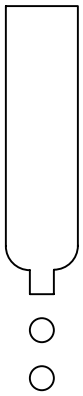
Supporting Information I

Principe of the Hummel-Dreyer-Method (HD)



HPLC-Column equilibration with the ligand

⇩

Incubation/equilibration of protein (P) and ligand (L) in the sample

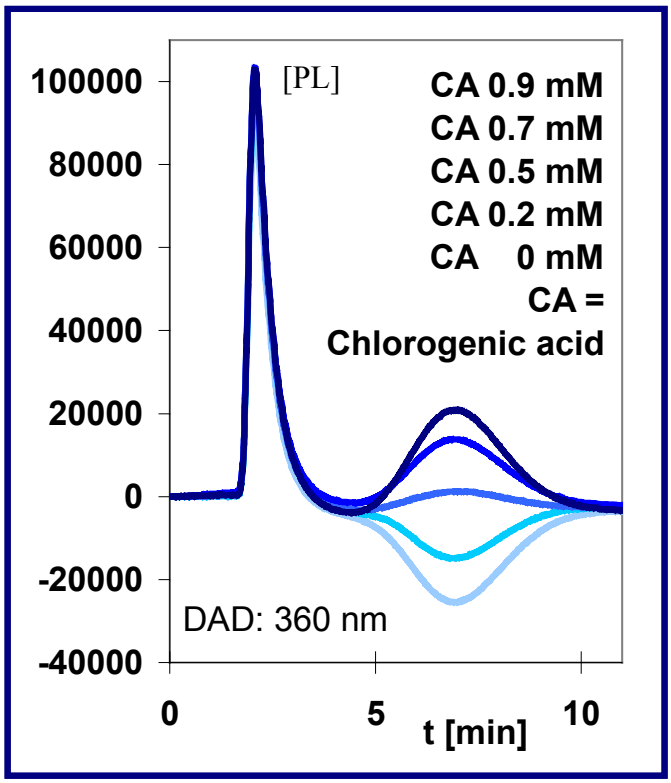
⇩

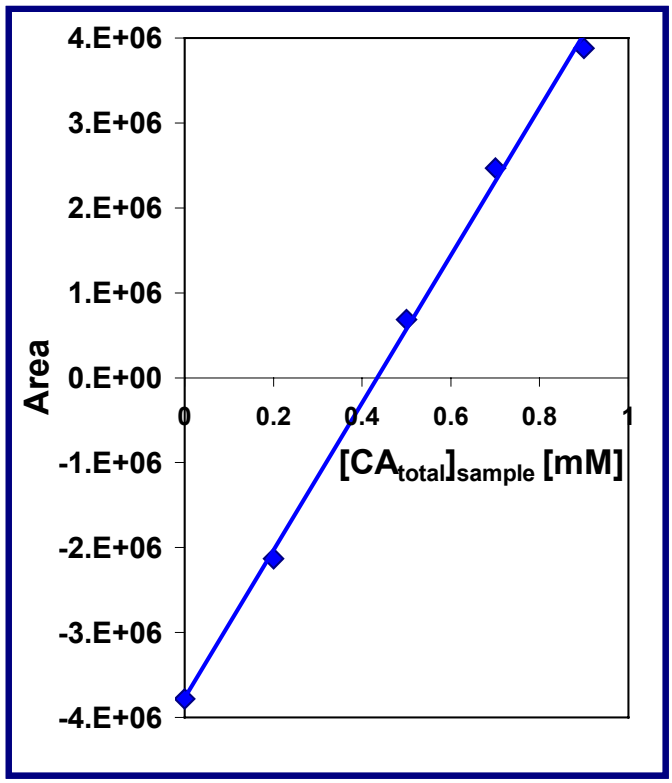
Injection of the sample onto a gel filtration column

⇩

Detection of the ligand in the eluent

Chromatogram





Chromatogram of the gel filtration HPLC according to Hummel-Dreyer-Method $[CA_{eluent}]$ = 0.24 mM, $[BSA]_{sample}$ = 0.18 mM, $[CA_{total}]_{sample}$ = 0 – 0.9 mM. The amount of the chlorogenic acid bound to BSA was obtained by using the values of the peak areas (t = 5-10 min). At area = 0 (x-intercept), we have the following situation: $[CA_{bound}] = [CA_{total}]_{sample} - [CA_{eluent}]$

Supporting Information II
Gel Filtration (Hummel – Dreyer – Method)

Binding studies with BSA

BSA – Ferulic acid, pH 4.8

Eluent A : 0.05 M Hepes Buffer, pH 4.8
Eluent B: : 0.5 mM ferulic acid dissolved in 0.05 M Hepes Buffer, pH 4.8
Elution : isocratic, with different proportions of Eluent B
Column : Bio-Rad P6 cartridge
Injection volume : 30 µl
Flow rate : 0.8 ml/min
Sample : Protein or Protein-Phenol-mixture
Detector : DAD

BSA - SL : 120 g/l, solubilized in 0.05 M Hepes Buffer, pH 4.8
Ferulic acid - SL : 0.5 mM ferulic acid dissolved in 0.05 M Hepes Buffer, pH 4.8
1 mM ferulic acid dissolved in 0.05 M Hepes Buffer, pH 4.8

Table 1: Composition of the samples for BSA-ferulic acid (pH 4.8) in dependence of the Ferulic acid concentration of the eluent

$[FA_{Eluent}]$ mM	$[FA_{Probe}]$ [µM]					$[BSA_{Probe}]$ [µM]
	b	c	d	e	f	Samples b-f
0.03 / 0.06 / 0.09	0	100	250	350	450	181
0.12 / 0.15 / 0.18 / 0.24 / 0.30 / 0.36 / 0.50	0	200	500	700	900	181
0.50	0	200	500	700	900	90

Sample a: only Buffer A

BSA – Ferulic acid, pH 7

Eluent A : 0.05 M Hepes Buffer, pH 7
Eluent B: : 0.5 mM ferulic acid dissolved in 0.05 M Hepes Buffer, pH 7
Elution : isocratic, with different proportions of Eluent B
Column : Bio-Rad P6 cartridge
Injection volume : 50 µl
Flow rate : 1 ml/min
Sample : Protein or Protein-Phenol-mixture
Detector : DAD

BSA - SL : 120 g/l, solubilized in 0.05 M Hepes Buffer, pH 7
Ferulic acid - SL : 0.5 mM ferulic acid dissolved in 0.05 M Hepes Buffer, pH 7
1 mM ferulic acid dissolved in 0.05 M Hepes Buffer, pH 7

Table 2: Composition of the samples for BSA-ferulic acid (pH 7) in dependence of the Ferulic acid concentration of the eluent

$[FA_{Eluent}]$ mM	$[FA_{Probe}]$ [µM]					$[BSA_{Probe}]$ [µM]
	b	c	d	e	f	Samples b-f
0.030 / 0.045 / 0.060 /0.075 / 0.090 / 0.120	0	100	250	350	450	181
0.150 / 0.180 / 0.240 / 0.500	0	200	500	700	900	181
0.500	0	200	500	700	900	90
	0	200	500	700	900	45

Sample a: only Buffer A

BSA – Chlorogenic acid, pH 4.8

Eluent A : 0.05 M Hepes Buffer, pH 4.8
Eluent B: : 0.5 mM chlorogenic acid dissolved in 0.05 M Hepes Buffer, pH 4.8
Elution : isocratic, with different proportions of Eluent B
Column : Bio-Rad P6 cartridge
Injection volume : 30 µl
Flow rate : 0.8 ml/min
Sample : Protein or Protein-Phenol-mixture
Detector : DAD

BSA - SL : 120 g/l, solubilized in 0.05 M Hepes Buffer, pH 4.8
Chlorogenic acid - SL : 0.5 mM chlorogenic acid dissolved in 0.05 M Hepes Buffer, pH 4.8
1 mM chlorogenic acid dissolved in 0.05 M Hepes Buffer, pH 4.8

Table 3: Composition of the samples for BSA-chlorogenic acid (pH 4.8) in dependence of the chlorogenic acid concentration of the eluent

$[CA_{Eluent}]$ mM	$[CA_{Probe}]$ [µM]					$[BSA_{Probe}]$ [µM]
	b	c	d	e	f	Samples b-f
0.03 / 0.06 / 0.09 /	0	100	250	350	450	181
0.12 / 0.15 / 0.18 / 0.24 / 0.30 / 0.36 / 0.50	0	200	500	700	900	181
0.50	0	200	500	700	900	90

Sample a: only Buffer A

BSA – Chlorogenic acid, pH 7

Eluent A : 0.05 M Hepes Buffer, pH 7
Eluent B: : 0.5 mM chlorogenic acid dissolved in 0.05 M Hepes Buffer, pH 7
Elution : isocratic, with different proportions of Eluent B
Column : Bio-Rad P6 cartridge
Injection volume : 50 µl
Flow rate : 1 ml/min
Sample : Protein or Protein-Phenol-mixture
Detector : DAD

BSA - SL : 120 g/l, solubilized in 0.05 M Hepes Buffer, pH 7
Chlorogenic acid - SL : 0.5 mM chlorogenic acid dissolved in 0.05 M Hepes Buffer, pH 7
1 mM chlorogenic acid dissolved in 0.05 M Hepes Buffer, pH 7

Table 4: Composition of the samples for BSA-chlorogenic acid (pH 7) in dependence of the chlorogenic acid concentration of the eluent

$[CA_{Eluent}]$ mM	$[CA_{Probe}]$ [µM]					$[BSA_{Probe}]$ [µM]
	b	c	d	e	f	Samples b-f
0.030 / 0.045 / 0.060 /0.075 / 0.090 / 0.120	0	100	250	350	450	181
0.150 / 0.180 / 0.240 / 0.500	0	200	500	700	900	181
0.500	0	200	500	700	900	90
	0	200	500	700	900	45

Sample a: only Buffer A

BSA – Gallic acid, pH 3.5

Eluent A : 0.05 M Na-acetate Buffer, pH 3.5
Eluent B: : 0.5 mM gallic acid dissolved in 0.05 M Na-acetate Buffer, pH 3.5
Elution : isocratic, with different proportions of Eluent B
Column : Bio-Rad P6 cartridge
Injection volume : 20 µl
Flow rate : 1 ml/min
Sample : Protein or Protein-Phenol-mixture
Detector : DAD

BSA - SL : 60 g/l, solubilized in 0.05 M Na-acetate Buffer, pH 3.5
Gallic acid - SL : 0.5 mM gallic acid dissolved in 0.05 M Na-acetate Buffer, pH 3.5
1 mM gallic acid dissolved in 0.05 M Na-acetate Buffer, pH 3.5

Table 5: Composition of the samples for BSA-gallic acid (pH 3.5) in dependence of the gallic acid concentration of the eluent

$[GA_{Eluent}]$ mM	$[GA_{Probe}]$ [µM]					$[BSA_{Probe}]$ [µM]
	b	c	d	e	f	Samples b-f
0.025 / 0.050 / 0.075 / 0.100	0	100	250	350	450	90
0.125 / 0.150 / 0.175 / 0.200 / 0.225 / 0.250 / 0.275 / 0.300 / 0.400 / 0.500	0	200	500	700	900	90

Sample a: only Buffer A

BSA – Gallic acid, pH 4.8

Same conditions as pH 3.5, except pH 4.8 was applied and gallic acid – SL solution was 1 mM dissolved in 0.05 M Na-acetate Buffer

Table 6: Composition of the samples for BSA-gallic acid (pH 4.8) in dependence of the gallic acid concentration of the eluent

$[GA_{Eluent}]$ mM	$[GA_{Probe}]$ [µM]					$[BSA_{Probe}]$ [µM]
	b	c	d	e	f	Samples b-f
0.025 / 0.050 / 0.075 / 0.100 / 0.125 / 0.150 / 0.175 / 0.200 / 0.225 / 0.250 / 0.275 / 0.300 / 0.325 / 0.350 / 0.375 / 0.400 / 0.425 / 0.450 /0.475 / 0.500	0	200	500	700	900	90

Sample a: only Buffer A

BSA – Gallic acid. pH 6

Same conditions as pH 4.8, except pH 6 was applied

BSA-Rutin, pH 7, 1% DMSO

In order to retain rutin in a soluble form for at least 24 h, it was necessary to add 1 % DMSO to the buffer.

Eluent A : 0.05 M Hepes Buffer + 1% DMSO, pH 7
Eluent B: : 0.05 mM Rutin dissolved in 0.05 M Hepes Buffer + 1% DMSO, pH 7
Elution : isocratic, with different proportions of Eluent B
Column : Bio-Rad P6 cartridge
Injection volume : 50 µl
Flow rate : 1 ml/min
Sample : Protein or Protein-Phenol-mixture
Detector : DAD

BSA - SL : 12 g/l, dissolved in 0.05 M Hepes Buffer, pH 7
Rutin - SL : 0.05 mM Rutin dissolved in 0.05 M Hepes Buffer + 1% DMSO, pH 7
0.1 mM Rutin dissolved in 0.05 M Hepes Buffer + 1% DMSO, pH 7

Table 7: : Composition of the samples for BSA-rutin (pH 7) in dependence of the rutin concentration of the eluent

$[R_{Eluent}]$ µM	$[R_{Probe}]$ µM					$[BSA_{Probe}]$ µM
	b	c	d	e	f	Samples b-f
3 / 4.5 / 6 / 7.5 / 9 / 12	0	10	25	35	45	18.1
15 / 18 / 24 / 30 / 36 / 50	0	20	50	70	90	18.1
50	0	20	50	70	90	9.0
50	0	20	50	70	90	4.5

Sample a: only Buffer A

BSA-Isoquercetin, pH 7, 1% DMSO

In order to retain isoquercetin in a soluble form for at least 24 h, it was necessary to add 1 % DMSO to the buffer; otherwise same conditions as for rutin

BSA-Quercetin, pH 7, 20% Ethanol

In order to retain quercetin in a soluble form for at least 24 h, it was necessary to add 20 % ethanol to the buffer. Excepting for the buffer containing ethanol and quercetin, all other conditions were same as for rutin.

Determination of ferulic acid bound

Protein mix – physiological conditions

Eluent A :	: 0.24mM ferulic acid dissolved in 0.067 M Na-phosphate – Buffer ($HPO_4^{2-}/H_2PO_4^-$), pH 7.4	
Elution	: isocratic, with 100% Eluent A	
Column	: Bio-Rad P6 cartridge	
Injection volume	: 30 µl	
Flow rate	: 0.8 ml/min	
Sample	: Different proteins	
Detector	: DAD	
Protein / Concentration in Sample	: Gelatin	12 g/l
	Milk whey proteins	12 g/l
	Soy glycinin	12 g/l
	α – Amylase	12 g/l
	Lysozyme	12 g/l
	BSA	12 g/l
	HSA 1	12 g/l
	HSA 2	7.71 g/l
	Serum (N Protein standard SL)	12 g/l Total protein content

Protein mix – conditions as found in many food systems

Eluent A :	: 0.24mM ferulic acid dissolved in 0.05 M Hepes-Buffer. pH 4.8	
Elution	: isocratic, with 100% Eluent A	
Column	: Bio-Rad P6 cartridge	
Injection volume	: 30 µl	
Flow rate	: 0.8 ml/min	
Sample	: Different proteins	
Detector	: DAD	
Protein / Concentration in Sample	: Gelatin	12 g/l
	Milk whey protein	12 g/l
	Soy glycinin	12 g/l
	α – Amylase	12 g/l
	Lysozyme	12 g/l
	BSA	12 g/l
	HSA 1	12 g/l
	HSA 2	7.71 g/l

Supporting Information III

Theory to calculation of the binding parameters

Considering the interactions between protein and phenol as a bimolecular association and phenol as the Ligand = L, than a single step thermodynamic equilibrium according to the following scheme can be described:

Plant phenol (L) + Protein (P) ↔ Plant phenol-protein-complex (PL)

The association- or dissociation constants (K_A or K_D) for this equilibrium are than defined by the equation 1:

Equation 1
$$K_A = \frac{1}{K_D} = \frac{[PL]}{[L] \cdot [P]}$$

If the specific binding B (equation 2) is defined as the number of moles of ligand bound per mol protein,

Equation 2
$$B = \frac{[PL]}{[P_{total}]}$$

than since $[P_{total}] = [PL] + [P]$ we get following equation:

Equation 3
$$B = \frac{[PL]}{[P] + [PL]}$$

By substitution of equation 1 in equation 3, we get:

Equation 4
$$B = \frac{[L]}{K_D(1 + 1/K_D[L])} = \frac{[L]}{K_D + [L]}$$

In equation 4, attention is drawn to [L], which means here the free concentration of the plant phenol. Since the plant phenols are small molecules (180 - 700 Da) and proteins comparatively very large (14 000 - 350 000 Da), it is possible that more than one phenol molecule can bind to one protein molecule. Further, taking in account that the phenol is bound at specific binding sites, a new variable n for the number of such sites is needed:

Equation 5
$$B = n \cdot \frac{[L]}{K_D \cdot [L]}$$

Equation 5 applies only under those conditions, when the phenol binds to all the binding sites with the same affinity. In case more than one class of binding sites are possible, than we have:

Equation 6
$$B = \sum_{i=1}^{z} \frac{n_i \cdot [L]}{K_{D_i} + [L]}$$

Where variable z is the number of the classes of specific binding sites. For every class, a particular number of binding sites n is possible for which in turn a specific dissociation constant K_D applies.
Equation 6 also has a drawback, since it applies only under those conditions, where the binding sites do not interact with one another. But it is possible that the binding of a phenol

molecule may influence the binding of further molecules. In this case, we have cooperative binding mechanism. A positive cooperative binding mechanism is observed, if the binding of one molecule promotes the binding of further molecules: $P + nL \leftrightarrow PL_n$
A negative binding mechanism occurs in case the binding of further molecules is prevented. The dissociation constant can be calculated as follows:

Equation 7

$$K_D = \frac{[P] \cdot [L]^n}{[PL_x]^n}$$

And the number of binding sites with:

Equation 8

$$B = \sum_{i=1}^{z} \frac{n_i \cdot [L]^n}{K_{D_i}{}^n + [L]^n}$$

A further magnitude for binding equilibrium experiments is given by the standard Gibbs free energy (ΔG):

Equation 9

$$\Delta G = -R \cdot T \cdot \ln(K_X)$$

The equilibrium constant K_X in this equation depending on the mol fractions X and considers the presence of water and the hydration shell of the proteins as per:

$Protein(H_2O)_n + Ligand \leftrightarrow Protein\text{-}Ligand + n\ H_2O$

As a result K_X is defined as:

Equation 10

$$K_X = \frac{X(PL) \cdot X^n(H_2O)}{X(P(H_2O)_n) \cdot X(L)} \qquad X(n) = \frac{n_i}{\sum_{i=1}^{i} n}$$

Since was the major solvent, it is present in surplus and as result the mol fraction for water is 1. Therefore Σn is equal to $n(H_2O)$. This simplification leads to constant K_X^{eff}:

Equation 11

$$n(H_2O) \cdot K_X^{eff} = \frac{n(PL)}{n(P) \cdot n(L)}$$

Or to:

Equation 12

$$[H_2O] \cdot K_X^{eff} = \frac{[PL]}{[P] \cdot [L]}$$

The left side of the equation represents the association constant K_A (Equation 1). There for the standard Gibbs free energy (ΔG) can be calculated as follows:

Equation 13

$$\Delta G = -R \cdot T \cdot \ln\left(\frac{K_A}{[H_2O]}\right)$$

The calculation of the concentration of water is possible with the molecular weight (M = 18 g/mol) and the density ρ (ρ = 1 g/l) and is equal to 0.056 mmol/l.

The best-fit values for binding parameters were achieved by applying non-linear least-squares regression using the software: Microcal Origin 6.0 (Microcal Software Inc., Northampton, USA) according to (equation 14 for 1 set of binding sites and equation 15 in case of two sets of binding sites):

Equation 14

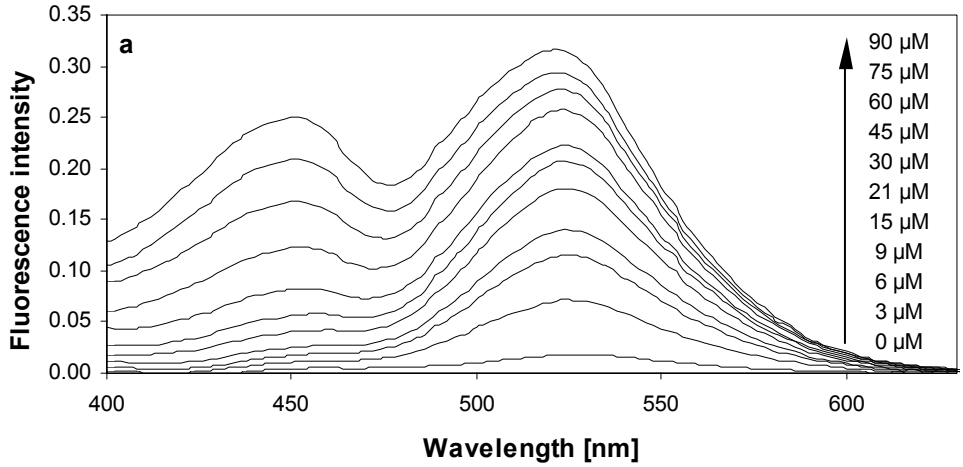
$$B = \frac{n \cdot L}{K_d + L}$$

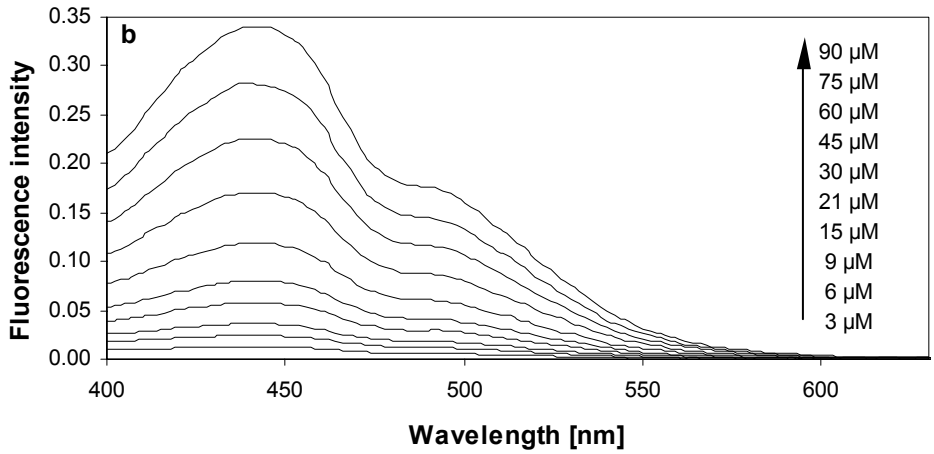
Equation 15

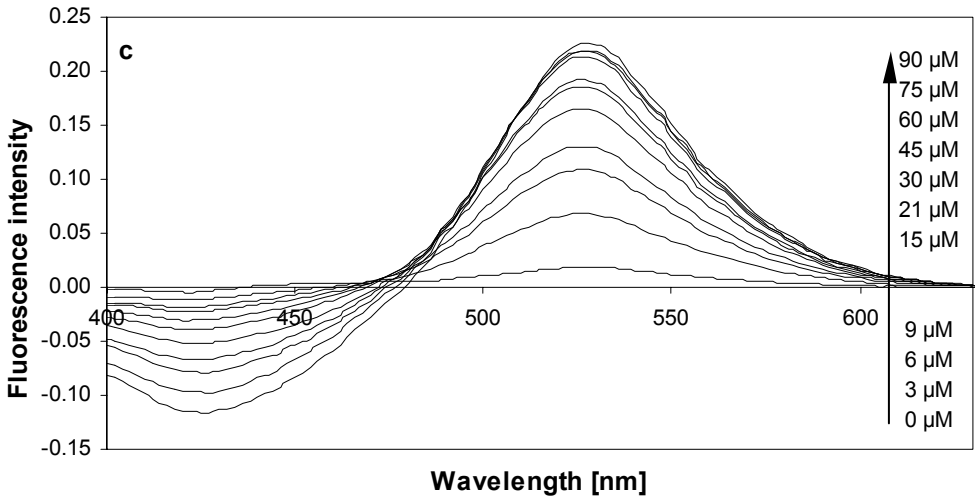
$$B = \frac{n_1 \cdot L}{K_{d1} + L} + \frac{n_2 \cdot L}{K_{d2} + L}$$

Supporting information IV

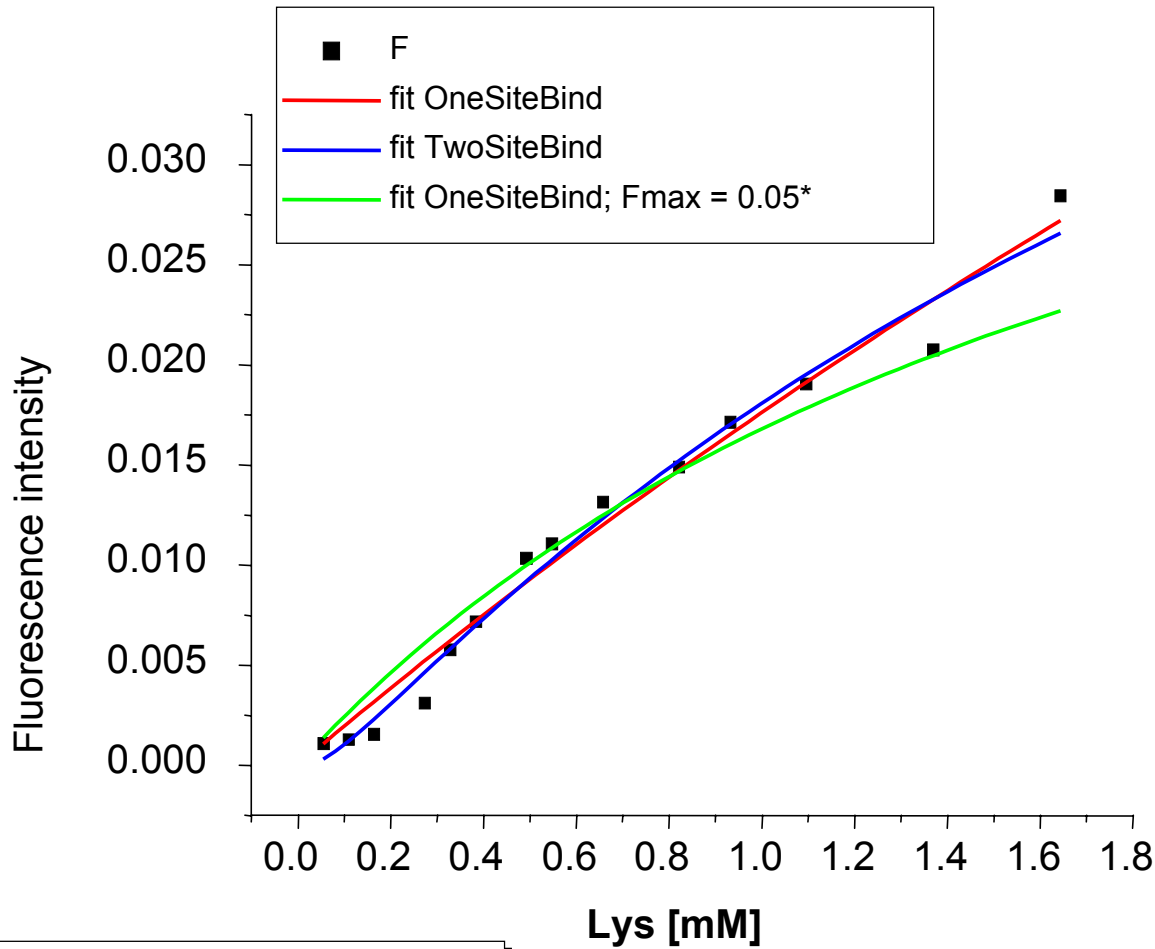
Fluorescence emission spectra in 0.05 M Hepes-buffer, pH 7, λ_{ex} = 370 nm. a: Change in quercetin fluorescence on addition to BSA (0-95 µM) b: Fluorescence of corresponding BSA-concentrations alone c: subtraction of the BSA – Fluorescence „b“ from that of the „a“.







Determination of K_D using enhancement of quercetin fluorescence when bound to lysozyme. [Quercetin] = 15 µM; [lysozyme] = 0-1.8 mM; model = one site binding.



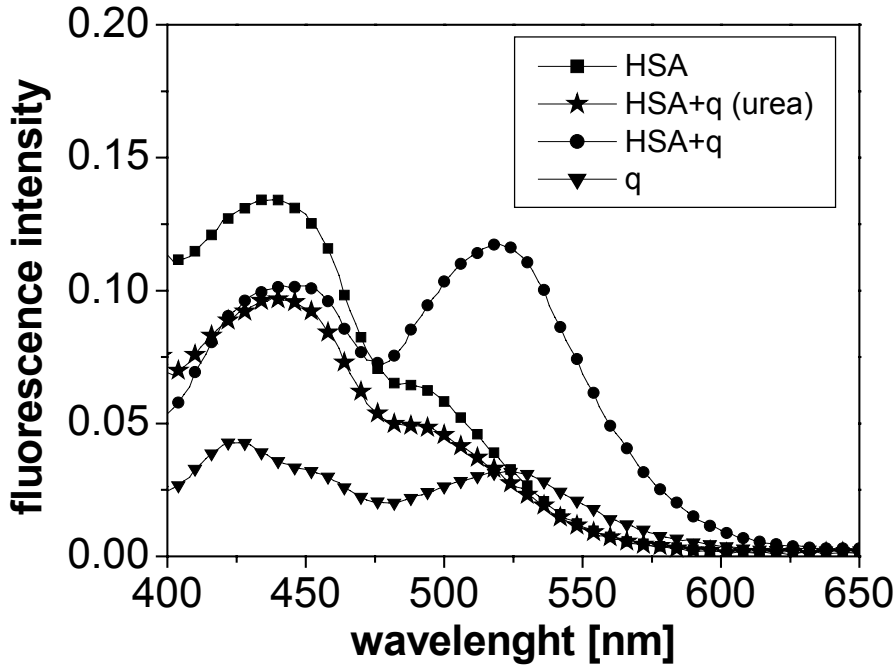
Data: DataLys_F
Model: OneSiteBind

Chi^2 = 2.0237E-6
R^2 = 0.9618

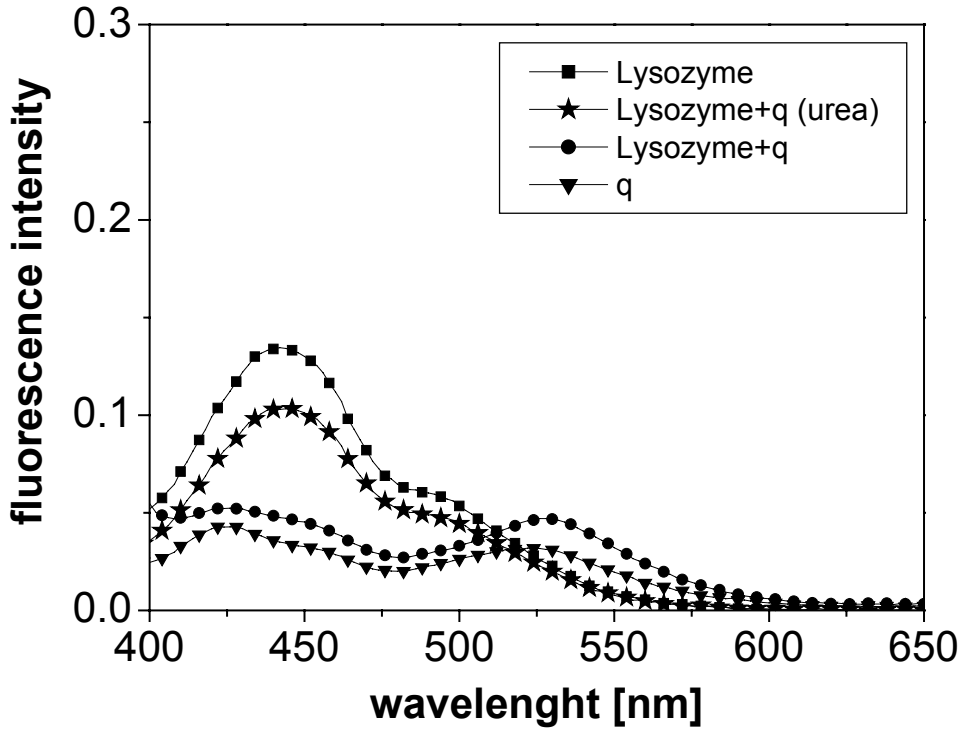
Bmax 0.05 ±0.01366
k1 1.97718 ±0.77246 mM

* the last data point was omitted, when applying this fit

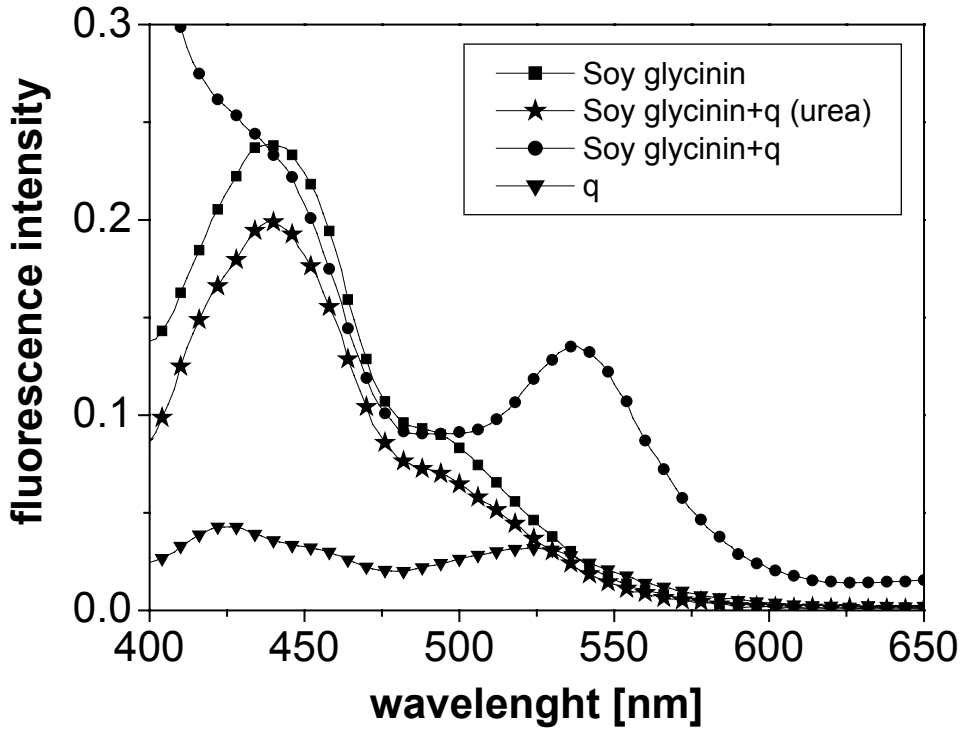
Effect of urea on the quercetin (q) fluorescence – confirmation of the non-covalent nature of the binding to protein
[Quercetin] = 15 μM; [HSA] = 30 μM.



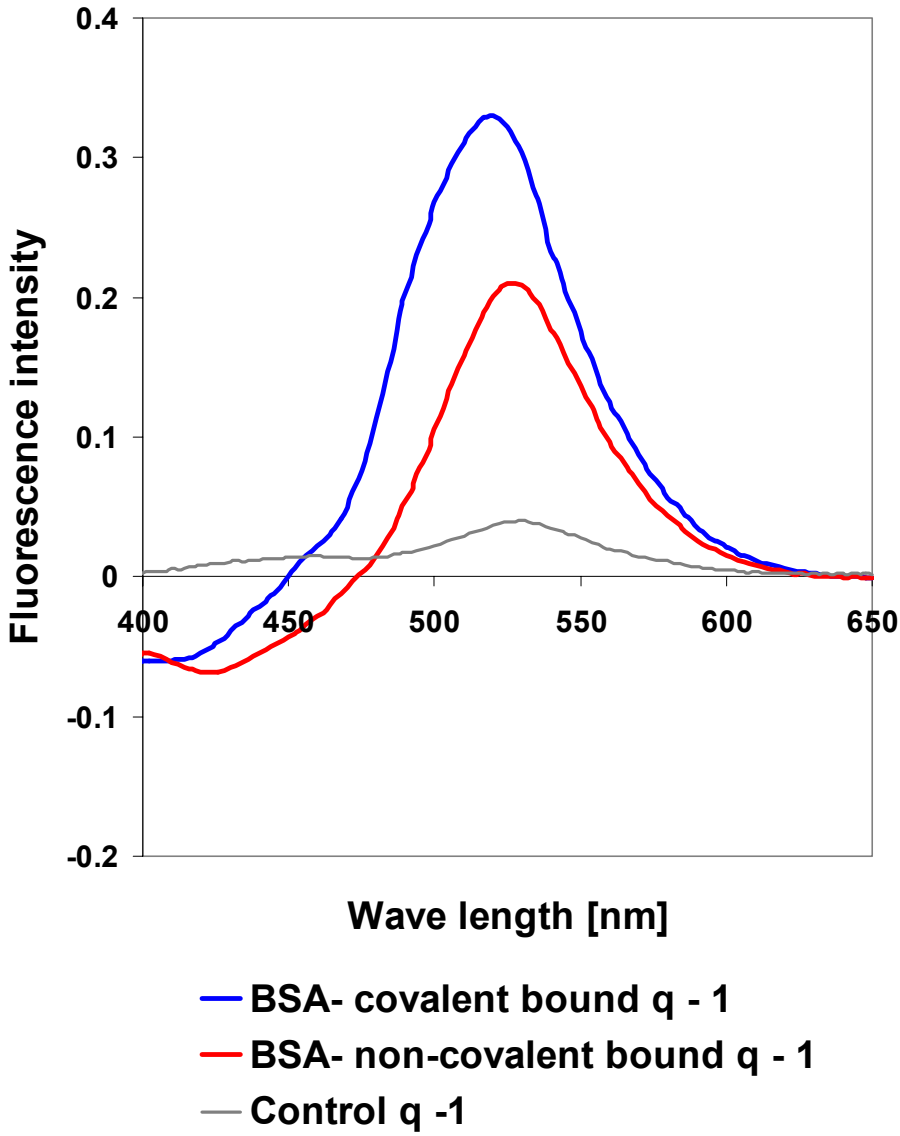
Effect of urea on the quercetin (q) fluorescence – confirmation of the non-covalent nature of the binding to protein
[Quercetin] = 15 µM; [Lysozyme] = 0.66 mM.



Effect of urea on the quercetin (q) fluorescence – confirmation of the non-covalent nature of the binding to protein
[Quercetin] = 15 μM; [Soy glycinin] = 6.98 μM.

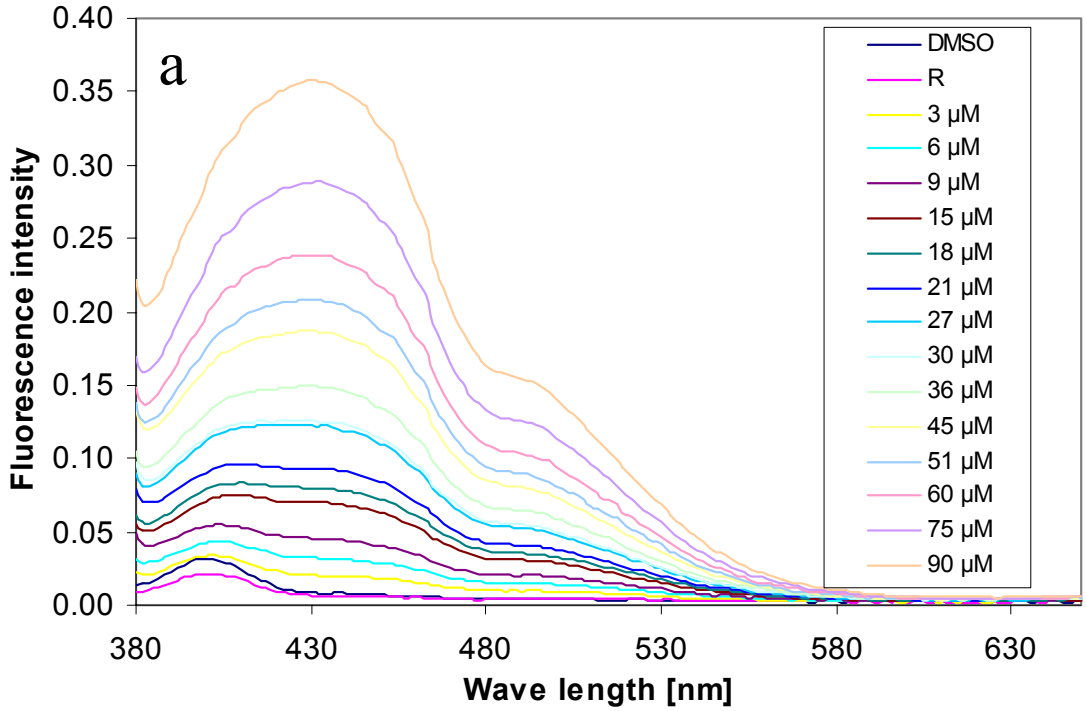


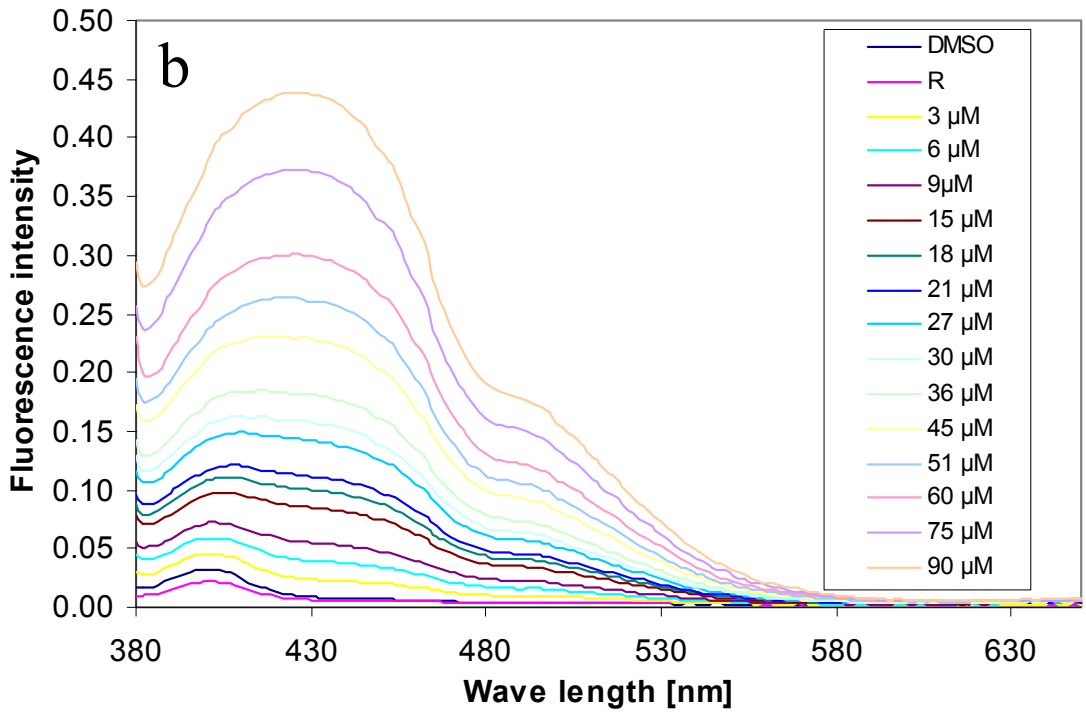
Resonance-Energy-Transfer Comparison of the covalent and non-covalent bound quercetin to BSA*



Quercetin was bound covalently as described in:
Rohn, S.; Rawel, H. M.; Kroll, J. Antioxidant activity of protein-bound quercetin. J Agric Food Chem 2004, 52, 4725-4729.
Whereby, q – 1 = [7.9 µg quercetin / mg protein];
control q –1 is the corresponding curve for quercetin alone.

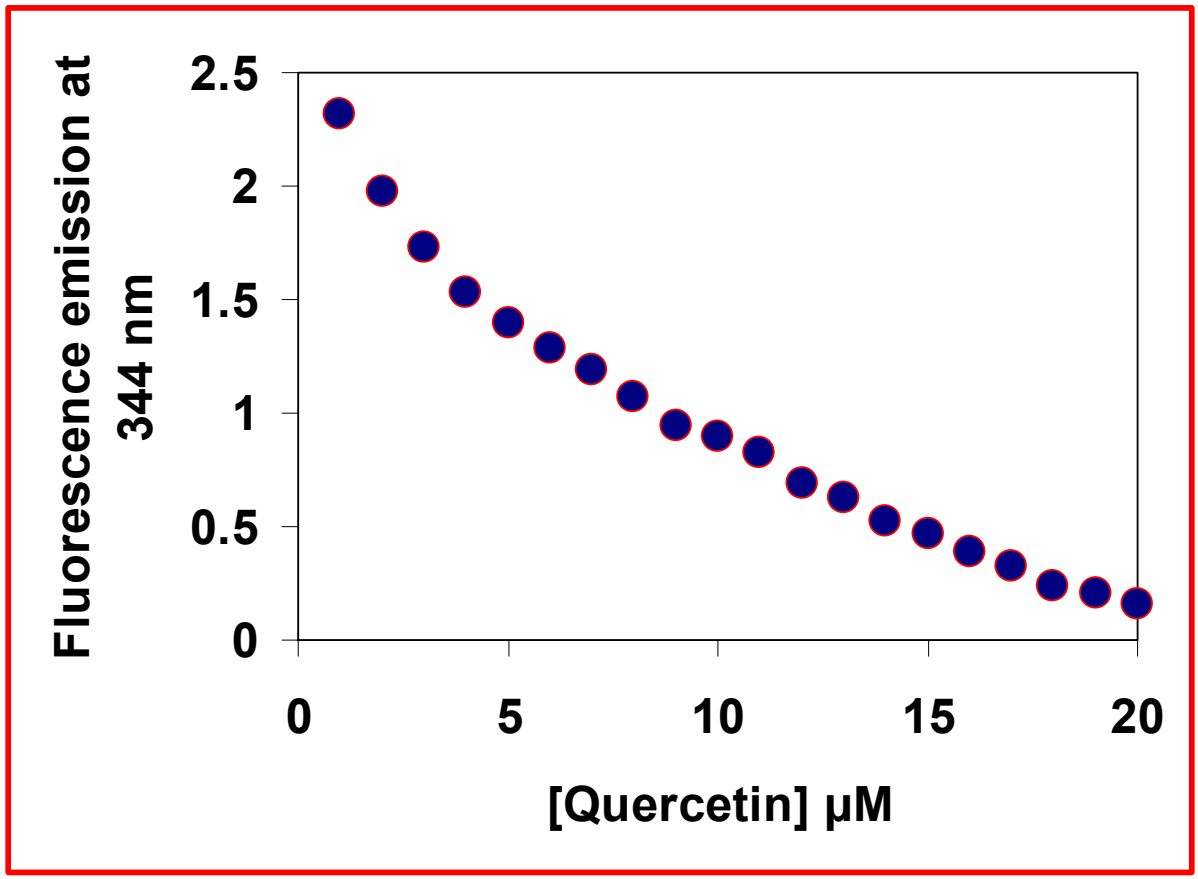
Fluorescence emission spectra in 0.05 M Hepes-buffer, pH 7, λ_{ex} = 354 nm. a: Change in rutin fluorescence on addition to BSA (0-95 µM) b: Fluorescence of corresponding BSA-concentrations alone; subtraction of the values of „b“ from that of the „a“ would give no net fluorescence enhancement





R = Rutin

Quenching of tryptophan fluorescence as induced by the binding of quercetin to BSA in 0.05 M Hepes-buffer, pH 7, λ_{ex} = 290 nm; λ_{em} = 344 nm



Assignment [%] of the secondary structures according to Sreerama & Woody (2000) by applying 48-protein reference set (190-240 nm) for addition of different concentrations of (a) quercetin, (b) rutin, and (c) isoquercetin to BSA using 1 = CONTIN/LL, 2 = SELCON3 and 3 = CDSSTR Methods. „Control“ samples were BSA + solvent (ethanol or ethanol:water - 1:1). The concentration of the reactants were: [BSA] = 3 µM and [Flavonol] = 15 (1:5) – 75 (1:25) µM.

(a) Quercetin	α-Helix			β-Strand			β-Turn			Unordered		
	1	2	3	1	2	3	1	2	3	1	2	3
BSA	64.8	61.9	67.1	2.8	5.5	4.2	12.2	11.0	9.8	20.3	22.1	18.8
B-Q 1:5	64.5	61.6	68.2	3.0	6.4	3.8	12.6	11.5	9.1	20.0	22.4	18.8
B-Q 1:10	64.4	59.3	73.6	3.4	6.6	3.2	13.3	12.9	8.1	18.9	23.8	15.1
B-Q 1:15	65.5	59.1	74.5	3.0	6.7	4.2	12.4	12.8	7.0	19.2	23.8	14.6
B-Q 1:20	65.0	59.1	73.7	2.7	6.6	4.9	12.4	12.9	9.4	19.8	23.8	13.0
B-Q 1:25	66.2	59.3	75.3	3.2	6.4	3.9	12.6	12.7	6.2	18.0	23.8	15.5
Control 1:25	65.8	60.7	75.4	3.4	5.7	3.1	12.0	12.3	7.5	18.8	23.0	14.3

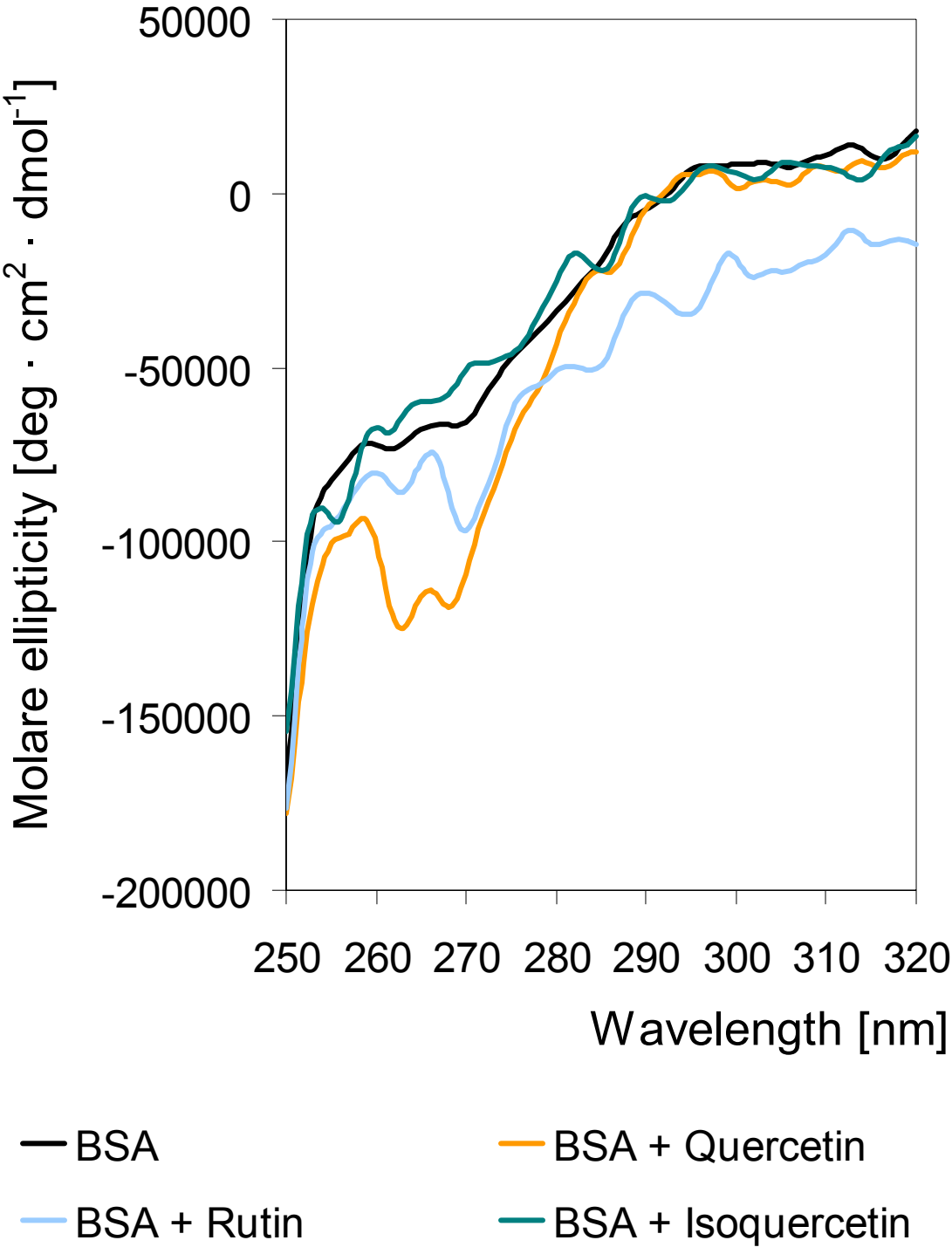
(b) Rutin	α-Helix			β-Strand			β-Turn			Unordered		
	1	2	3	1	2	3	1	2	3	1	2	3
BSA	64.8	61.9	67.1	2.8	5.5	4.2	12.2	11.0	9.8	20.3	22.1	18.8
B-R 1:5	66.6	66.6	68.7	3.1	3.1	4.1	12.3	12.3	9.0	18.1	18.1	18.0
B-R 1:10	62.7	62.8	68.1	4.4	4.9	5.1	13.4	11.3	8.7	19.5	21.8	18.4
B-R 1:15	64.0	62.8	68.5	4.2	4.9	4.7	13.6	11.4	9.4	18.3	21.5	17.5
B-R 1:20	64.8	64.2	69.8	4.0	4.9	4.8	11.9	11.0	8.1	19.4	22.0	17.0
B-R 1:25	66.1	63.5	68.7	3.7	4.8	4.1	13.0	11.1	8.9	17.1	21.4	18.2
Control 1:25	67.2	63.9	68.8	2.7	5.2	4.7	12.0	11.3	11.1	18.1	21.1	21.4

(c) Isoquercetin	α-Helix			β-Strand			β-Turn			Unordered		
	1	2	3	1	2	3	1	2	3	1	2	3
BSA	64.8	61.9	67.1	2.8	5.5	4.2	12.2	11.0	9.8	20.3	22.1	18.8
B-I 1:5	65.1	62.1	67.1	2.7	5.4	4.2	12.2	11.1	9.5	20.1	22.3	19.2
B-I 1:10	64.1	61.1	66.3	2.7	5.7	4.5	12.2	11.2	10.0	21.0	22.4	19.1
B-I 1:15	64.3	60.9	66.7	2.7	5.8	4.6	12.8	11.2	9.8	20.2	22.3	19.1
B-I 1:20	65.4	61.9	66.3	2.4	5.7	4.9	12.1	11.2	10.5	20.2	22.2	18.5
B-I 1:25	63.6	60.1	65.9	3.2	6.1	5.0	13.0	11.2	9.8	20.2	22.4	19.8
Control 1:25	64.7	62.3	67.5	3.4	5.5	4.4	12.2	10.9	9.6	19.6	22.0	18.5

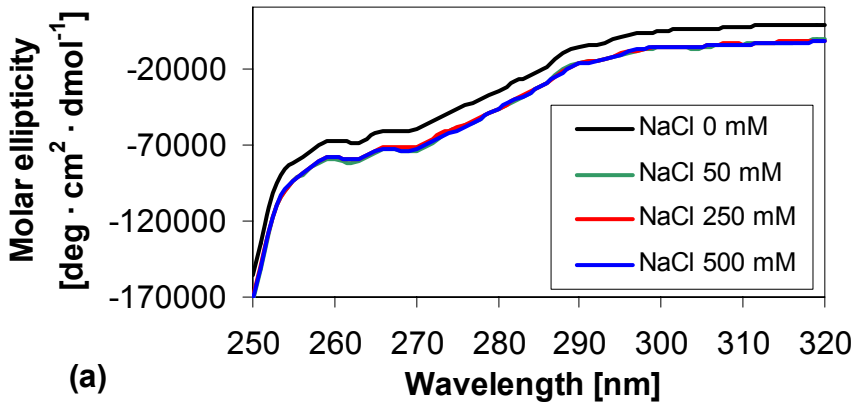
(1) Sreerama, N.; Woody, R. W. Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. Anal. Biochem. 2000, 287, 252-260.

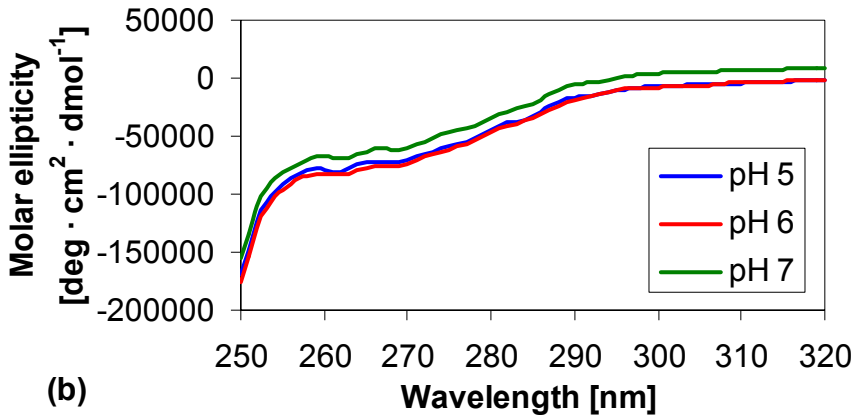
(2) Sreerama, N.; Venyaminov, S. Y.; Woody, R. W. Estimation of protein secondary structure from circular dichroism spectra: inclusion of denatured proteins with native proteins in the analysis. *Anal. Biochem.* **2000**, *287*, 243-251.

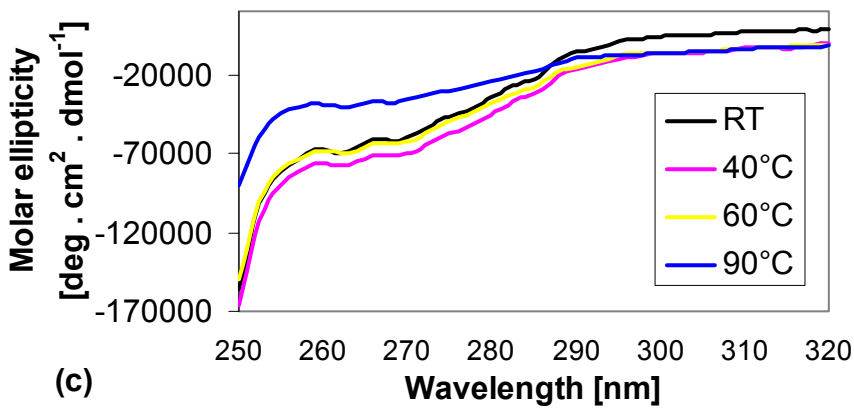
Near-UV-CD spectra of BSA (3 µM) in presence of phenolic compounds (90 µM).



Near-UV-CD spectra of BSA (3 µM) depending on buffer composition and temperature







RT = Room temperature