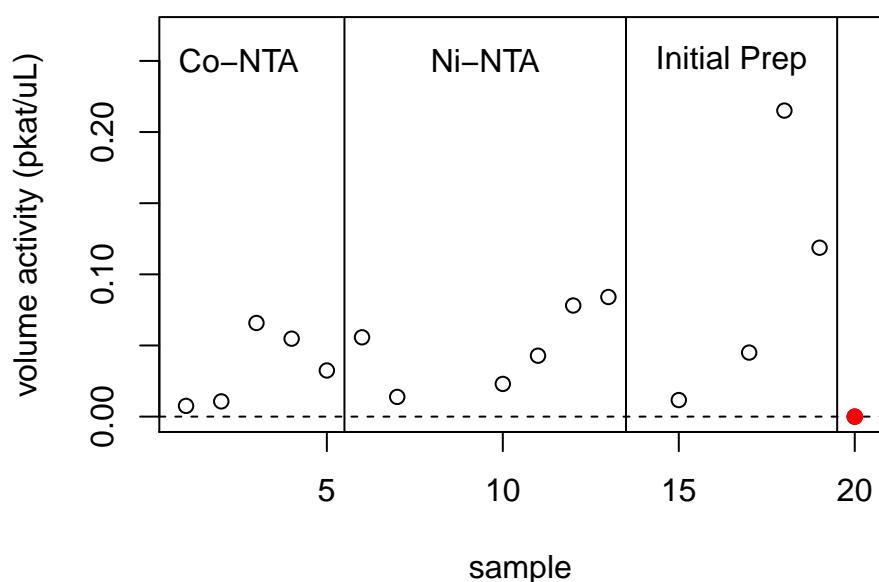


Figure 4: Volumetric activity of the COMT samples. Judging from the concentration of the sample 18 ($\sim 1 \frac{mg}{mL}$) the specific activity should be around $A_S \sim 0.2 \frac{nkat}{mg}$ ($\sim 1.25 \frac{nkat}{mg}$ for PFOMT wt).

5 Activity of COMT of SAE

Activity test according to 3.

Mastermix (10x):

volume	compound	final concentration
100 μ l	OMT-Buffer	1x
20 μ l	10 mM caffeic acid	0.2 mM
25 μ l	0.1 M GSH	2.5 μ M
181 μ l	0.55 mg/mL COMT	0.1 mg /mL
ad to 930 μ l H ₂ O (604 μ l)		

Reaction:

1. start reaction by addition of 7 μ l of SAM/SAE to 93 μ l mastermix
2. incubate at 30 °C and 100 rpm over night
3. stop reaction by addition of 50 μ l *catechol reagent* (2 mM FeCl₃, 1 mM Triton X-100 in 10 mM HCl)
4. measure OD⁵⁹⁵

WEB337 - *in vivo* biotransformation

using SOMT-2

Benjamin Weigel

February 14, 2015

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1 Introduction

Test whether different substrates available in-lab are converted by SOMT-2 *in vivo*. Use SOMT seed culture to inoculate main cultures. Add substrates after 4 hours of incubation at 30 °C. 16 substrates means 16 flasks. Take two samples from each flask at 0, 10, 20 and 30 hours. $16 \times 4 \times 2 = 128$ samples.

2 Experimental

2.1 seed culture

- 1) ~10 mL pre-culture in LB supplemented with proper AB (100 ug/mL kanamycin)
- 2) grow over night at 30 °C and 220 rpm

2.2 main culture

- 1) pellet cells (5 min @ 5000×g, 4 °C) and wash with 15 mL PBS
- 2) resuspend pellet in the 3 mL of PBS
- !! measure and record OD⁶⁰⁰
- 3) inoculate 200 mL of autoinduction medium (+ 100 ug/mL kan) to an OD⁶⁰⁰ = 0.1
- 4) aliquot 10 mL into new flasks for each sample (17 flasks) (*use 100 mL flasks*)
- 5) add 0.1 mM of flavonoid (see 4) from 10 mM stock in MeOH or DMSO to the cultures at 4 hours after inoculation (OD⁶⁰⁰ ~ 0.8)
- 6) take a 600 µl sample at 10, 20 and 30 hours after inoculation and divide as follows:
(on ice!)
 - a) measure OD⁶⁰⁰ (~100 µl)
 - b) 500 µl for HPLC (see 2.4)

2.3 OD⁶⁰⁰ measurements

- measure OD⁶⁰⁰ in MTP (all samples, 100 µl of sample) **!pathlength differs from cuvette!**
- measure OD⁶⁰⁰ of random samples in cuvette as reference

2.4 HPLC

1. extract 500 µl of culture **twice** with 500 µl ethyl acetate + 1% formic acid
2. vortex for 30 s to extract, centrifuge for 10 min @ 10.000×g, 4 °C to separate phases
3. pool organic phases and evaporate in SpeedVac (45 °C)
4. solve remainder in 200 µl MeOH
5. analyze via HPLC

Column:

Injection volume: 10 µl

Solvent A: H₂O + 0.2% formic acid

Solvent B: MeCN + 0.2% formic acid

Program: 5% B (hold 4 min) → 21 min ramp → 100% B (hold 5 min)

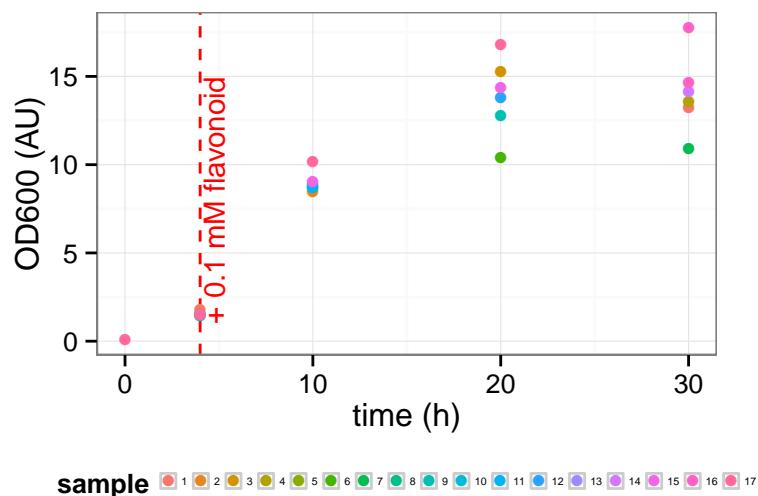


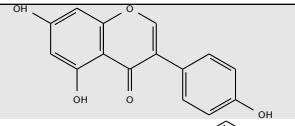
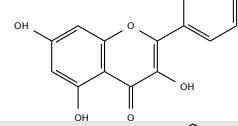
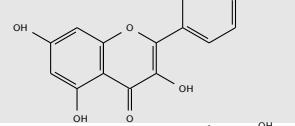
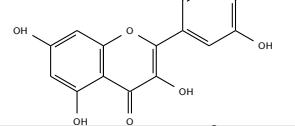
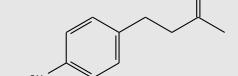
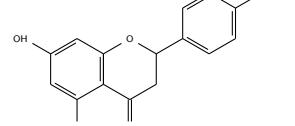
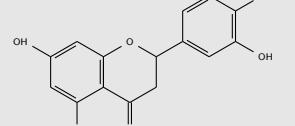
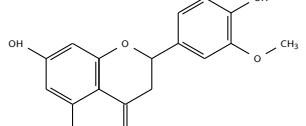
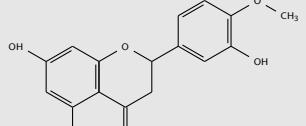
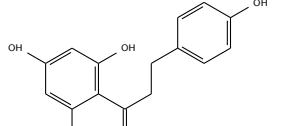
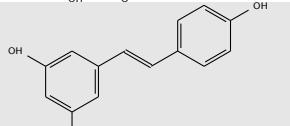
Figure 1: OD⁶⁰⁰ of samples.

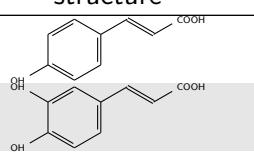
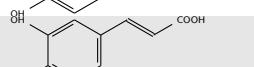
3 Results

3.1 OD⁶⁰⁰

4 Appendix

No.	substrate	moiety	structure
1	alizarin	anthrachinone	
2	purpurin	anthrachinone	
3	apigenin	flavone	
4	chrysins	flavone	

No.	substrate	moiety	structure
5	genistein	isoflavone	
6	galangin	flavonol	
7	kaempferol	flavonol	
8	quercetin	flavonol	
8	reosmin	homo-dihydro cinnamic keton	
9	naringenin	flavanon	
10	eriodictyol	flavanon	
11	homoeriodictyol	flavanon	
12	hesperetin	flavanon	
13	phloretin	chalcon	
14	resveratrol	stilbene	

No.	substrate	moiety	structure
15	<i>p</i> -coumaric acid	cinnamic acid	
16	caffeic acid	cinnamic acid	
17	none (blank)		

WEB338 - Comparison of PFOMT and COMT

or: the last rituals of the dying

Benjamin Weigel

March 9, 2015

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1 The final experiments - planning

1.1 ITC

PFOMT:

1. pH dependency of binding for (SAH, SAM, SAE)¹
2. binding of substrates (caffeic acid, ferulic acid)²
3. pfomt + ferulic acid in cell → titrate SAH → difference in binding (n)?
4. temperature dependency (entropic/enthalpic)
5. binding of metals³

COMT:

1. pH dependency of binding for (SAH, SAM, SAE)⁴
2. binding of substrates (caffeic acid, ferulic acid)

1.2 Catalysis

PFOMT:

1. pH dependency of methylation reaction (caffeic acid, catechol assay)⁵
2. influence of NADES on methylation reaction
3. combine the above (build statistic model)

COMT:

1. pH dependency of methylation reaction (caffeic acid, catechol assay)
2. influence of NADES on methylation reaction
3. combine the above (build statistic model)

2 ITC

2.1 PFOMT

2.1.1 Preparation

Multi pH Buffer-Mixture. Prepare a buffer mixture (L-malonic acid/Mes/Tris) to compare binding and catalysis reactions at different pHs. Newman described several three component buffer systems, which were optimized to give easy access to a broad pH range without the need to change buffer composition (2). A high and low pH counterpart of each system was prepared and the final pH was achieved by simply mixing both components in different ratios (e.g. 0:1, 0.1:0.9, . . . , 1:0).

¹use buffer mixture with wide pH-range (e.g. Mes/acetate/Tris, pH 4-9; see Biological Buffers by AppliChem)

²NADES might be needed to increase solubility

³if time permits → might require de- and refolding?

⁴if enough "clean" COMT can be prepared

⁵requires tuning of catechol assay (due to instability of complex at low pH) → use buffered catechol reagent (e.g. 2 mM FeCl₃, 1 mM Triton X-100 in 1 M Tris pH 7.5)

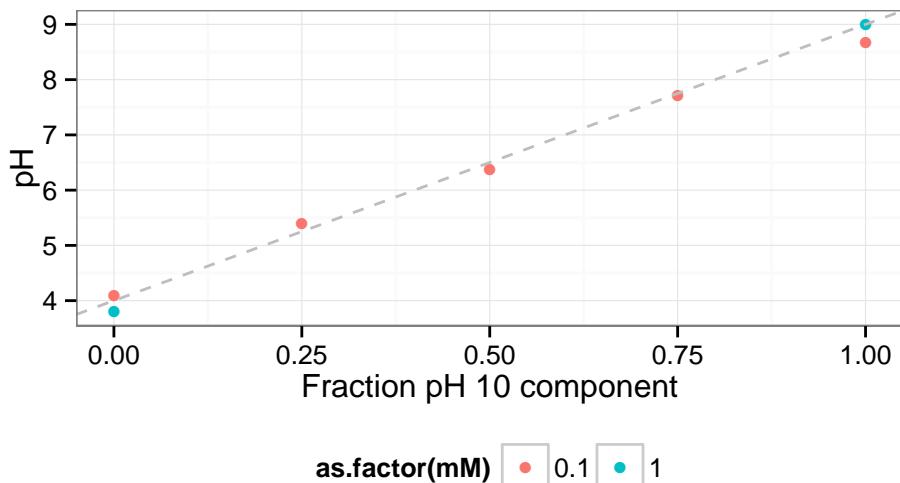


Figure 1: pH of different mixtures of MMT

MMT-Buffer stock solution:

- the MMT-Buffer recipe was prepared according to Newman
- Five different 0.1 M buffers with different fractions of the low pH component $x_A = (1, 0.75, 0.5, 0.25, 0)$ were prepared from the low and high pH 10×stocks

2.2 Assay

- full factorial design using pH and DES content:
 - (i) **5 pH values** (4.1, 5.4, 6.4, 7.7, 8.7) → (100, 75, 50, 25 and 0% MMT pH 4)
 - (ii) **4 DES contents** (0, 10, 20, 30 % (V/V))
- 20 different buffers (100 mM, each DES/pH combination, see tab. 1)
- use catechol assay for determination of activity
 - (i) **5 time points**(0, 5, 10, 15, 20 min)
 - $5 \times 4 \times 5 = 100$ samples in total

Before measuring the actual assays, the suitability of the catechol assay in each buffer was determined (fig. 4). The full factorial design of this experiment is shown in figure 3, where each point corresponds to one measurement. From the absorption (response) versus time (z-axis) the slope and consequently activity can be calculated.

2.2.1 Measurability of different fractions of ferulic and caffeic acid in MMT-Buffers

- measured for 5 different fractions of ferulic acid to caffeic acid, $\frac{[FA]}{[CA]} = f_{FA} = (0, 0.25, 0.5, 0.75, 1)$, in 20 different buffers $pH = (4, \dots, 9)$ and $DES = (0, 10, 20, 30)$

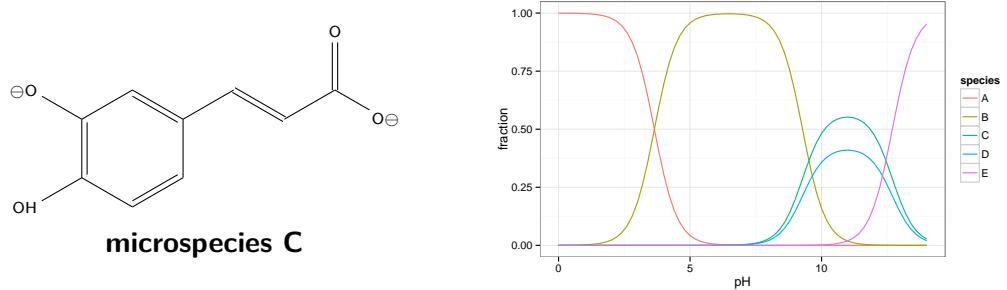


Figure 2: The deprotonated microspecies of caffeic acid, denoted as C, which can nucleophilically attack the methyl-group of SAM. The fraction of this microspecies at different pHs was calculated using ChemAxons MarvinBeans.

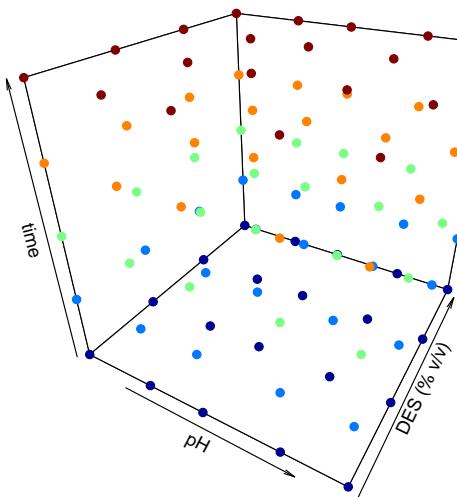


Figure 3: Full factorial design of PFOMT activity test.

Table 1: The buffers used for the activity test experiments. Each buffer was 100 mM. The fraction of the deprotonated microspecies $f_{CA^{2-}}$ (2) was calculated using ChemAxons MarvinBeans.

No.	pH	DES	$f_{CA^{2-}}$
1	4.1	0	0
2	4.1	10	0
3	4.1	20	0
4	4.1	30	0
5	5.4	0	0.0001
6	5.4	10	0.0001
7	5.4	20	0.0001
8	5.4	30	0.0001
9	6.37	0	0.0006
10	6.37	10	0.0006
11	6.37	20	0.0006
12	6.37	30	0.0006
13	7.72	0	0.0118
14	7.72	10	0.0118
15	7.72	20	0.0118
16	7.72	30	0.0118
17	8.68	0	0.12
18	8.68	10	0.12
19	8.68	20	0.12
20	8.68	30	0.12
9.2	6.37	0	0.0006
5.2	5.4	0	0.0001

→ makes $20 \times 5 = 100$ measurements in total

prepare:

1. 5 different flavonoid mixtures, each containing different ferulic acid fractions, with a total concentration of $c_{FA} + c_{CA} = 10 \text{ mM}$
2. 5 mastermixes containing *flavonoid mixes*, GSH, PFOMT and MgCl_2

22× **mastermix:** one for each flavonoid mix

volume	compound	final concentration
88 μl	10 mM flavonoid mix	0.4 mM
55 μl	0.1 M GSH	2.5 μM
22 μl	1 M MgCl_2	10 mM
440 μl	1 mg/ml PFOMT	0.2 $\mu\text{g}/\mu\text{l}$
ad to 1100 μl H_2O (495 μl)		

Assay procedure:

50 μ l of the mastermix and 50 μ l of each buffer (tab. 1) were mixed in a MTP and incubated at 30 °C for 20 minutes while shaking. Afterwards 10 μ l of 1 M Tris-HCl pH 8.5 and 50 μ l of catechol reagent were added. The complexation reaction was incubated for 5 minutes and the absorption at 595 nm was recorded.

Analysis of the results:

The data (OD^{595} , pH, DES, etc.) were plotted against each other in a scatterplot matrix, to get a feel for the data. Afterwards linear models were calculated in R and simplified to obtain a model that best described the relationship between the absorption and fraction of ferulic acid.

Results of the catechol assays. The data obtained by measuring different fractions of ferulic acid by the catechol assay was plotted in a scatterplot matrix (Fig. 4). The scatterplots already show, that the absorption OD^{595nm} is strongly dependent on the fraction of ferulic acid, f_{FA} , and possibly on the pH.

To clarify, which factor (DES, pH, f_{FA}) has an influence on OD^{595nm} general linear models were fitted to the data. 10-fold cross validation was carried out to select an appropriate model. First the most complex model $A^{595} \sim DES * pH * f_{FA}$ was calculated. This was then simplified by the step() function in R.

The following models were used for the cross validation procedure:

$$A \sim DES * pH * f_{FA} \quad (1)$$

$$A \sim f_{FA} + DES + pH + f_{FA} : pH \quad (2)$$

$$A \sim f_{FA} + DES + pH \quad (3)$$

$$A \sim f_{FA} + pH \quad (4)$$

$$A \sim f_{FA} \quad (5)$$

Plot 5 shows the performance of five differently complex models (1) in estimating the value of OD^{595nm} . Models 1 to 4 each show the same low test error, with model 2 being slightly better than the other three. However, model 5, which only looks at the f_{FA} variable for prediction performs slightly worse. Nonetheless, in the light of relative values even this model performs well with an error of less than 1%.

Overall the data shows, that this procedure can reliable measure the caffeic acid content in all the buffers and is suited for the following experiments.

The formula of model 4

$$A = \beta_0 + \beta_1 \times FA + \beta_2 \times pH \quad (6)$$

$$FA = \frac{A - \beta_0 - \beta_2 \times pH}{\beta_1} \quad (7)$$

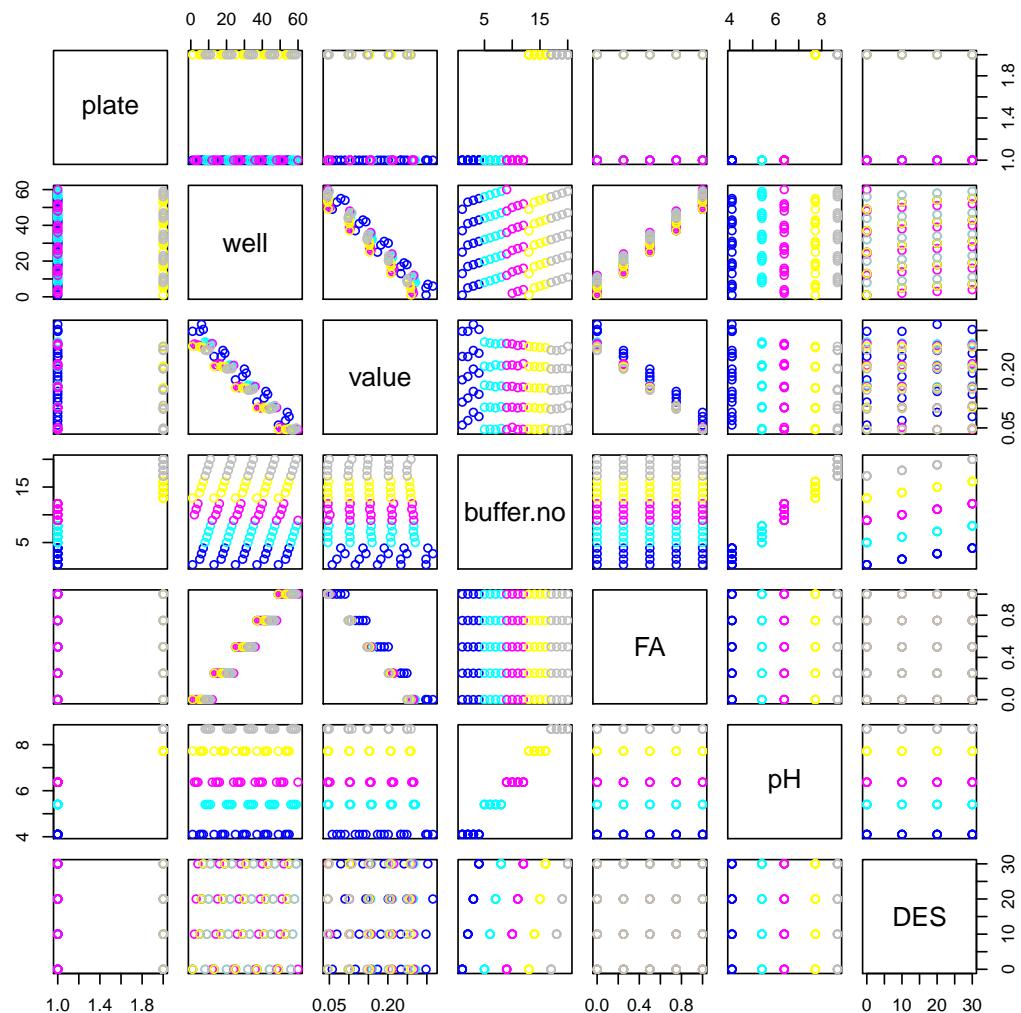


Figure 4: Scatterplot matrix of catechol assay validation.

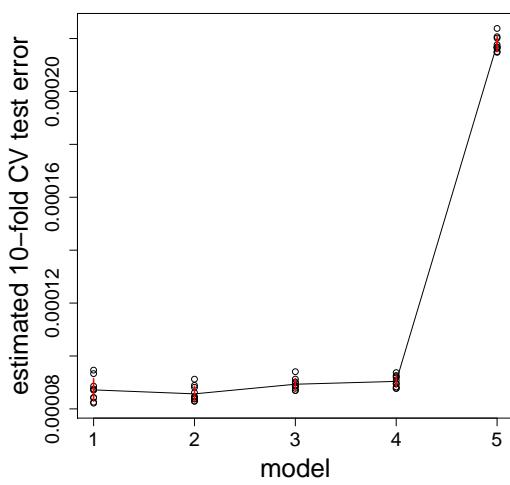


Figure 5: The prediction error of the selected models. The prediction error was estimated 10 times for each model (points), using 10-fold cross validation. The mean of the test error is shown as a line. Models 1 to 4 perform equally well showing very low test error. Model 2 seems to be the best candidate of the first four. However, model 5, which only contains f_{FA} as a predictor performs significantly worse in predicting OD^{595nm} . The simplest model within one standard error (red vertical bars) of model 2 is model 4 .

Figure 6: Model 4 summary. From the formula below the ferulic acid concentration can be calculated.

```
##  
## Call:  
## glm(formula = value ~ FA + pH, data = data)  
##  
## Deviance Residuals:  
##      Min       1Q     Median       3Q      Max  
## -0.0152416 -0.0064423  0.0000475  0.0039486  0.0309365  
##  
## Coefficients:  
##             Estimate Std. Error t value Pr(>|t|)  
## (Intercept)  0.3126000  0.0040355   77.46 <2e-16 ***  
## FA          -0.2156020  0.0026354  -81.81 <2e-16 ***  
## pH          -0.0068626  0.0005731  -11.97 <2e-16 ***  
## ---  
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1  
##  
## (Dispersion parameter for gaussian family taken to be 8.68195e-05)  
##  
## Null deviance: 0.6019234 on 99 degrees of freedom  
## Residual deviance: 0.0084215 on 97 degrees of freedom  
## AIC: -646.43  
##  
## Number of Fisher Scoring iterations: 2  
##  
## Call:  
## glm(formula = value ~ FA + DES + pH, data = data)  
##  
## Deviance Residuals:  
##      Min       1Q     Median       3Q      Max  
## -0.0149597 -0.0067591 -0.0005094  0.0044842  0.0302169  
##  
## Coefficients:  
##             Estimate Std. Error t value Pr(>|t|)  
## (Intercept) 3.104e-01 4.181e-03  74.253 <2e-16 ***  
## FA          -2.156e-01 2.608e-03 -82.667 <2e-16 ***  
## DES         1.439e-04 8.248e-05   1.745  0.0842 .  
## pH          -6.863e-03 5.671e-04 -12.100 <2e-16 ***  
## ---  
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1  
##  
## (Dispersion parameter for gaussian family taken to be 8.502687e-05)  
##  
## Null deviance: 0.6019234 on 99 degrees of freedom  
## Residual deviance: 0.0081626 on 96 degrees of freedom  
## AIC: -647.55  
##  
## Number of Fisher Scoring iterations: 2
```

2.2.2 Assays without addition of Mg²⁺

Because of an error (MgCl₂ was forgotten), the first round of assays was performed without addition of MgCl₂ to the reactions. This has been shown to have adverse effects on the PFOMT activity at a physiological pH around 7.5. After all PFOMT is dependent on divalent cations for its activity. However there seems to be no data regarding the pH dependence of catalysis without addition of MgCl₂.

The standard reaction conditions for a total volume of 100 µl are 50 mM buffer, 0.4 mM caffeic acid, 2.5 µM GSH, 0.5 mM SAM and 20 µg PFOMT.

OMT-Reaction (100 µl):

volume	compound	final concentration
50 µl	100 mM buffer	50 mM
4 µl	10 mM caffeic acid	0.4 mM
2.5 µl	0.1 M GSH	2.5 µM
13.6 µl	73.5% 5 mM SAM (3.675 mM)	0.5 mM
20 µl	1 mg/ml PFOMT	0.2 µg/ µl
ad to 100 µl H ₂ O (9.9 µl)		

A standard substrate mastermix was prepared first. This mastermix was then added to an appropriate amount of buffer. 80 µl of this resulting *reaction buffer* was pipetted into an MTP and the reaction was started by addition of PFOMT.

substrate mastermix (130 × 30 µl):

volume	compound	final concentration
520 µl	10 mM caffeic acid	1.33 mM
325 µl	0.1 M GSH	8.33 µM
1768 µl	73.5% 5 mM SAM	1.66 mM
ad to 3900 µl H ₂ O (1287 µl)		

reaction buffer preparation:

1. add 186 µl *substrate-MM* to 310 µl *buffer (1-20)*

Reaction:

1. 80 µl of *reaction buffer* is pipetted into an MTP well, the plate is allowed to heat to room temperature for 2 minutes
2. reaction is of the 20 minutes timepoint is started by addition of 20 µl 1 mg/mL PFOMT
3. incubate at 30 °C, 100 rpm
4. every 5 minutes the reaction in a new row (new timepoint) is started by addition of enzyme, meanwhile the plate is incubated at 30 °C and 100 rpm
5. stop reaction(s) after 2 minutes by addition of 10 µl 1 M Tris-HCl pH 8.5 and 50 µl *catechol reagent* (2 mM FeCl₃, 1 mM Triton X-100 in 10 mM HCl)

Table 2: The timepoints of PFOMT addition to start the reaction.

sample time t_i (min)	addition of PFOMT ($t_0 + \dots$ min)	stop time (min)
$t_0 = 0$	20	20
5	15	20
10	10	20
15	5	20
20	0	20

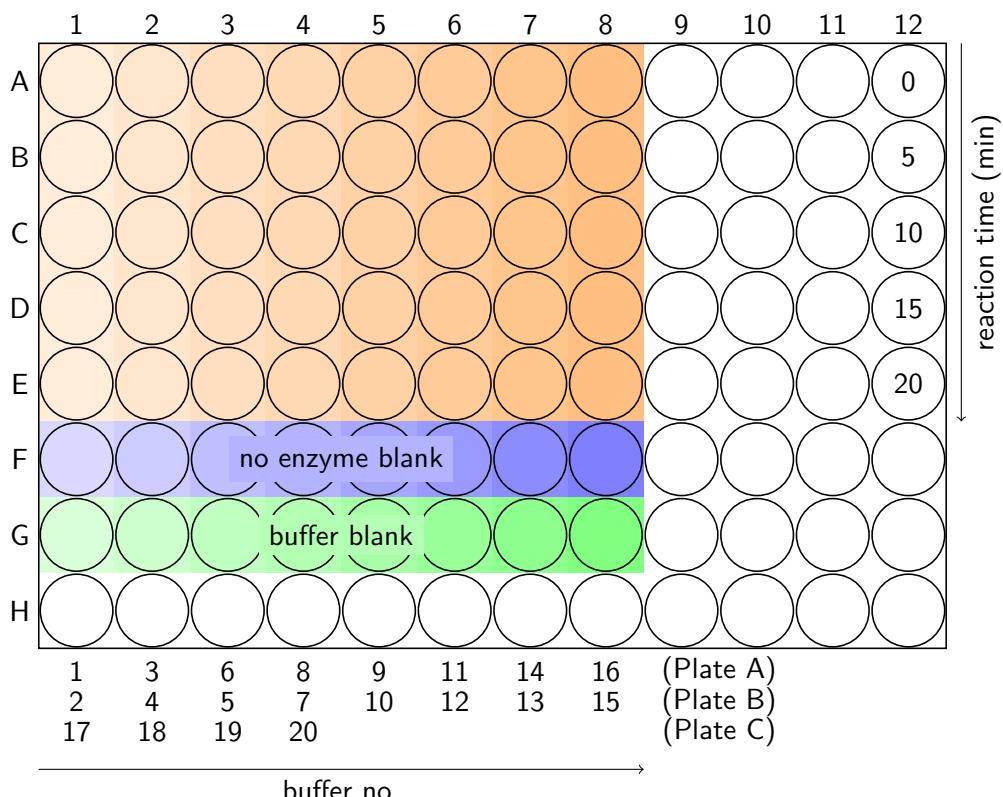


Figure 7: Layout of the MTP-plates to measure PFOMT activity.

2.2.3 Assays with addition of Mg²⁺

After noticing the aforementioned error, the assays were repeated and this time contained 10 mM MgCl₂. Here, the final reaction volume was 100 µl containing 50 mM buffer, 0.4 mM caffeic acid, 2.5 µM GSH, 0.5 mM SAM, **10 mM MgCl₂** and 20 µg PFOMT.

OMT-Reaction (100 µl):

volume	compound	final concentration
50 µl	100 mM buffer	50 mM
4 µl	10 mM caffeic acid	0.4 mM
2.5 µl	0.1 M GSH	2.5 µM
13.6 µl	73.5% 5 mM SAM (3.675 mM)	0.5 mM
1 µl	1 M MgCl ₂	10 mM
20 µl	1 mg/ml PFOMT	0.2 µg/µl
ad to 100 µl H ₂ O (8.9 µl)		

Only 4 timepoints, $t_i = (0, 5, 10, 15 \text{ min})$, are used for measuring these progress-curves. Therefore only $4 \times 4 \times 5 = 80$ measurements are required.

substrate mastermix (110 × 30 µl):

volume	compound	final concentration
440 µl	10 mM caffeic acid	1.33 mM
275 µl	0.1 M GSH	8.33 µM
1496 µl	73.5% 5 mM SAM	1.66 mM
110 µl	1 M MgCl ₂	
ad to 3300 µl H ₂ O (979 µl)		

reaction buffer preparation (5.2x):

1. add 156 µl substrate-MM to 260 µl buffer (1-20)

Reaction:

1. start reaction by addition of 20 µl 1 mg/mL PFOMT to 80 µl of reaction buffer
2. incubate at 30 °C for 20 min
3. stop reaction by addition of 10 µl 1 M Tris-HCl pH 8.5 and 50 µl catechol reagent (2 mM FeCl₃, 1 mM Triton X-100 in 10 mM HCl)

3 Solubility of ferulic acid in different DES concentrations

It has been previously shown, that the solubility of phenolic compounds in aqueous media is poor (1). For example the solubility of ferulic acid in water at room temperature was reported to be 0.78 g/L, or 4.02 mM.

To see, whether the solubility of ferulic acid could be increased by the addition of *natural deep eutectic solvents* (NADES), the solubility of ferulic acid in the buffers 13 to 16 (10 mM malate-mes-tris pH 7.6 and 0, 20, 40, 60 % DES) was measured. Therefore a spatula tip of

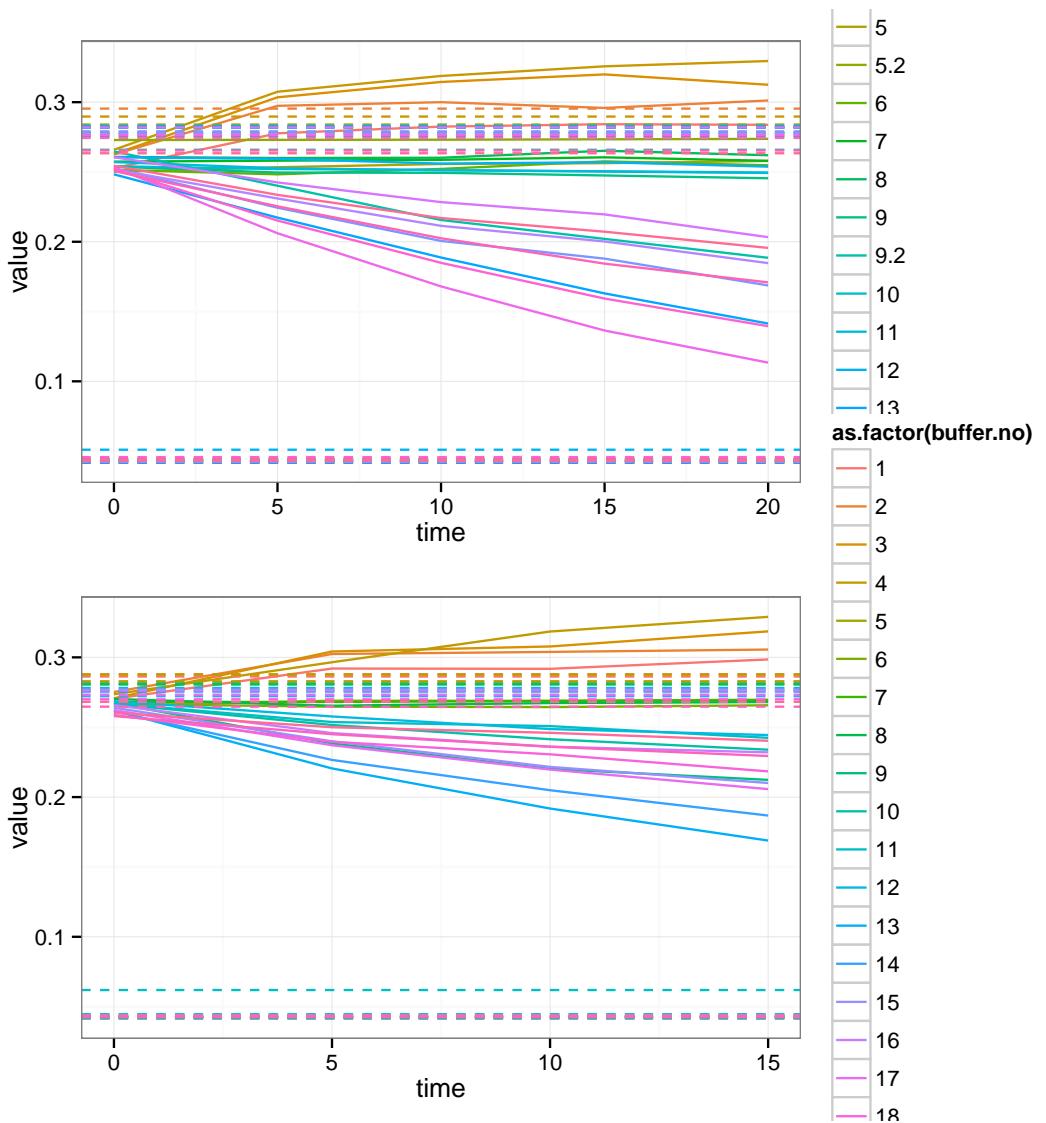


Figure 8: Progress curves of PFOMT activity tests.

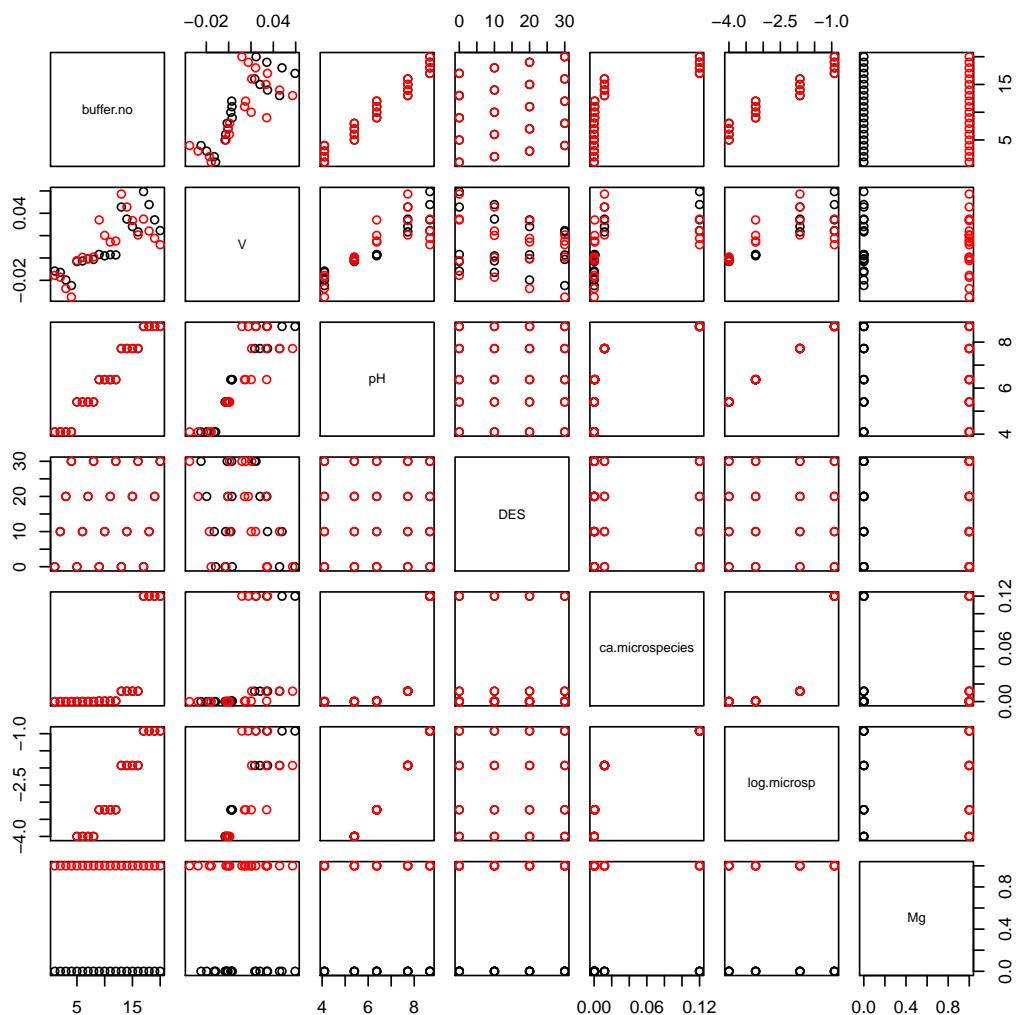


Figure 9: Scatterplot Matrix for activities of PFOMT with (red) and without (black) MgCl_2

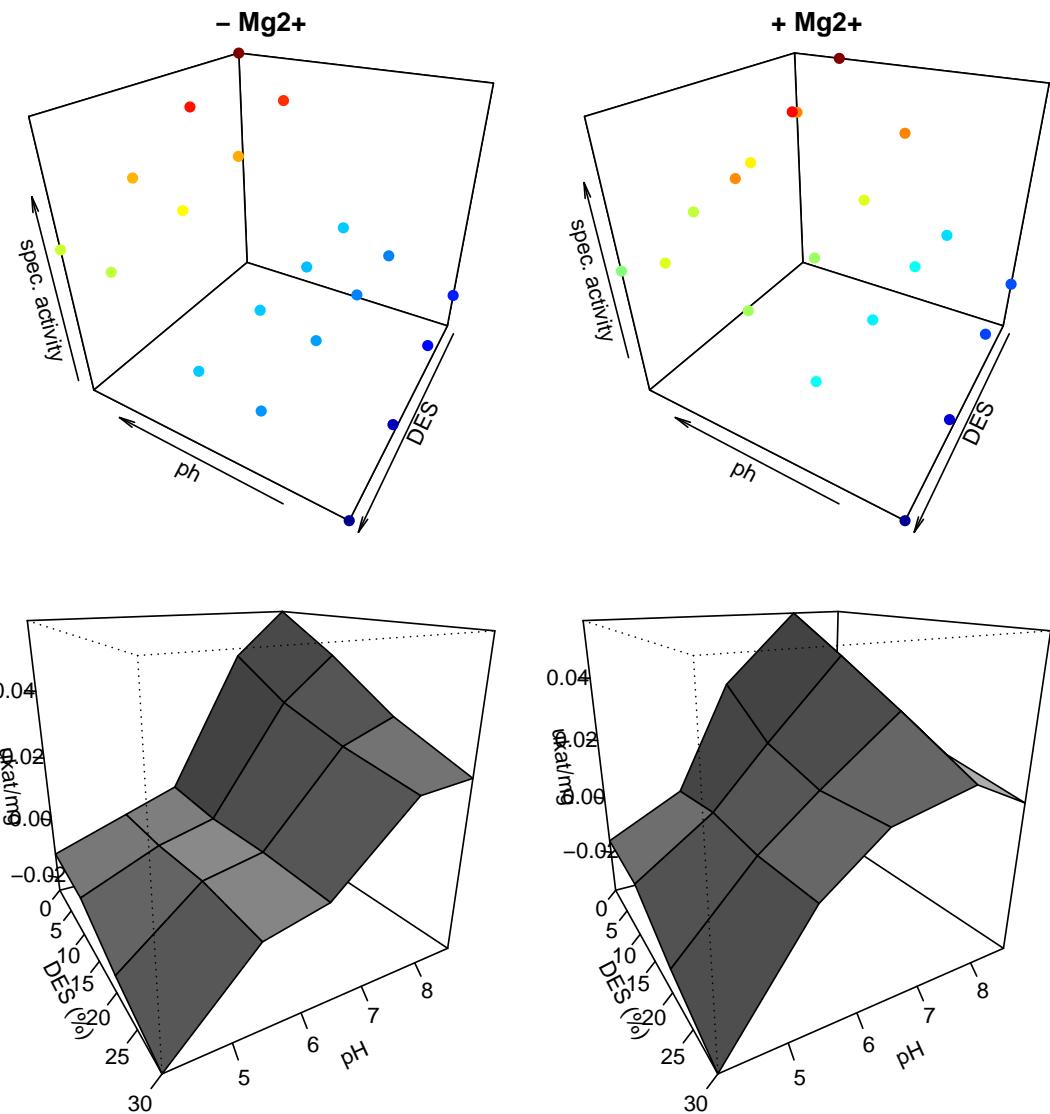


Figure 10: Activity of PFOMT without and with Mg²⁺

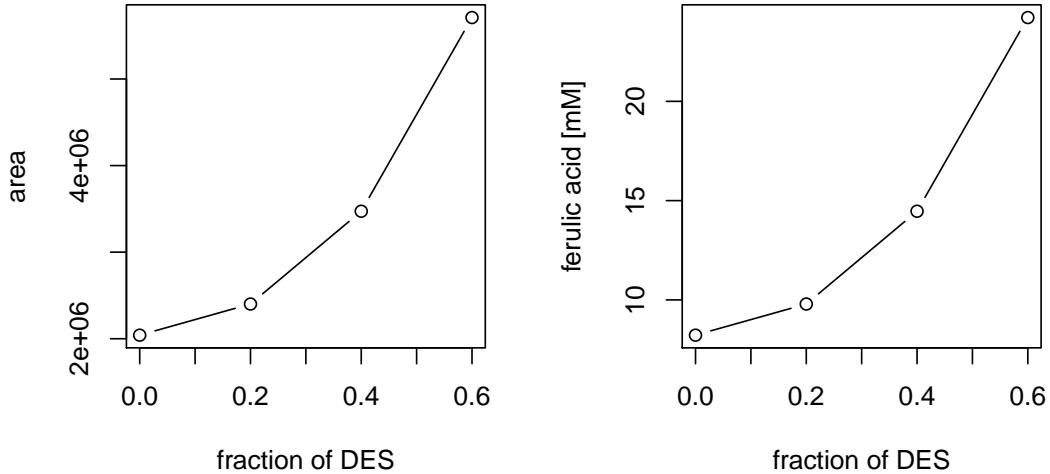


Figure 11: Solubility test of ferulic acid in different DES concentrations.

ferulic acid was dissolved in 250 μl buffer by shaking at 1700 rpm and RT for 1 hour. After 1 hour the suspension was centrifuged to collect undissolved ferulic acid. The supernatant was diluted 1:100 into methanol. Afterwards the samples were analyzed by HPLC. 1 μl of each sample was injected.

The concentration of ferulic acid dissolved was calculated by the calibration curve of ferulic acid (*WEB290*):

$$c_{mM} = \frac{area - 3.104 \cdot 10^5}{4.5867 \cdot 10^7} \quad (8)$$

This result was multiplied by 2 (1 μl injected here versus 2 μl injected for WEB290) and 100 (dilution factor). The data in figure 11 show, that the solubility of ferulic acid increases with increasing concentration of DES in the buffer. There seems to be a higher order (non-linear) relationship.

4 ITC measurements

The cells should be cleaned with a detergent soak (0.5% Hellmanex, 50 °C for 30 min) and water rinse every two days. To obtain an optimally low background.

Table 3: ITC measurements.

No.	filename (*.itc)	cell	syringe	temp (°C)
1	150304_PFOMT.vs.MgCl2_Run1	48.7 μM PFOMT	1 mM MgCl ₂	20
2	150304_PFOMT.vs.SAH_Run2	48.7 μM PFOMT	520 μM SAH	20
3	150304_PFOMTSAH.vs.MgCl_Run3	40.8 μM PFOMT 63.7 μM SAH (remainder of No.2)	1 mM MgCl ₂	20
4	150304_PFOMTSAH.vs.FA_Run4	44.3 μM PFOMT 47.3 μM SAH	0.5 mM ferulic acid	20
5	150305_PFOMTSAHMg.vs.CA	50 μM PFOMT 100 μM SAH 500 μM MgCl ₂	509.8 μM caffeic acid $A^{312} = 5.71$	20
6	150305_PFOMTSAHMg.vs.iFA	50 μM PFOMT 100 μM SAH 500 μM MgCl ₂	500 μM iso-ferulic acid, $A^{310} = 8.18$	20
7	150305_PFOMTSAHMg.vs.CA_2	50 μM PFOMT 100 μM SAH 500 μM MgCl ₂	509.8 μM caffeic acid	20
8	150305_SAHMg.vs.CA	100 μM SAH 500 μM MgCl ₂	5 mM caffeic acid	20
9	150305_SAHMgCl2.vs.CA_2	100 μM SAH 500 μM MgCl ₂	509.8 μM caffeic acid	20
10	150305_Mg.vs.CA	50 μM MgCl ₂	5 mM caffeic acid	20
11	150306_PFOMTSAHMgCl2.vs.CAMgCl2	50 μM PFOMT 100 μM SAH 500 μM MgCl ₂	433 μM caffeic acid 500 μM MgCl ₂	20
12	150306_PFOMTSAHMgCl2.vs.-CAMgCl2_Run2	50 μM PFOMT 100 μM SAH 500 μM MgCl ₂	433 μM caffeic acid 500 μM MgCl ₂	30
13	150306_PFOMTCAMgCl2.vs.SAHMgCl2	42.3 μM PFOMT 84.6 μM caffeic acid 423 μM MgCl ₂	500 μM SAH 500 μM MgCl ₂	20
14	150307_PFOMTMgCl2.vs.CAMgCl2	47.9 μM PFOMT 500 μM MgCl ₂	433 μM caffeic acid 500 μM MgCl ₂	30
15	150307_PFOMTMgCl2.vs.CAMgCl2_Run2	47.9 μM PFOMT 500 μM MgCl ₂	433 μM caffeic acid 500 μM MgCl ₂	30
16	150307_PFOMTMgCl2CA.vs.SAH	40.1 μM PFOMT 53.1 μM caffeic acid 500 μM MgCl ₂ (remainder of No. 14)	500 μM MgCl ₂ 500 μM SAH	30
17	150307_PFOMT.vs.CA	46 μM PFOMT	406 μM caffeic acid	30

References

- [1] Mota, F. L., Queimada, A. J., Pinho, S. a. P., and Macedo, E. a. (2008). Aqueous solubility of some natural phenolic compounds. *Industrial and Engineering Chemistry Research*, 47:5182–5189.
- [2] Newman, J. (2004). Novel buffer systems for macromolecular crystallization. *Acta Crystallographica Section D: Biological Crystallography*, 60:610–612.

WEB339

optimization of the catechol assay for all pHs

Benjamin Weigel

February 24, 2015

1 Problem

The catechol assay by Dippe is an excellent tools for activity testing OMTs which methylate catecholic OHs. However, due to the instability of the Fe^{3+} -catechol complex this assay is only suitable at pHs of 7 or higher.

2 Experimental

2.1 Catechol Reagents

different versions:

- a. catechol reagent by DIM
- b. catechol reagent by WEB (2 mM FeCl_3 , 1 mM Triton X-100 in 0.5 M HEPES pH 7.5)
- c. addition of 10 μl 1M Tris-HCl pH 8.5, subsequent addition of 50 μl catecholic reagent

2.2 Experimental Setup

1. 0.2 mM caffeic acid in 100 μl of different pH (buffers concentrated to 50 mM, pH range 4–9)
2. add 50 μl of different versions of catechol reagent
3. incubate for 5 min
4. measure OD⁵⁹⁵

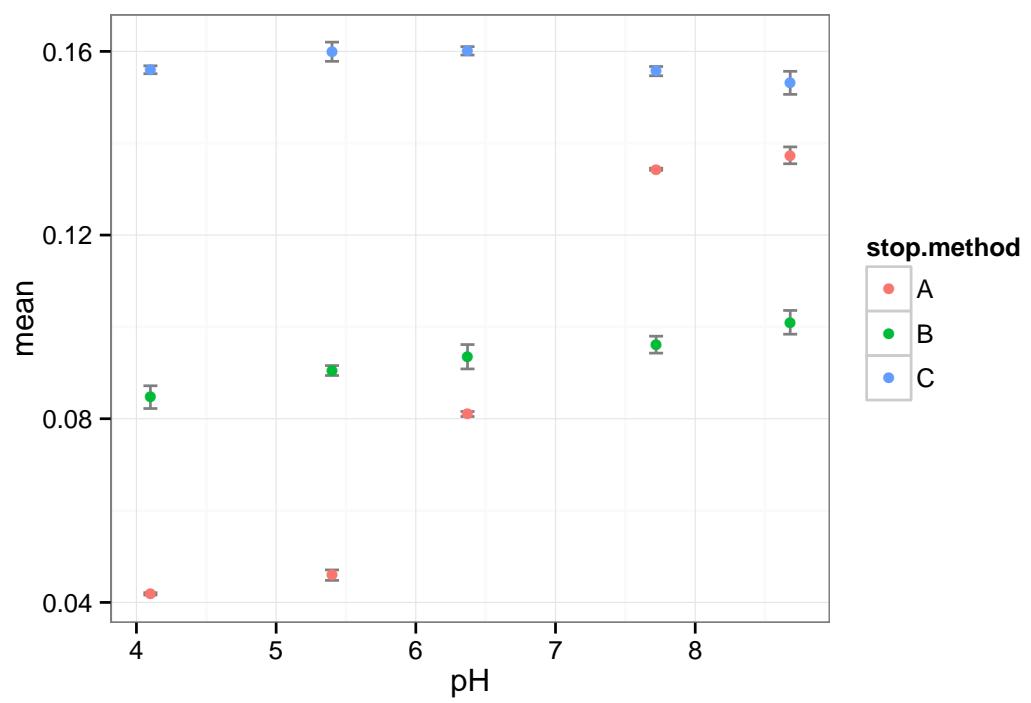


Figure 1: Catechol Assay Trials

WEB314

Comparing SOMT and COMT

Benjamin Weigel

February 18, 2015

1 Active Site Residues

1.1 1kyz (modelling template)

1.2 COMT

1.3 SOMT-2

front lining (towards flavonoid) K258-W264

- F155-W156

back lining (adenine): L223-R225

back lining (ribose): V200-T204, V228, F169-N170

back lining (methionine): M173

WEB342

Assessment of PFOMT for non-catecholic methylation

Benjamin Weigel

March 9, 2015

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1 Introduction

PFOMT without addition of MgCl₂ to the reaction buffer displays increasing methylation activity towards caffeic acid with increasing pH (see [WEB338](#)). With increasing pH, the deprotonated microspecies C (fig. 1) also increases. Thus the PFOMT activity is also correlated to the amount of deprotonated microspecies.

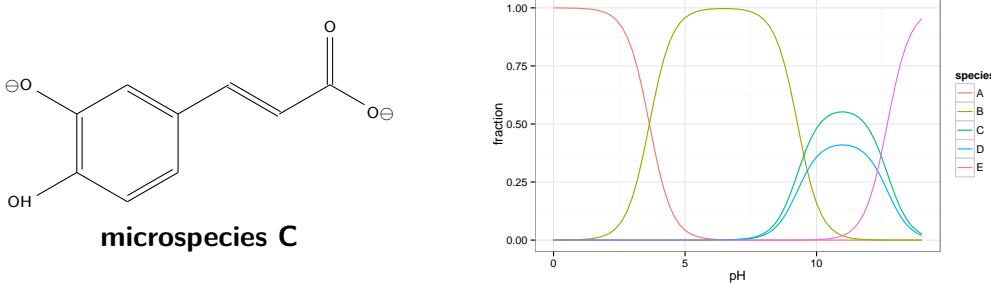


Figure 1: The deprotonated microspecies of caffeic acid, denoted as C, which can nucleophilically attack the methyl-group of SAM. The fraction of this microspecies at different pHs was calculated using ChemAxons MarvinBeans.

The idea was to use this to maybe methylate non-catechols, since the pKa of the phenol hydroxyl-groups are in the same range for a lot of similar compounds such as *m*-coumaric acid, iso-ferulic acid, naringenin and quercetin. The pKa values of these compounds were calculated using ChemAxons MarvinBeans Suite cxcalc commandline tool for batch calculations (tab. 1).

ID	substrate	type	structure	pKa
1	<i>m</i> -coumaric acid	cinnamic acid		9.85
2	Iso-ferulic acid	cinnamic acid		9.43
3	naringenin	flavanon		9.79
4	quercetin	flavonol		12.82

Table 1: Substrates used for testing non-catecholic methylation.

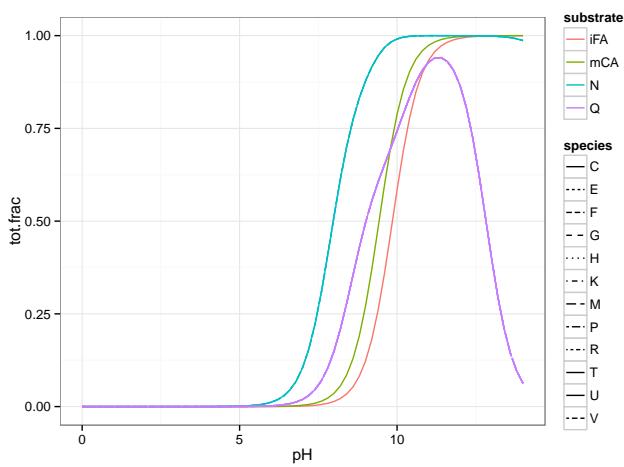
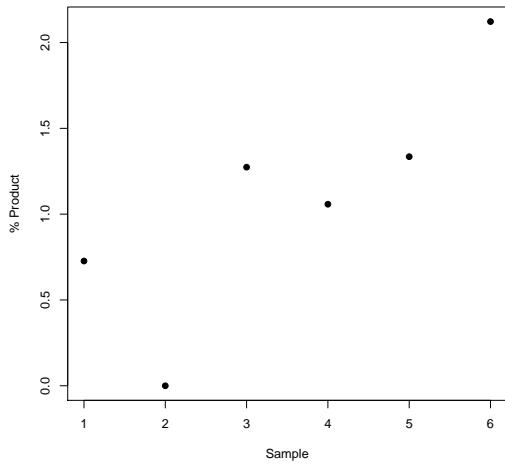


Figure 2: Correctly deprotonated microspecies of m-coumaric acid, iso-ferulic acid, narin-genin and quercetin.



No.	PFOMT	Mg	pH	subst-rate	co-subs
1	T	F	8.6	iFA	SAM
2	F	F	8.6	iFA	SAM
3	T	F	10	iFA	SAM
4	T	F	8.6	CA	SAE
5	T	F	10	CA	SAE
6	T	T	8.6	iFA	SAM

Figure 3: Activity of PFOMT with iso-ferulic acid and SAM, as well as caffeic acid and SAE.

WEB346

Assessment of PFOMT for non-catecholic methylation

Benjamin Weigel

April 7, 2015

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1 Introduction

Previous experiments (*WEB342*) showed minor amounts of methylation of iso-ferulic acid by PFOMT. This experiment was designed to answer three major questions – (a) influence of magnesium on methylation, (b) influence of pH on methylation, (c) dependency of methylation on pKas of flavonoids.

The pKas of "the same" hydroxyl groups in similar flavonoids (e.g. eriodictyol – luteolin – quercetin) depends on the chemical nature of the surrounding groups. Thus

2 PFOMT Assay - No extraction

The standard reaction conditions for a total volume of $50\ \mu\text{l}$ are 50 mM MMT-buffer¹, 0.4 mM caffeic acid, 2.5 μM GSH, 0.5 mM SAM and 20 μg PFOMT. **Reactions** were started by pipetting 48 μl of the corresponding mastermix (A–X) to 2 μl of flavonoid, which was already in the tube. The drops were mixed by tapping and collected by short burst centrifugation and the samples were incubated for 16 hours at 30 °C and 100 rpm in a shaking incubator. 0.3 volumes (15 μl) of **stop solution** (10% trichloro acetic acid in 50% acetonitrile) was added to the samples. The samples were incubated for ≥ 30 minutes on ice and centrifuged for 10 minutes at 4 °C, $10.000\times g$ to collect protein precipitate. The supernatants were transferred to analysis vials and analysed by HPLC.

The chosen **HPLC program** (5% ACN, 0.2% formic acid to 100% ACN, 0.2% formic acid) was sufficient to separate SAM and SAH ($t_R \approx 4\text{ min}$), as well flavonoids ($12\text{ min} \leq t_R \leq 22\text{ min}$).

2.1 LCMS - Extraction of samples

LCMS measurements made extraction of samples necessary. The samples ($V < 50\ \mu\text{l}$) were pipetted from the HPLC inlets into 1.5 mL tubes. The inlets were rinsed with water, to get all the sample. The aqueous phase was extracted two times with 500 μl 1% formic acid in ethyl acetate. The ethyl acetate was evaporated in a SpeedVac and the remainder was resuspended in 75 μl of methanol.

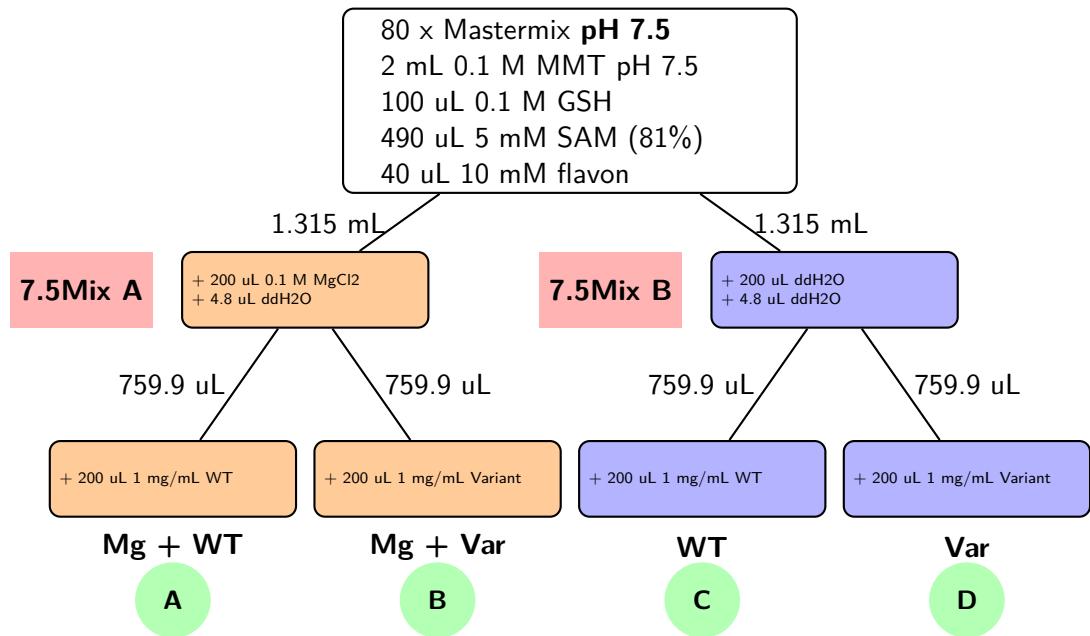
3 Results

After the HPLC runs the automatically detected peaks were integrated and placed in an external ASCII file. The resulting files were loaded into R and arranged into a dataframe for analysis (Fig. 2).

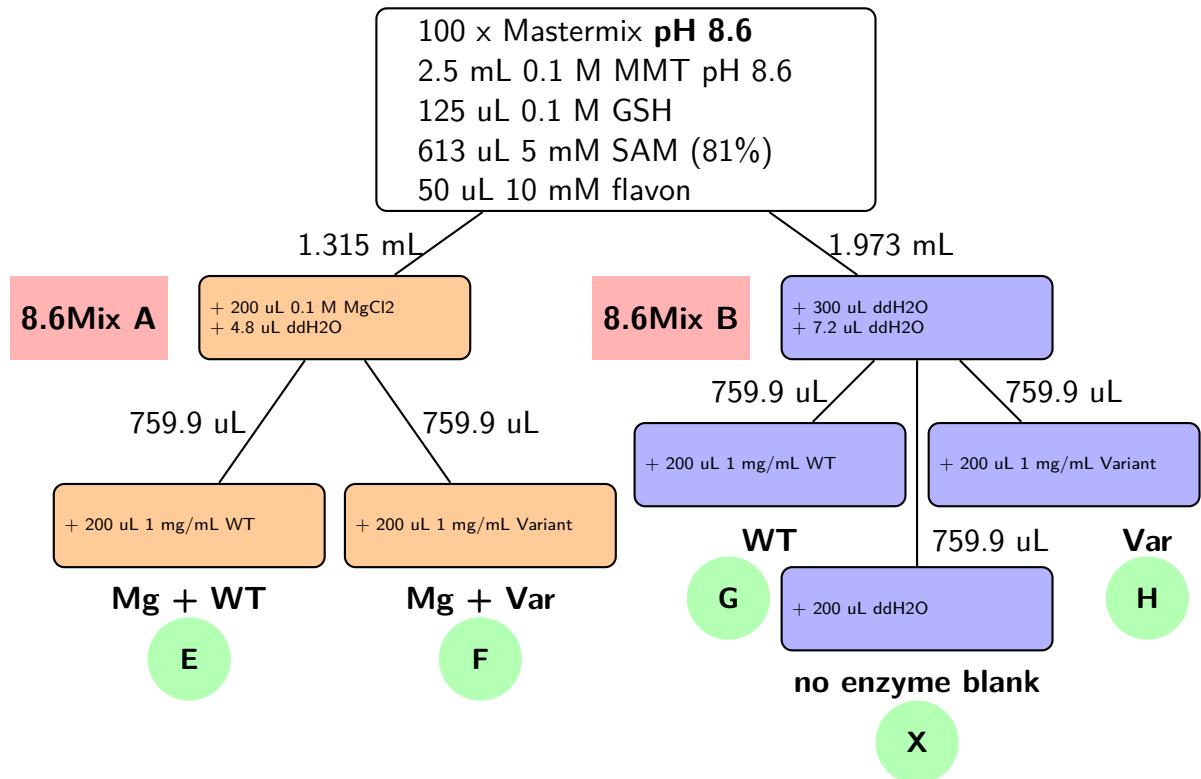
The produced SAH was used as a measure for conversion. Therefore the integral of SAH was divided by the integral of the ISTD flavone to afford the relative Area A_r .

$$A_r = \frac{A_{SAH}}{A_{flavone}} \quad (1)$$

¹MMT - L-malic acid/Mes/Tris



(a) Pipetting scheme for pH 7.5



(b) Pipetting scheme for pH 8.6

Figure 1: Pipetting schemes for the enzymatic reactions. The reactions are started by pipetting 48 μ L of the corresponding mastermix to 2 μ L of substrate. Var - Y51R N202W

```

## for every file ...
for(i in files){
  ## read every line
  tmp <- readLines(paste(PATH, i, sep="/"))

  ## remove commented lines and split lines by tabs
  tmp <- tmp[-(tmp %>% grep(pattern=COM))]
  tmp %>>% str_split(pattern = "\t") %>% unlist %>%
    str_replace(pattern = ",", replacement = ".") %>%
    ## finally arrange unlisted data in a matrix and subsequently df
    matrix(ncol = 6, byrow = T) %>%
    as.data.frame

  tmp <- tmp[-1]
  ## transform data to numerics
  tmp %>>% transform(V1 = as.character(V1) %>% as.numeric,
                      V2 = as.character(V2) %>% as.numeric,
                      V3 = as.character(V3) %>% as.numeric)

  if(exists("HPLC.data")) HPLC.data <- rbind(HPLC.data, tmp)
  else HPLC.data <- tmp
}
names(HPLC.data) <- c("rt", "area", "area.pc", "identified", "trace", "sample")
save(HPLC.data, file="HPLC.RDa")

```

Figure 2: Code to read and arrange HPLC data into a dataframe.

3.1 Data fluctuations and outliers.

Firstly the fluctuations of the retention times of peaks that need always be present (SAM, SAH and flavon) were compared (see Fig. 3). There are some outliers in the retention times, however for the most part the retention times seem normally distributed.

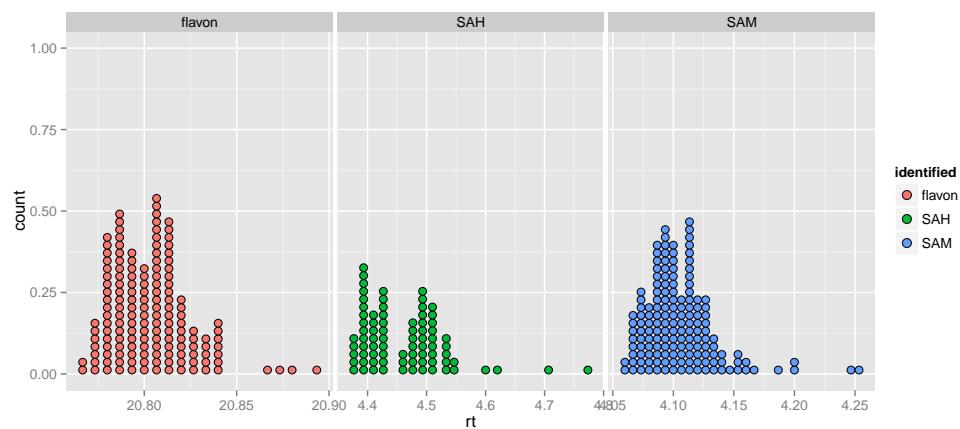
The integration results of the internal standard (ISTD, flavone) were used to detect outliers. Herein outliers are defined as samples, where the integral of flavone was orders of magnitude lower than the mean. The *local outlier factor* (LOF) was used as a measure for the outliers (see Fig. 4). Samples with $LOF \geq 3.5$ were declared as outliers.

3.2 Graphical data exploration

At a first glance some HPLC chromatograms show unexpectedly low intensity peaks. Especially in the samples of substrate 17 there is a lot of decomposition of the starting material, which consequently leads to "fences" in the chromatogram. It has also been shown by other groups, that the flavononols (e.g. quercetin and myricetin) are quiet unstable, especially at elevated pH and temperature (1).

Notably all samples containing substrate **8** suffered from bad solubility after the 16-hours reaction time. In each tube there was precipitate at the bottom. To circumvent the problem of deteriorating substrates the area of the "co-product" SAH was used for quantification of conversion.

As a first undirected approach to data analysis the data was prepared graphically. To get a broad overview of the conversions A_r was plotted for each substrate and buffer. As expected



(a) Dotplot of retention times.

```
##          flavon            SAH            SAM
##  Min.   :20.77   Min.   :4.373   Min.   :4.060
##  1st Qu.:20.79  1st Qu.:4.407   1st Qu.:4.087
##  Median :20.80  Median :4.467   Median :4.100
##  Mean   :20.80  Mean   :4.462   Mean   :4.106
##  3rd Qu.:20.81 3rd Qu.:4.503   3rd Qu.:4.120
##  Max.   :20.89  Max.   :4.773   Max.   :4.253
##          NA's    :74
```

(b) Summary

Figure 3: Fluctuations in the retention times of flavon, SAM and SAH

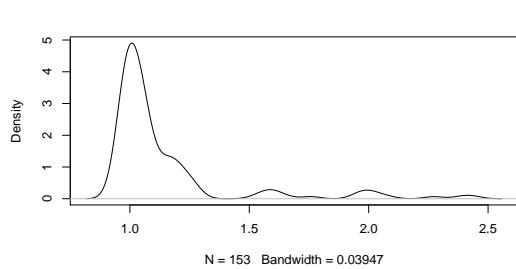
```

## rearrange data for ISTD (flavon)
flavon <- HPLC.data %>%
  filter(identified == "flavon" | identified == "SAH" | identified == "SAM") %>%
  select(identified, area, sample) %>%
  tidyverse::spread(key = identified, value = area)

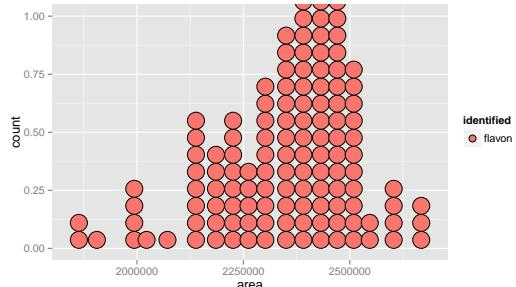
## calculate local outlier factor (LOF) with k is the number of neighbors to use
outliers.scores <- lofactor(data = flavon[,2], k=10)
## order outliers
outliers <- which(outliers.scores >= 3.5)

```

(a) Code used for outlier detection.



(b) Local Outlier factor density. Outliers are marked as red crosses.



(c) Dotplot.

(d) Excluded outliers.

Figure 4: Outlier detection in internal standard (ISTD) flavone via the *local outlier factor* (LOF) using the DMwR package.

the conversion was maximum with known substrates with catecholic moieties (Fig. 5). The substrates that showed the highest conversion were eriodictyol (2), luteolin (6), caffeic acid (12), quercetin (16) and myricetin (17).

3.3 General trends

- conversion drops from catechol to 3'-methylated to 4'-methylated (9)
- conversion better with 10 mM MgCl₂
- if **no Mg²⁺** is supplied conversion is better at high pH (Fig. 8)
- *worst performance:* no Mg, low pH

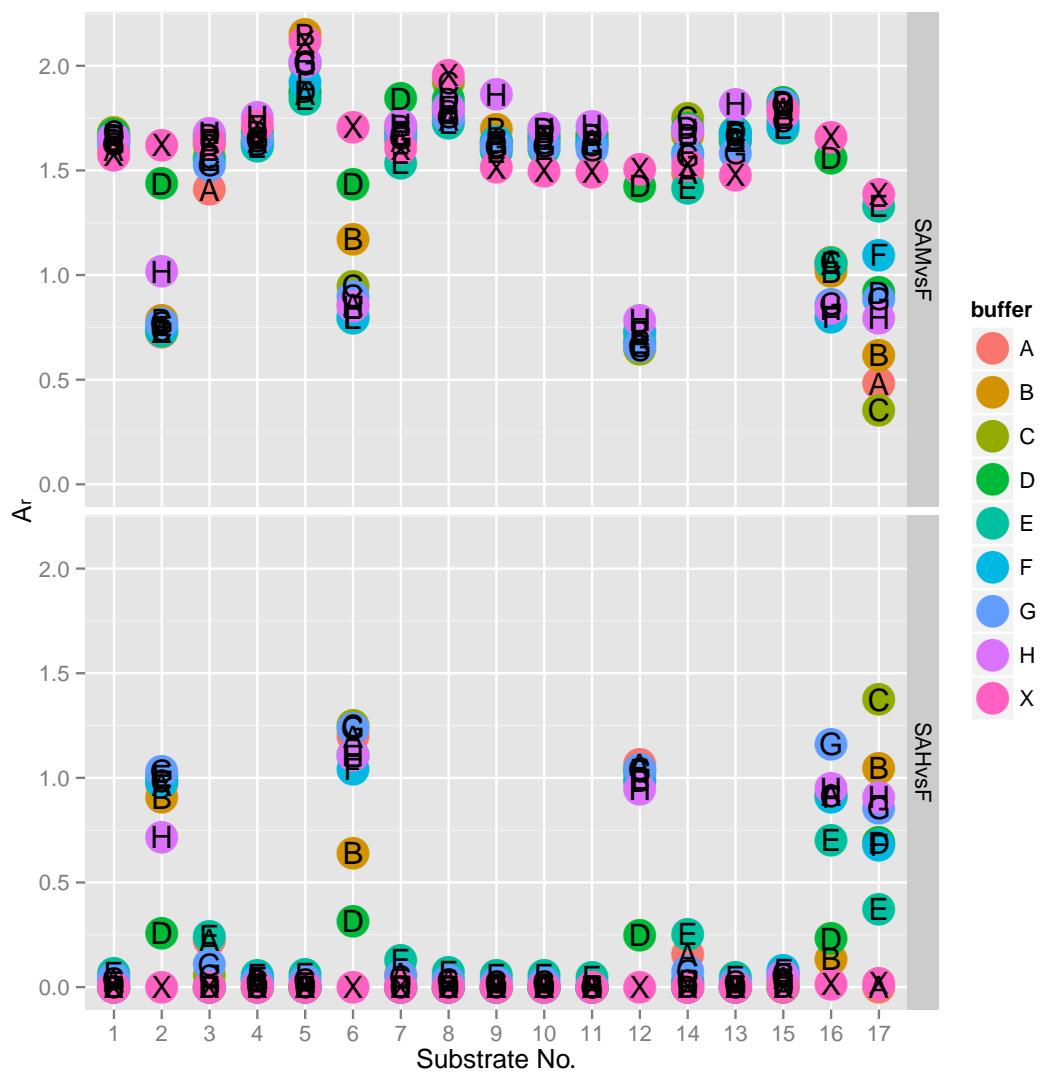


Figure 5: Fluctuations in the measured areas of flavon, SAM and SAH

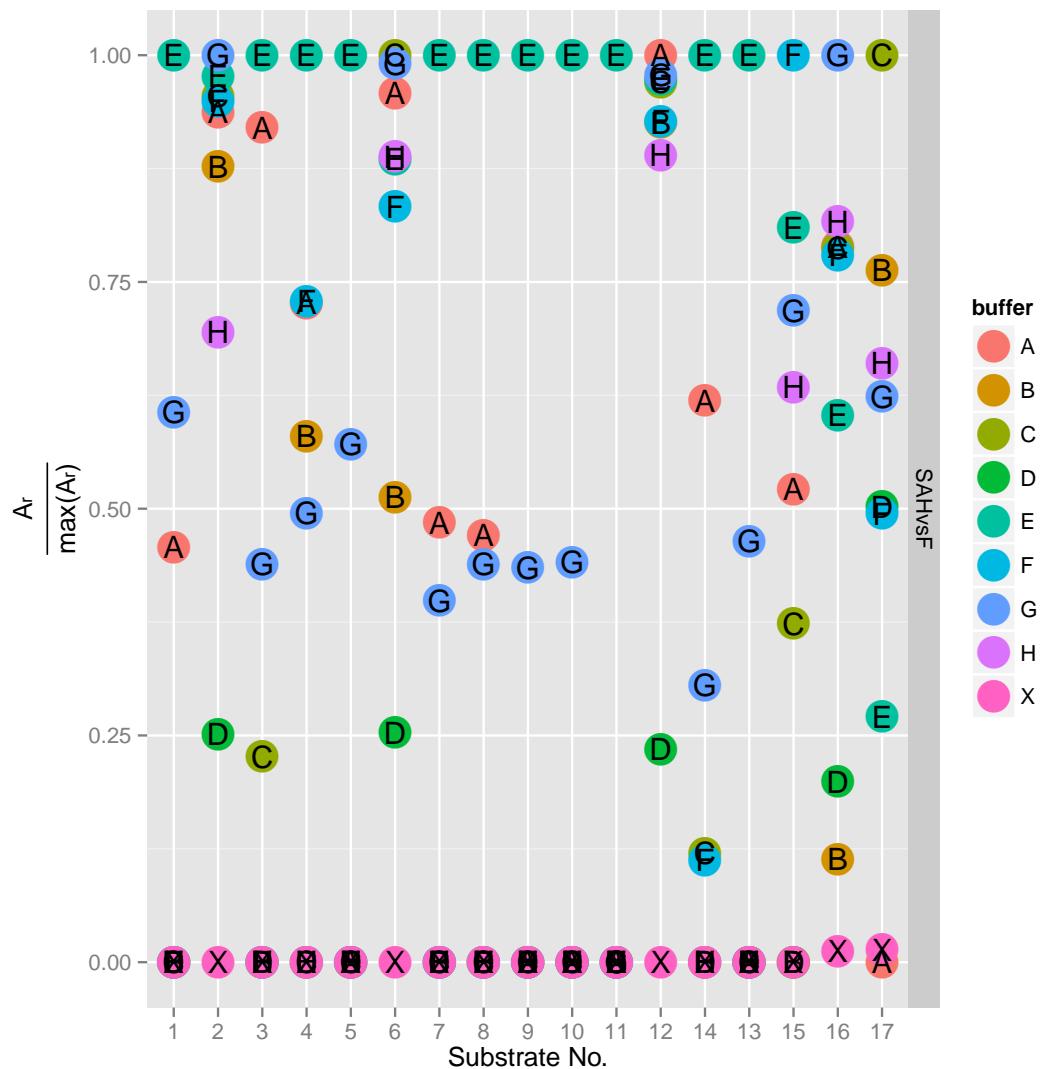


Figure 6: For each substrate the buffer that afforded maximum conversion was set to 1 (100%). The other conversions are given relative to the maximum of 100%.

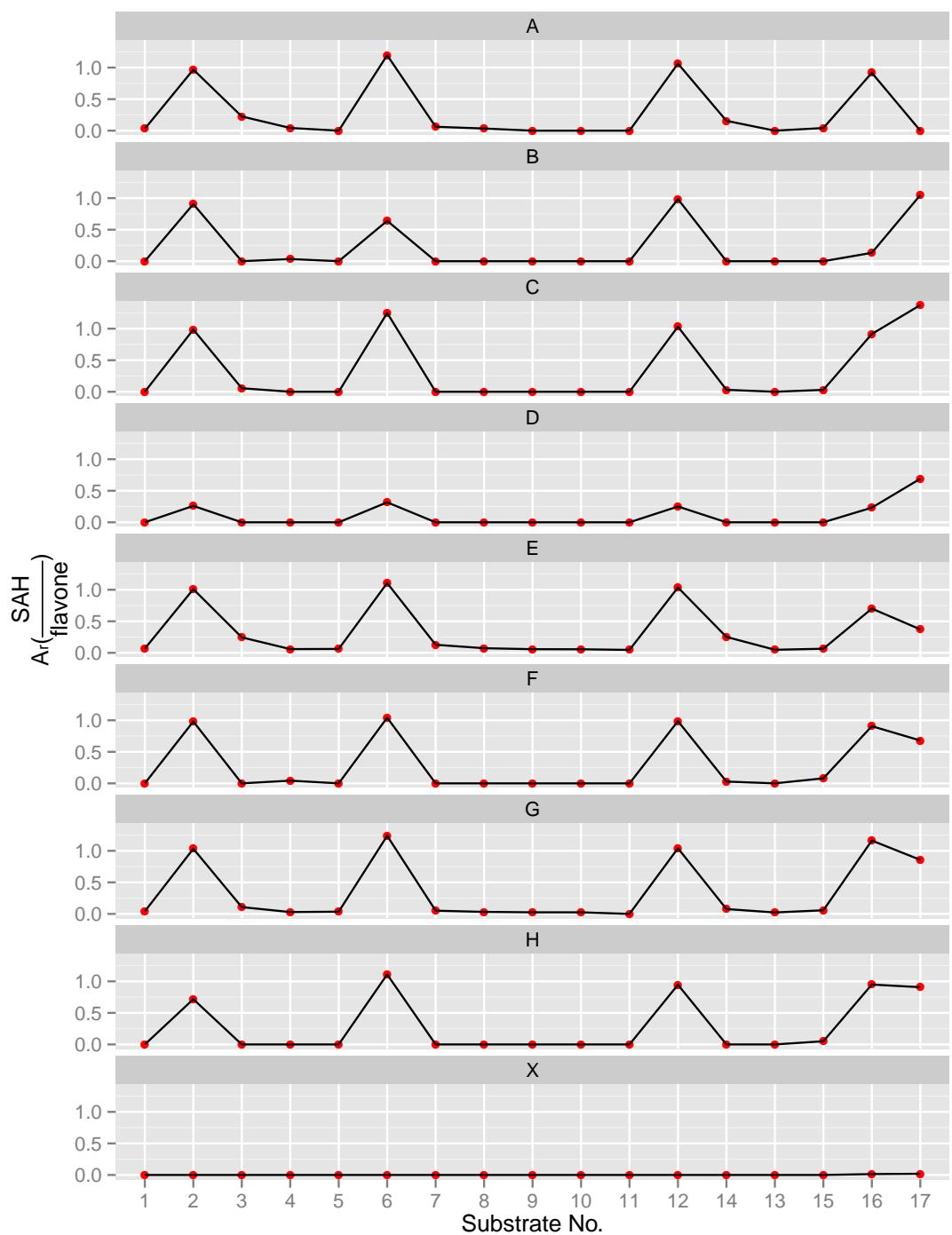


Figure 7: Relative amount of SAH produced during incubation. The production of SAH is pronounced in true substrates (eriodictyol (2), luteolin (6), caffeic acid (12), quercetin (16), myricetin (17)).

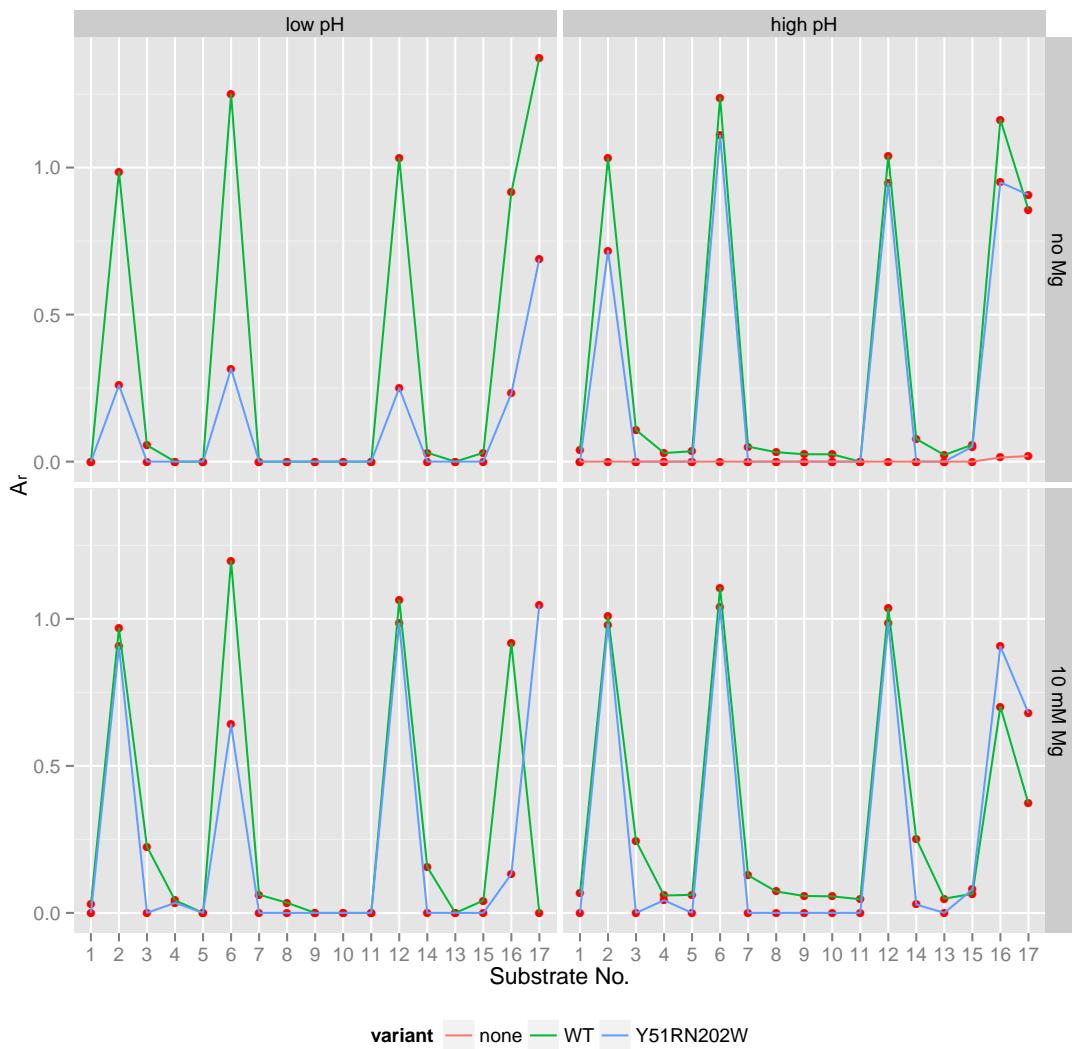


Figure 8: Relative amount of SAH produced compared by pH and Mg.

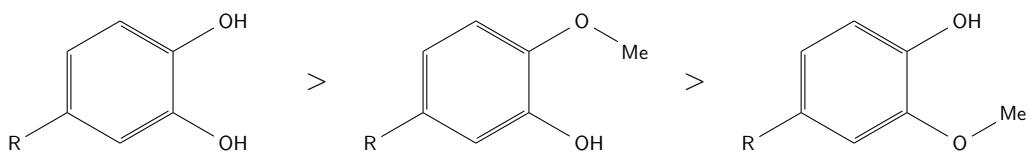


Figure 9: General efficiency of the conversion of phenylpropanoids. See substrates (2,3,4), (6,7,8) and (12,14,13).

References

- [1] Yao, Y., Lin, G., Xie, Y., Ma, P., Li, G., Meng, Q., and Wu, T. (2014). Preformulation studies of myricetin: A natural antioxidant flavonoid. *Pharmazie*, 69:19–26.

WEB347

Determination of Magnesium in dialyzed PFOMT samples

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March 23, 2015

1 Introduction

The amount of magnesium bound in PFOMT after extensive dialysis is of interest. The best (e.g. easiest, quickest and most available) method for this job is *atomic absorption spectrometry* (AAS). The food chemistry department of the MLU happens to possess a flame-AAS (FAAS). Contact was established through Tobias Jost and Christian ??. The consensus was, that the most feasable way is to first disintegrate the organic matrix (e.g. protein and buffer substances) by wet-ashing (e.g. high temperature hydrolysis by nitric acid) (1, 3, 2).

References

- [1] Alfassi, Z. and Wai, C. M. (1991). *Preconcentration Techniques For Trace Elements*. CRC Press.
- [2] Kingston, H. and Jassie, L. (1988). Microwave acid sample decomposition for elemental analysis. *Journal of Research of the National Bureau of Standards*, 93(3):269.
- [3] Würfels, M. and Jackwerth, E. (1985). Untersuchungen zur Kohlenstoffbilanz beim Aufschlußbiologischer Probenmaterialien mit Salpetersäure. *Fresenius Zeitung für Analytische Chemie*, 322:354–358.

WEB348

measuring pH Optima for PFOMT wildtype in different magnesium conditions

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April 15, 2015

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1 Introduction

Determination of the pH optimum of PFOMT with different substrates (caffeic acid, eriodictyol and iso-ferulic acid) and in low and high magnesium conditions. Measure progresscurve using six (6) timepoints (0, 3, 6, 9, 30 min and 2 h). Two different Mg^{2+} -concentrations (0 and 10 mM), as well as five pH-points (5.5, 6.5, 7.5, 8.5, 9.5). For each substrate this amounts to $6 \times 5 \times 2 = 60$ measurements.

2 Methods

All source-files (*.Rnw, *.R, *.RDa) are archived on the ocean.ipb-halle.de server.

2.1 Assays

The standard reaction conditions for a total volume of $50\ \mu l$ are 50 mM MMT-buffer, 0.4 mM caffeic acid, $2.5\ \mu M$ GSH, 0.5 mM SAM, $\pm 10\text{ mM MgCl}_2$ and $20\ \mu g$ PFOMT, plus 0.1 mM flavon as ITSD.

OMT-Reaction ($50\ \mu l$):

volume	compound	final concentration
$25\ \mu l$	100 mM buffer	50 mM
$2\ \mu l$	10 mM caffeic acid	0.4 mM
$1.25\ \mu l$	0.1 M GSH	$2.5\ \mu M$
$6.13\ \mu l$	81% 5 mM SAM (4.05 mM)	0.5 mM
$10\ \mu l$	1 mg/ml PFOMT	$0.2\ \mu g/\ \mu l$
$0.5\ \mu l$	10 mM flavone	0.1 mM
ad to $50\ \mu l H_2O$ (5.62 μl)		

A standard substrate mastermix was prepared first. This mastermix was then added to an appropriate amount of buffer. $40\ \mu l$ of this resulting *reaction buffer* was pipetted into an 1.5 mL centrifuge tube and the reaction was started by addition of PFOMT.

substrate mastermix A ($40 \times 15\ \mu l$):

volume	compound	final concentration
$80\ \mu l$	10 mM eriodictyol	1.33 mM
$50\ \mu l$	0.1 M GSH	$8.33\ \mu M$
$245.25\ \mu l$	81% 5 mM SAM	1.655 mM
$20\ \mu l$	10 mM flavone	0.33 mM
ad to $600\ \mu l H_2O$ (204.75 μl)		

substrate mastermix B ($40 \times 15\ \mu l$):

volume	compound	final concentration
80 μ l	10 mM eriodictyol	1.33 mM
50 μ l	0.1 M GSH	8.33 μ M
245.25 μ l	81% 5 mM SAM	1.655 mM
20 μ l	1 M MgCl ₂	33.3 mM
20 μ l	10 mM flavone	0.33 mM
ad to 600 μ l H ₂ O (184.75 μ l)		

substrate mastermix C (40 \times 15 μ l):

volume	compound	final concentration
80 μ l	10 mM iso-ferulic acid	1.33 mM
50 μ l	0.1 M GSH	8.33 μ M
245.25 μ l	81% 5 mM SAM	1.655 mM
20 μ l	10 mM flavone	0.33 mM
ad to 600 μ l H ₂ O (204.75 μ l)		

substrate mastermix D (40 \times 15 μ l):

volume	compound	final concentration
80 μ l	10 mM iso-ferulic acid	1.33 mM
50 μ l	0.1 M GSH	8.33 μ M
245.25 μ l	81% 5 mM SAM	1.655 mM
20 μ l	1 M MgCl ₂	33.3 mM
20 μ l	10 mM flavone	0.33 mM
ad to 600 μ l H ₂ O (184.75 μ l)		

substrate mastermix E (40 \times 15 μ l):

volume	compound	final concentration
80 μ l	10 mM caffeic acid	1.33 mM
50 μ l	0.1 M GSH	8.33 μ M
245.25 μ l	81% 5 mM SAM	1.655 mM
20 μ l	10 mM flavone	0.33 mM
ad to 600 μ l H ₂ O (204.75 μ l)		

substrate mastermix F (40 \times 15 μ l):

volume	compound	final concentration
80 μ l	10 mM caffeic acid	1.33 mM
50 μ l	0.1 M GSH	8.33 μ M
245.25 μ l	81% 5 mM SAM	1.655 mM
20 μ l	1 M MgCl ₂	33.3 mM
20 μ l	10 mM flavone	0.33 mM
ad to 600 μ l H ₂ O (184.75 μ l)		

reaction buffer preparation:

1. add 105 μ l (7 \times 15) substrate-MM to 175 μ l (7 \times 25) of each 100 mM buffer (pH 5.4, 6.5, 7.5, 8.5, 9.5)

Reaction:

1. add 70 μ L of 1 mg/mL PFOMT to the 7x reaction buffer (2.1)
2. mix and place at 30 °C, 100 rpm
3. take 50 μ L samples after (0, 3, 6, 9, 30, 120 minutes)
4. pipett the sample into 15 μ L stop solution (10% TCA in 50% ACN)
5. vortex and place on ice for at least 30 minutes
6. centrifuge for 10 min at 10.000 $\times g$ and 4 °C
7. transfer supernatant to HPLC vial for analysis

Table 1: Sample times for activity test.

sample / sample time	0	3	6	9	30	120	ON
A1	0	3	6	9	30	120	
A2	0.5	3.5	6.5	9.5	30.5	120.5	
A3	1	4	7	10	31	121	
A4	1.5	4.5	7.5	10.5	31.5	121.5	
A5	2	5	8	11	32	122	
B1	12	15	18	21	42	132	
B2	12.5	15.5	18.5	21.5	42.5	132.5	
B3	13	16	19	22	43	133	
B4	13.5	16.5	19.5	22.5	43.5	133.5	
B5	14	17	20	23	44	134	

Table 2: Buffer/pH key.

key	substrate	MgCl ₂	key	pH
A	ED	F	1	5.5
B	ED	T	2	6.5
C	iso-FA	F	3	7.5
D	iso-FA	T	4	8.6
E	CA	F	5	9.45
F	CA	T		

2.2 HPLC

The samples were analyzed by HPLC (A: H₂O + 0.2% formic acid, D: acetonitrile + 0.2% formic acid) using a linear gradient A → B in 30 minutes. Because the reaction was not extracted with organic solvent, it was also possible to analyze the amount of SAM and SAH in the reaction. This gave additional information on the progress of the reaction.

2.3 Data analysis

The HPLC raw data was automatically analyzed (e.g integration of peaks, naming of peaks) using the software provided by the instrument (EZ-Chrom). Only the 280 nm traces were used for analysis.

The processed data was exported into comma-separated-value (csv) format and analyzed using the R statistical software package. However, prior to analysis the csv-files were checked for errors (e.g. wrong annotation of peaks). Graphical analysis of the primary data consisted of plotting of the progress curves (AUC vs. time).

The initial rates of the reactions were determined by linearly fitting the progress curves using the lm() function in R. A self-developed packages named KayNetics <https://bitbucket.org/IPBweigel/kaynetics> was used as a wrapper to speed up the process.

3 Results

After the

```
## for every file ...
for(i in files){
  ## read every line
  tmp <- readLines(paste(PATH, i, sep="/"))

  ## remove commented lines and split lines by tabs
  tmp <- tmp[-(tmp %>% grep(pattern=COM))]
  tmp %<>% str_split(pattern = "\t") %>% unlist %>%
    str_replace(pattern = ",", replacement = ".") %>%
    ## finally arrange unlisted data in a matrix and subsequently df
    matrix(ncol = 6, byrow = T) %>%
    as.data.frame

  tmp <- tmp[-1,]
  ## transform data to numerics
  tmp %<>% transform(V1 = as.character(V1) %>% as.numeric,
                      V2 = as.character(V2) %>% as.numeric,
                      V3 = as.character(V3) %>% as.numeric)

  if(exists("HPLC.data")) HPLC.data <- rbind(HPLC.data, tmp)
  else HPLC.data <- tmp
}

names(HPLC.data) <- c("rt", "area", "area.pc", "identified", "trace", "sample")
save(HPLC.data, file="HPLC.RDa")
```

Figure 1: Code to read and arrange HPLC data into a dataframe.

4 Results

The progresscurves that were obtained from the integrated HPLC data can be found in the appendix (A).

Explorative analysis of the progress-curves clearly shows, that there is a pH dependence of

the PFOMT reaction, that also varies with the Mg^{2+} content. The catecholic substrates eriodictyol and caffeic acid are favourable substrates in terms of rates. The non-catecholic iso-ferulic acid is converted rather slowly. However, it seems that the rate of methylation is increased when magnesium is added. This is rather strange, since the rate of methylation decreased when magnesium was added to the catecholic substrates. The pH, at which the rate of reaction was maximal shifted towards values around 6.5, when Mg^{2+} . When no magnesium was added the rate increased steadily with increasing pH (for catecholics) (see fig. 3).

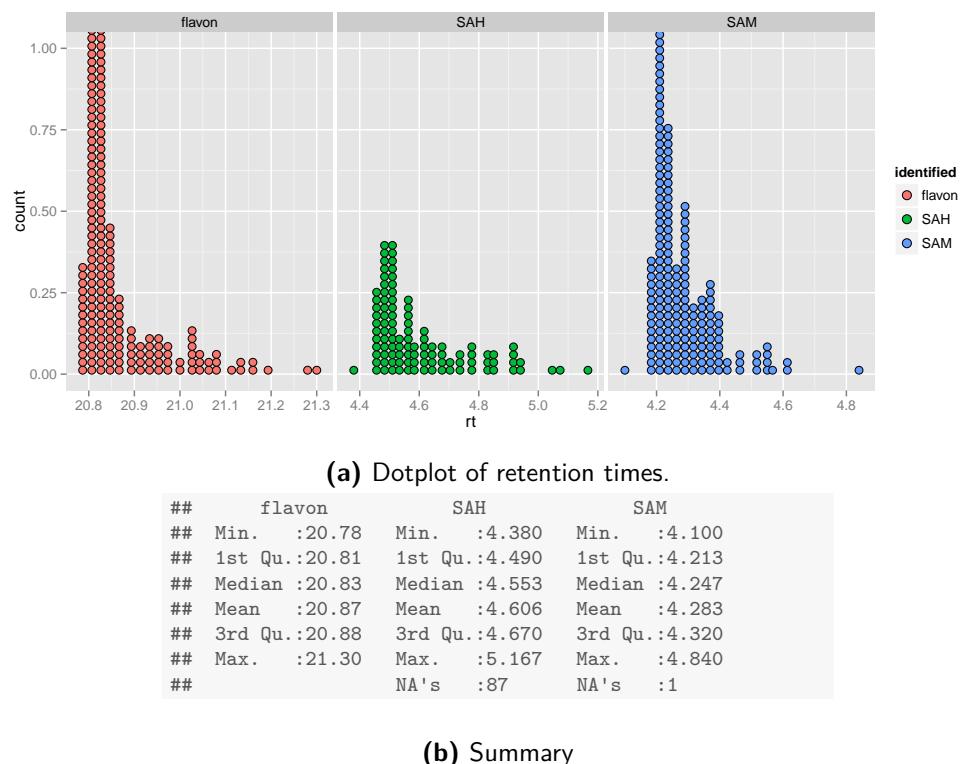


Figure 2: Fluctuations in the retention times of flavon, SAM and SAH

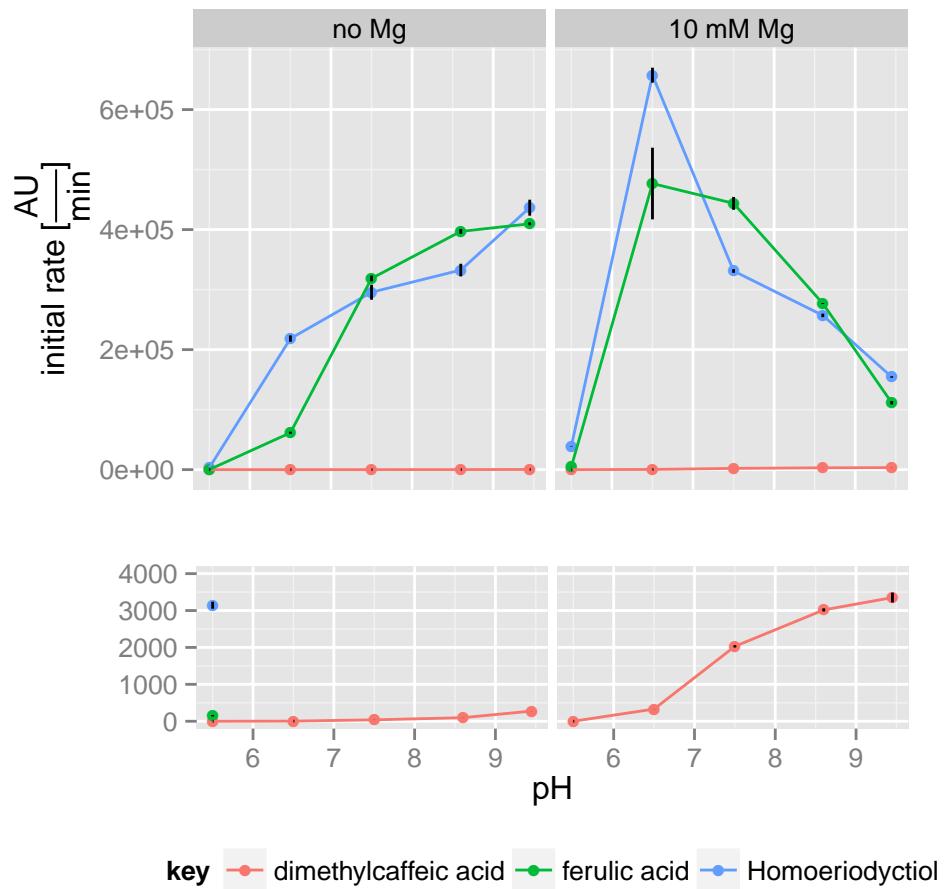


Figure 3: pH-profiles for the appearance of product (as initial rate of appearance).

Appendix

A.1 Progresscurves

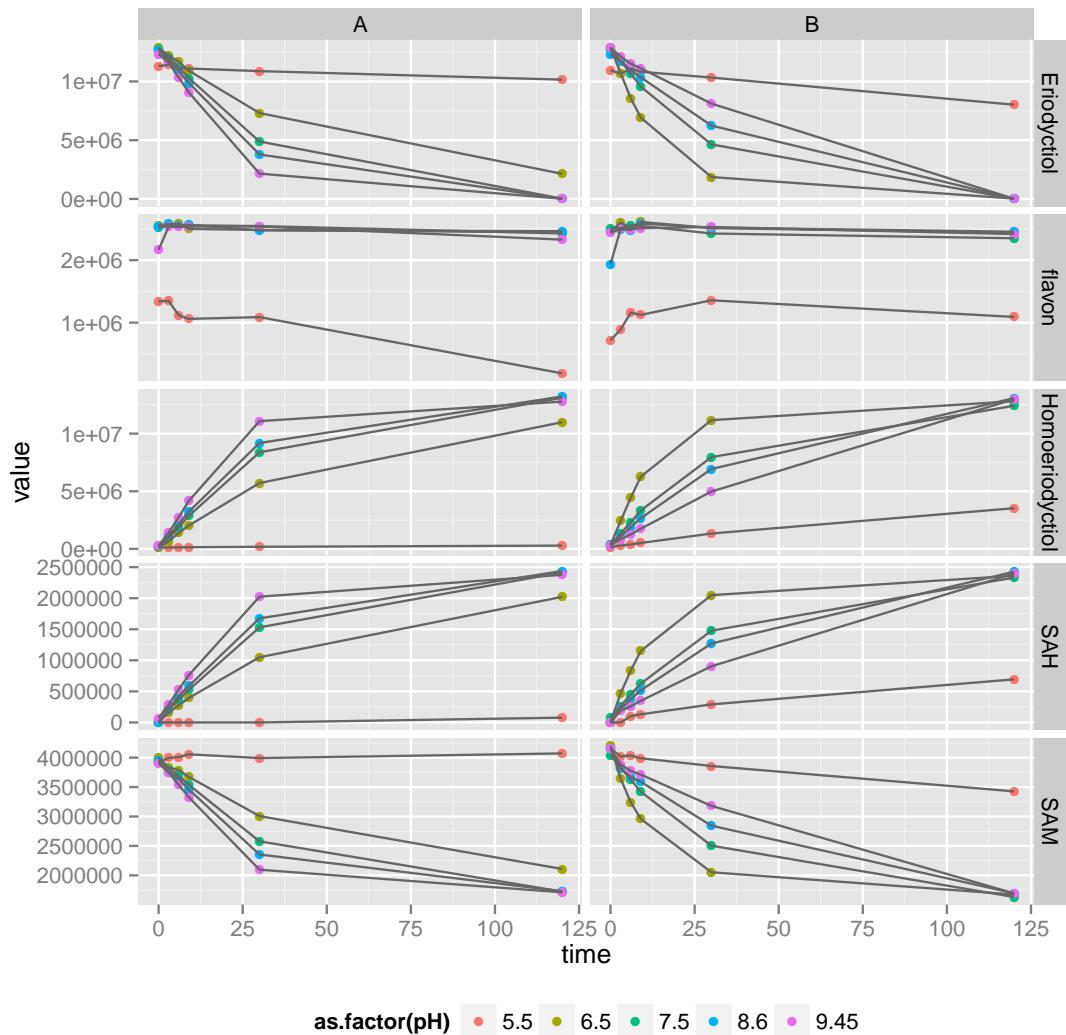
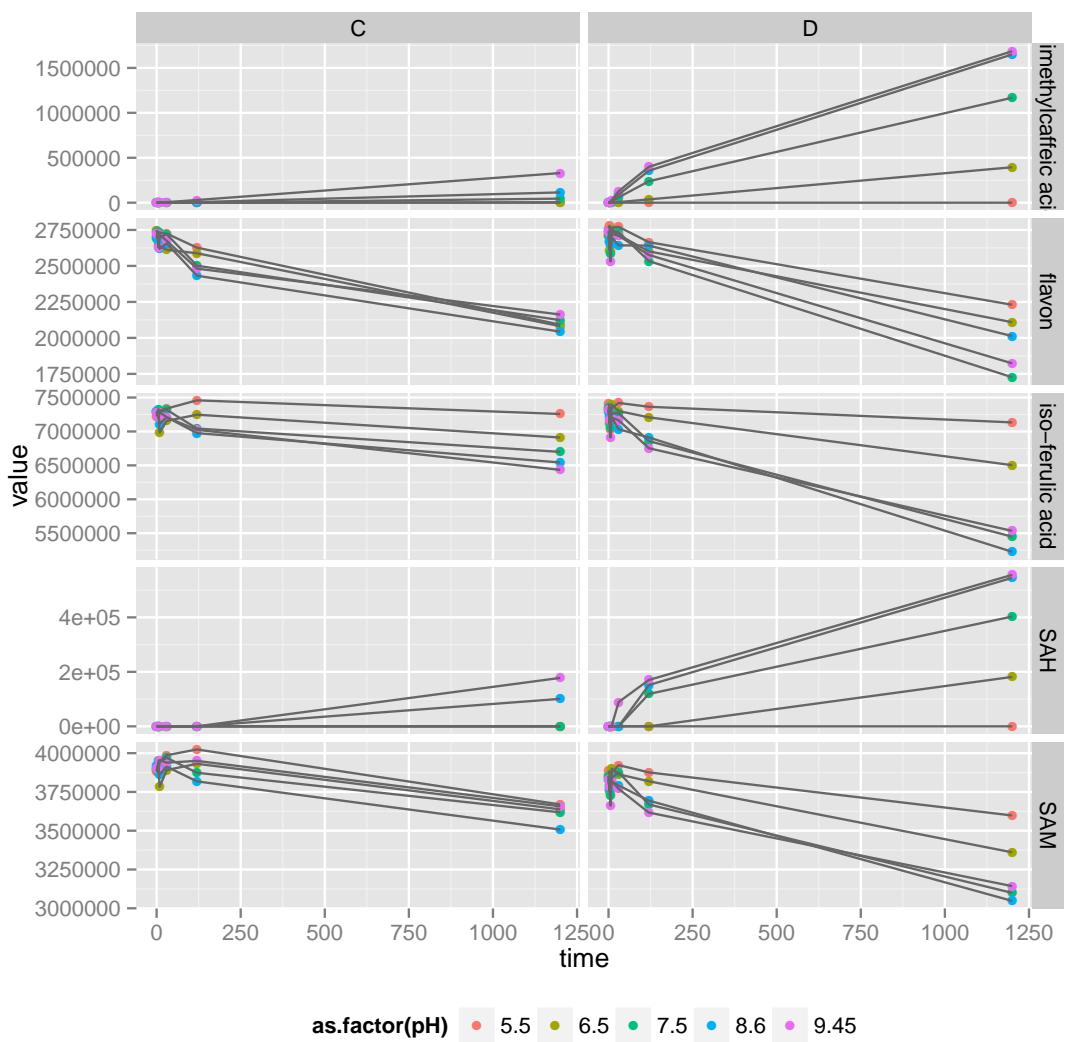


Figure 4: Progresscurves of eriodictyol conversion



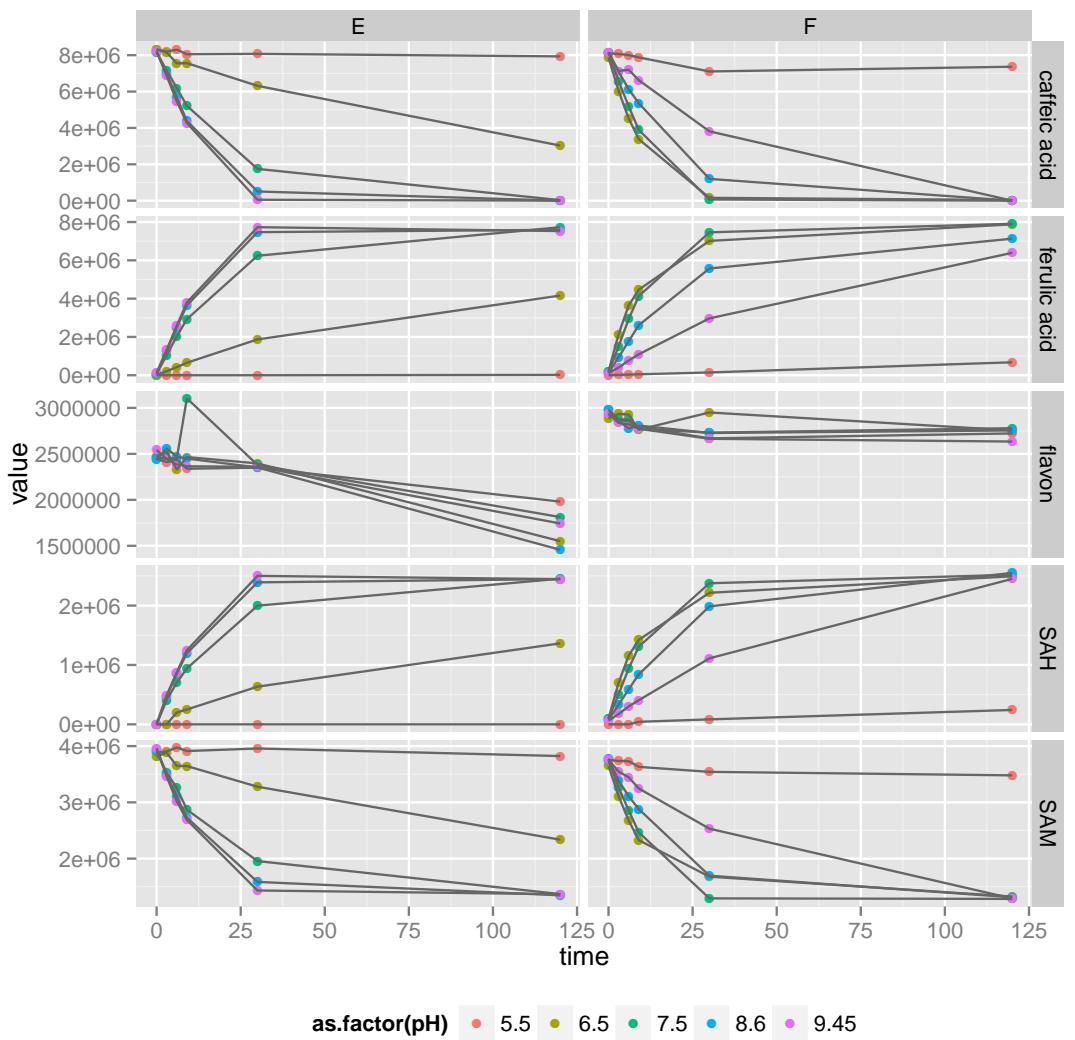


Figure 6: Progresscurves of caffeic acid conversion

A.2 pH Profiles

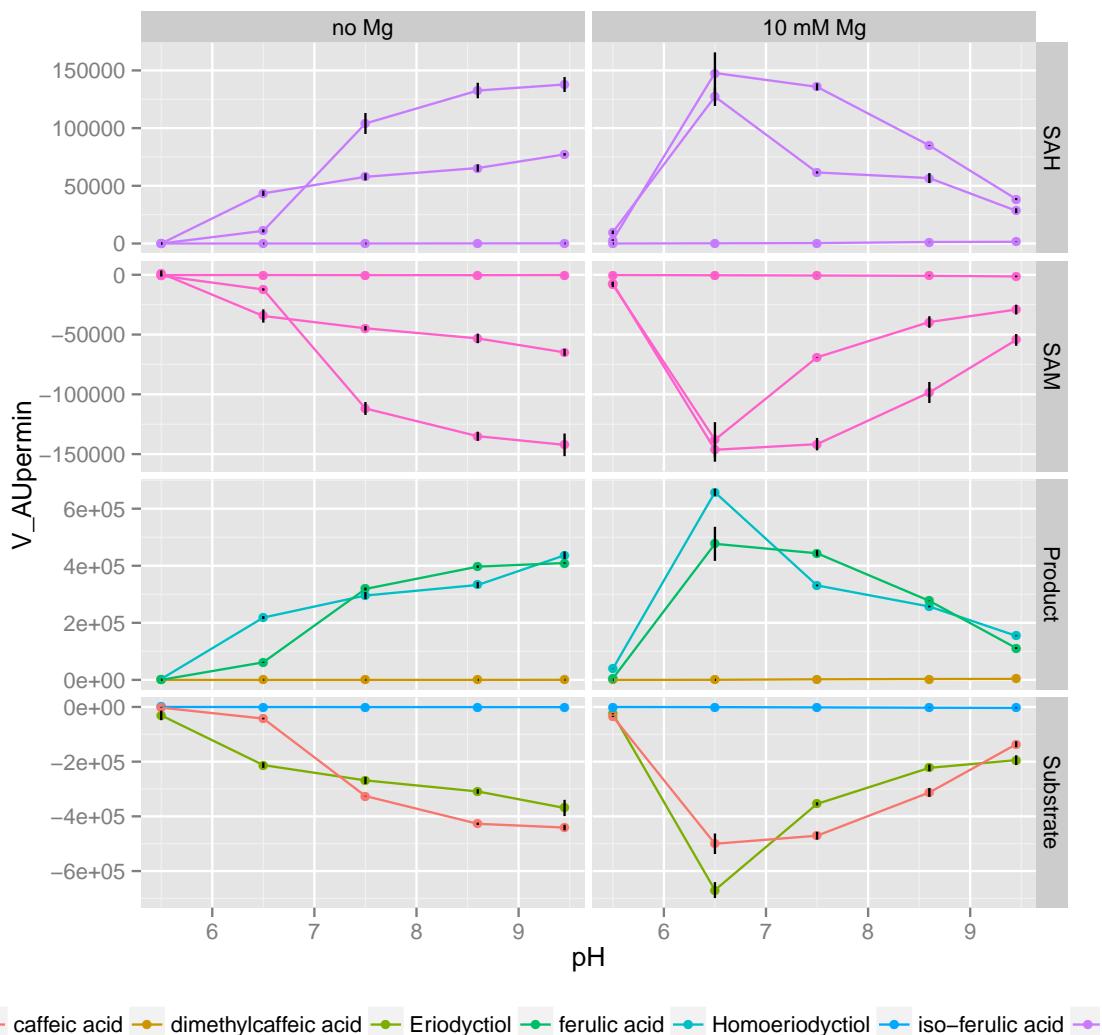


Figure 7: Overview of all pH-profiles. Each progresscurve for each detectable substance was fitted linearly. The resulting initial velocities are plotted against pH.