Recent Trends in Lipid Oxidation and Antioxidants in Omega-3 PUFA Rich Food Emulsions

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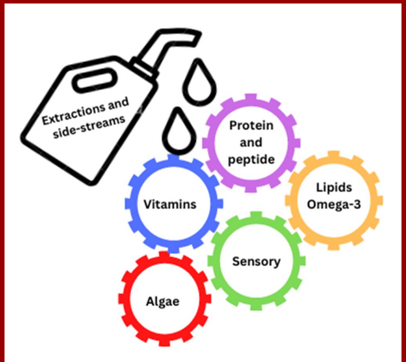
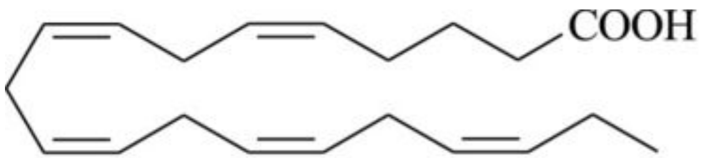


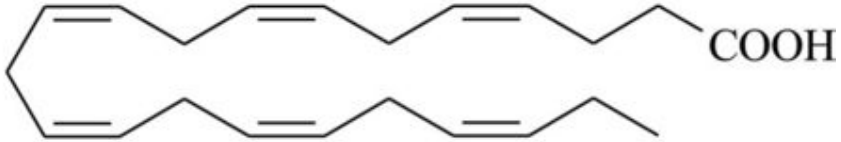
Outline

- Introduction to omega-3 PUFA
- Introduction to lipid oxidation
- Factors affecting lipid oxidation in food emulsions
  – Role of interface and emulsifier
- Peptides and protein hydrolysates as antioxidants – a new approach
- Effect of peptides in emulsions at different pH
- Screening of metal chelating peptides
- Conclusions

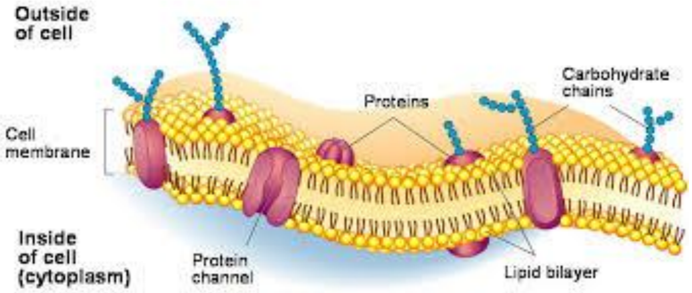
Health effects of omega-3 PUFA



Eicosapentaenoic acid
(EPA, $C_{20}H_{30}O_2$)

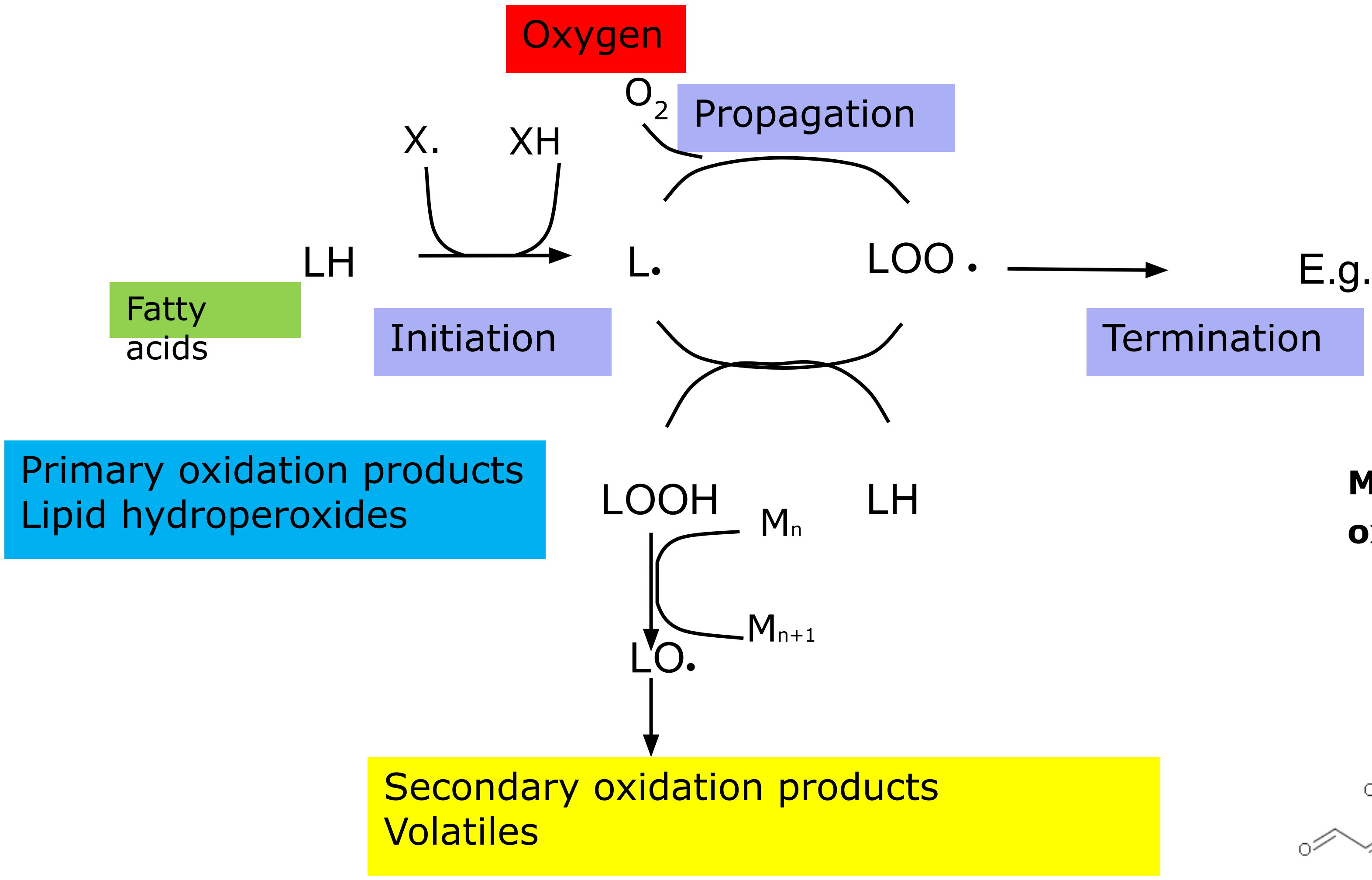


Docosahexaenoic acid
(DHA, $C_{22}H_{32}O_2$)

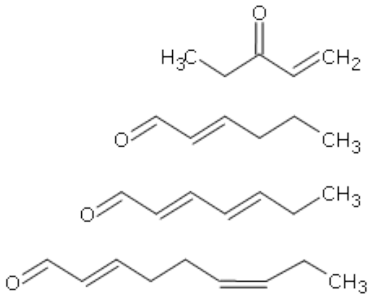


Prof. Calder: "We may need at least 1 g/day of EPA and DHA"
European Lipid Science Award lecture 2021

Lipid oxidation reactions: The basic reactions



Markers for n-3 PUFA oxidation (e.g.)



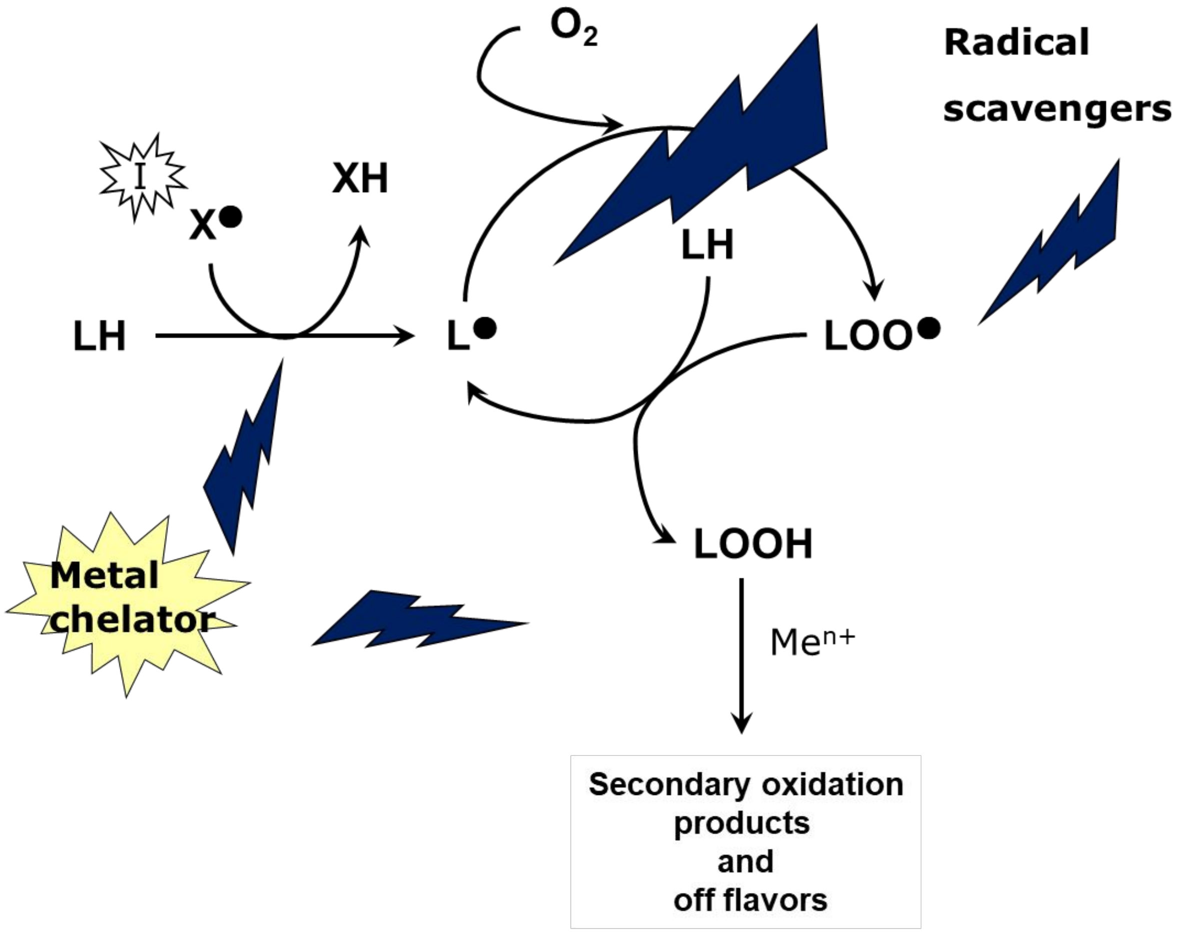
1-penten-3-one

(E)-2-hexenal

(E,E)-2,4-heptadienal

(E,Z)-2,6-nonadienal

Antioxidant mechanisms



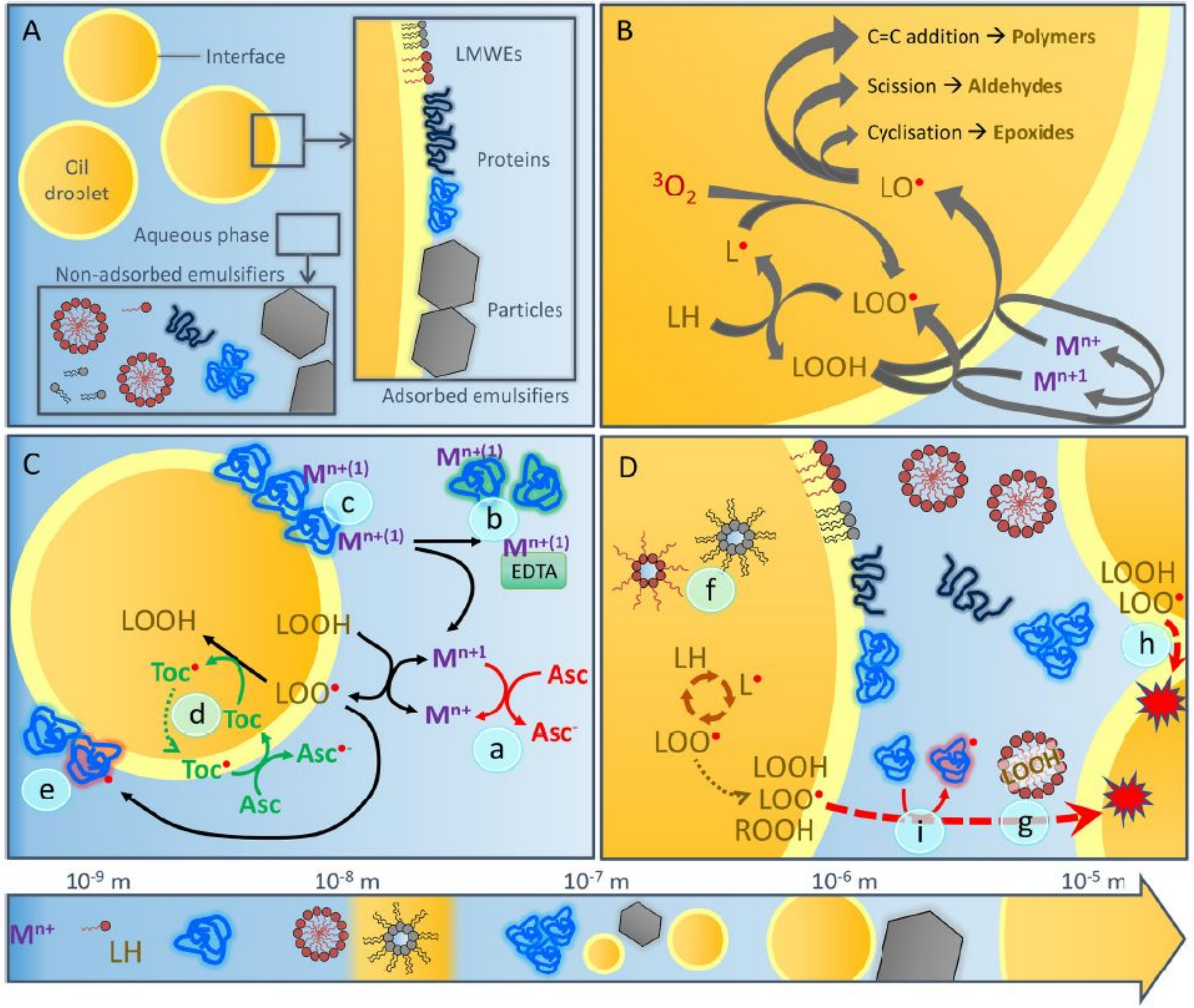


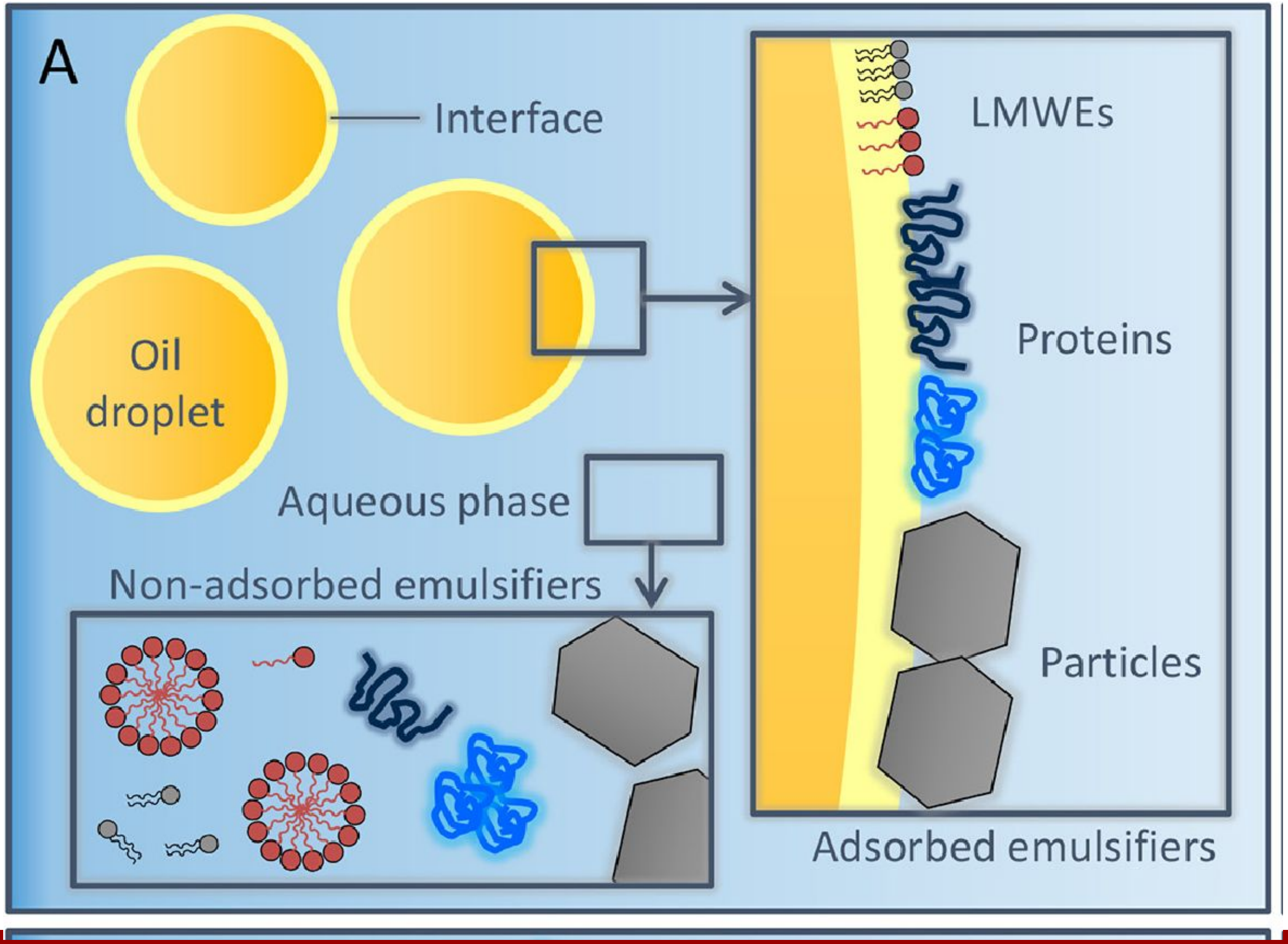
Fig. 1. (A) General structure of an O/W emulsion and of various types of food emulsifiers. (B) Simplified overview of dominant chemical pathways of lipid oxidation in emulsions, with an emphasis on the redox cycling engine involving transition metals (reduced form M^{n+}, oxidized form M^{n+1}). (C) Pro- (red) and antioxidant (green) mechanisms as influenced by the surface of oil droplets. (D) Possible transport routes (red arrows) of lipid oxidation intermediates (e.g., lipid oxidation products bearing a hydroperoxide group) between emulsion droplets. LH, unsaturated lipid; L•, alkyl radical; LOO•, peroxyl radical; LOOH, lipid hydroperoxide; LO•, alkoxyl radical; ROOH, molecule bearing a hydroperoxide group (e.g., hydroperoxy-alkenal); Asc, ascorbate; Toc, tocopherol. The letters (a) to (i) point to mechanisms referred to and further explained in the text. The large arrow at the bottom depicts typical dimensions (m) of the molecules and structures discussed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Aspects of lipid oxidation in emulsions

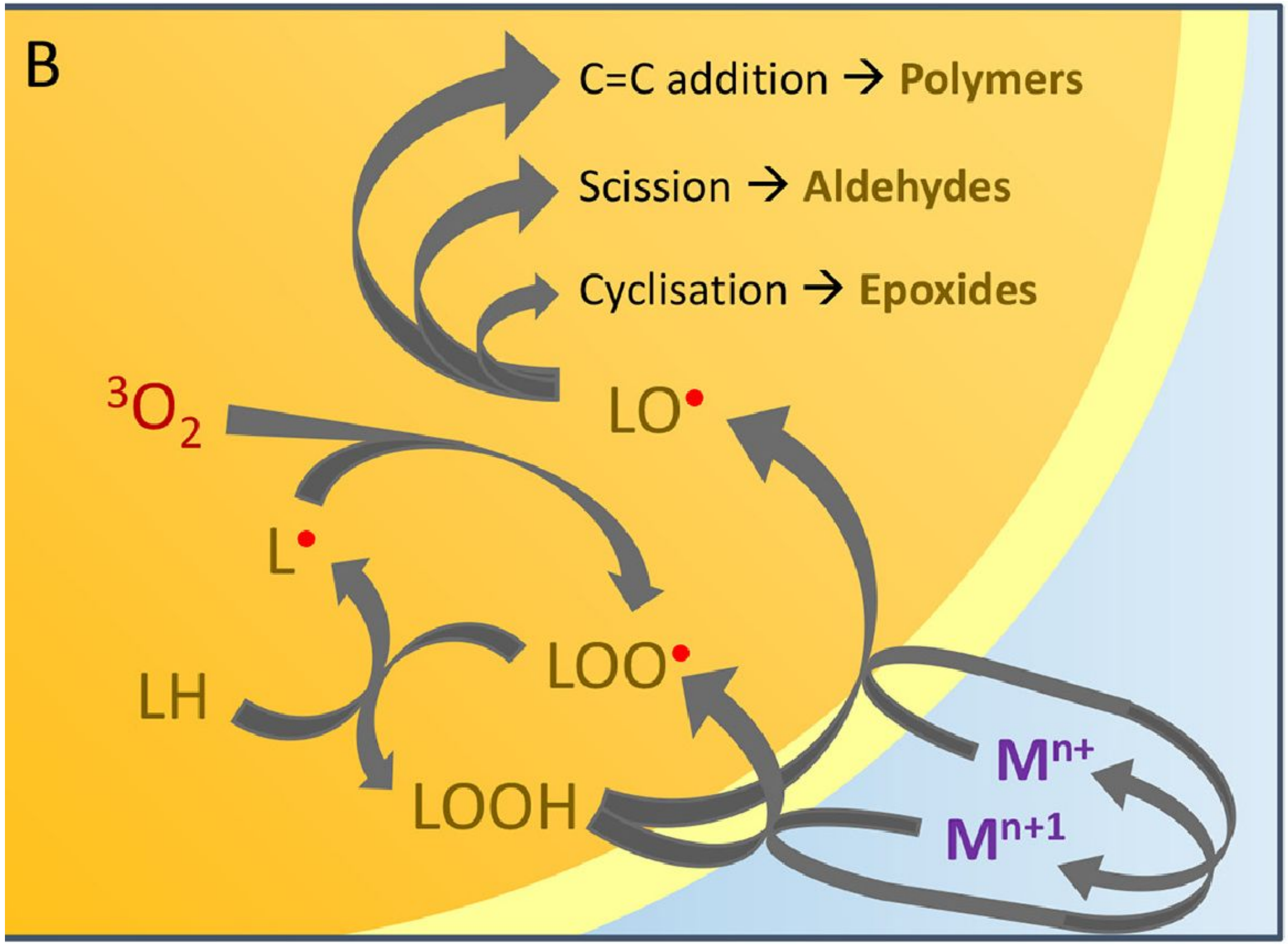
Berton-Carabin, C., Villeneuve; P, Durand, E., Lecomte, J., van Duynhoven, J., Meynier; A., Yesiltas, B., Jacobsen, C., Hennebelle, M. (2024).

Lipid oxidation in emulsions: New insights from the past two decades. Progress Lipid Res. 94, 101275

Structure of emulsions and types of emulsifiers



Location of chemical pathways of lipid oxidation in emulsions



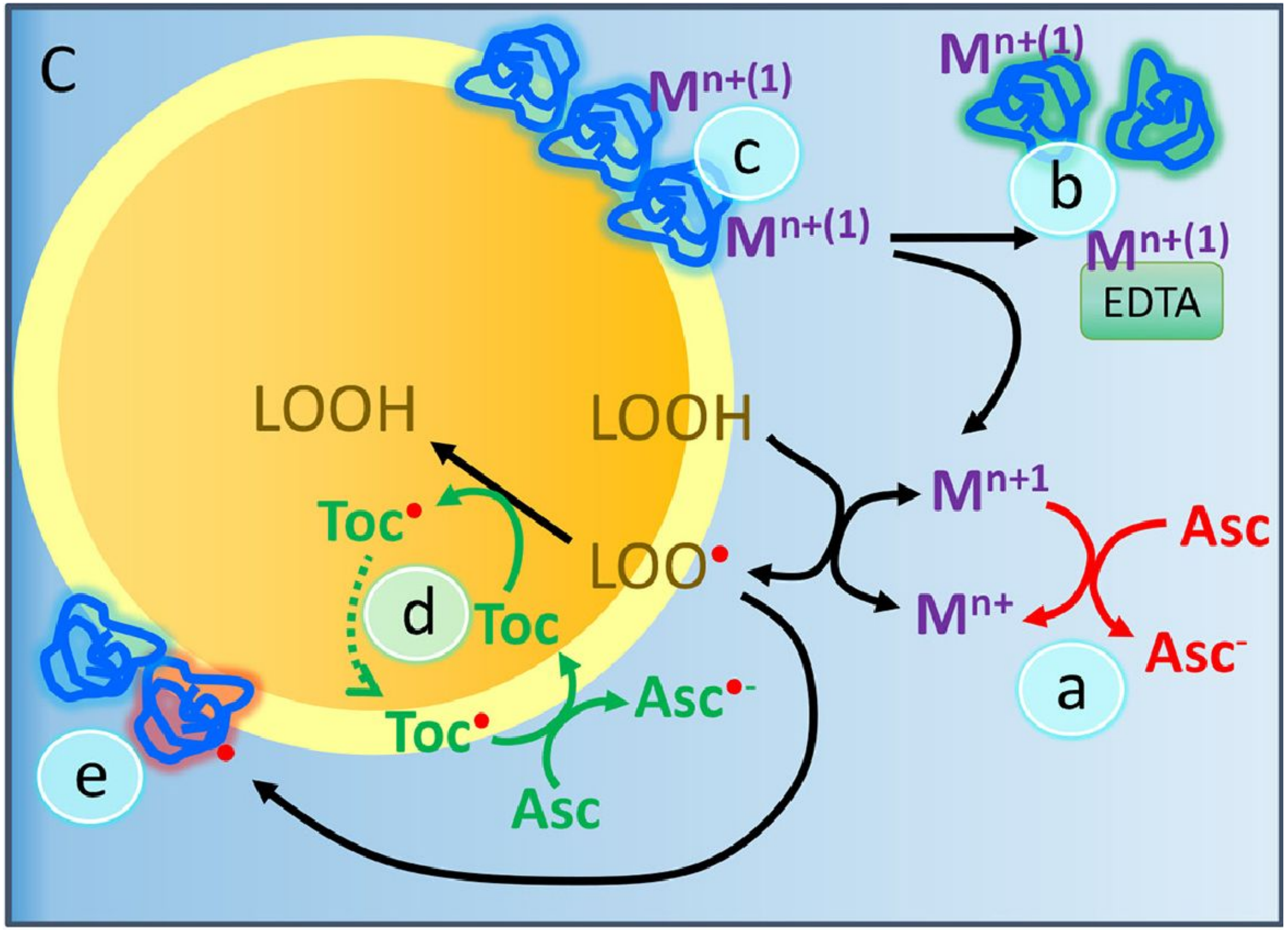
Pro- (red) and antioxidant (green) mechanisms as influenced by the surface of oil droplets

a: Reduction of metal ions by Asc

b and c: Binding of metal ions at the interface or in the aqueous phase

d: Tocopherol as an antioxidant and regeneration by Asc

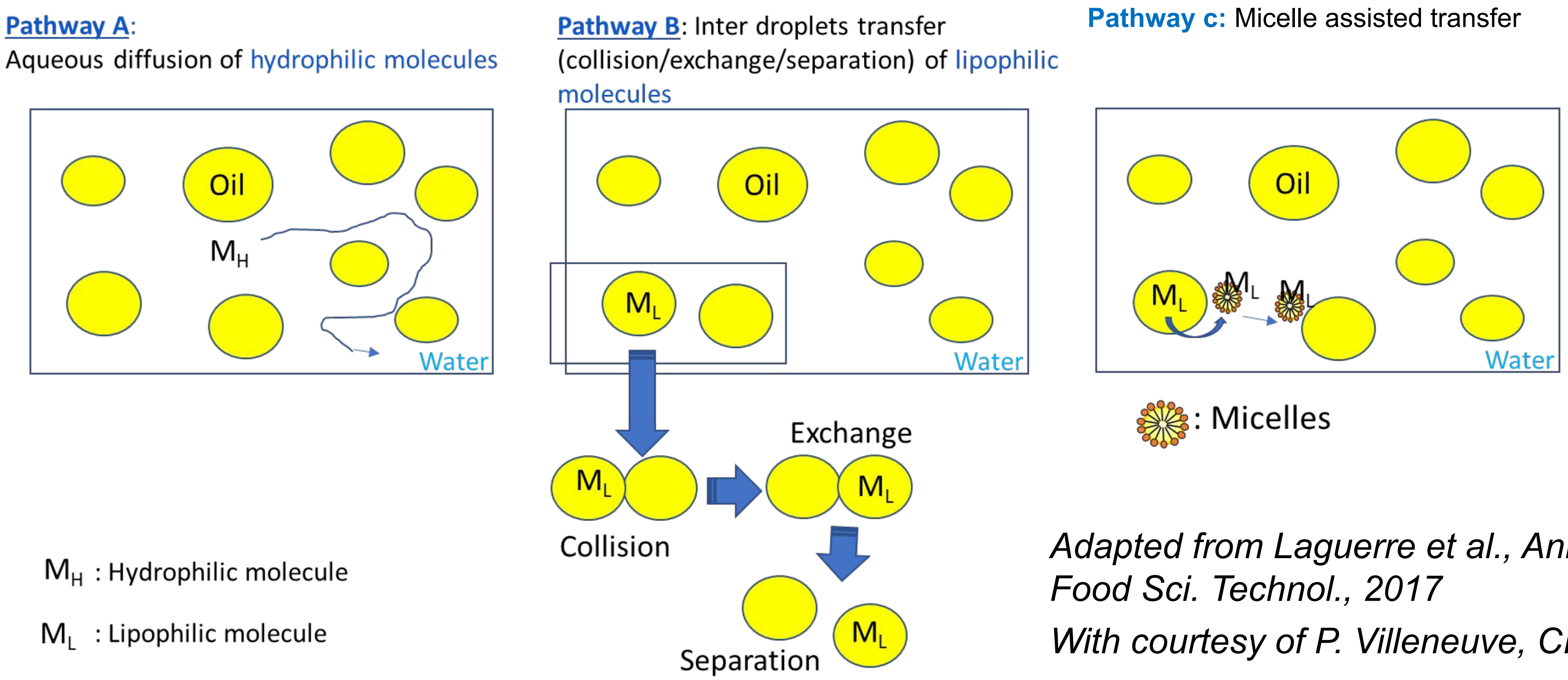
e: Co-oxidation of proteins



The role of mass transport in lipid oxidation

How does lipid oxidation spread within an emulsion ? How do lipid oxidation reactants transfer from lipid droplets to lipid droplets ?

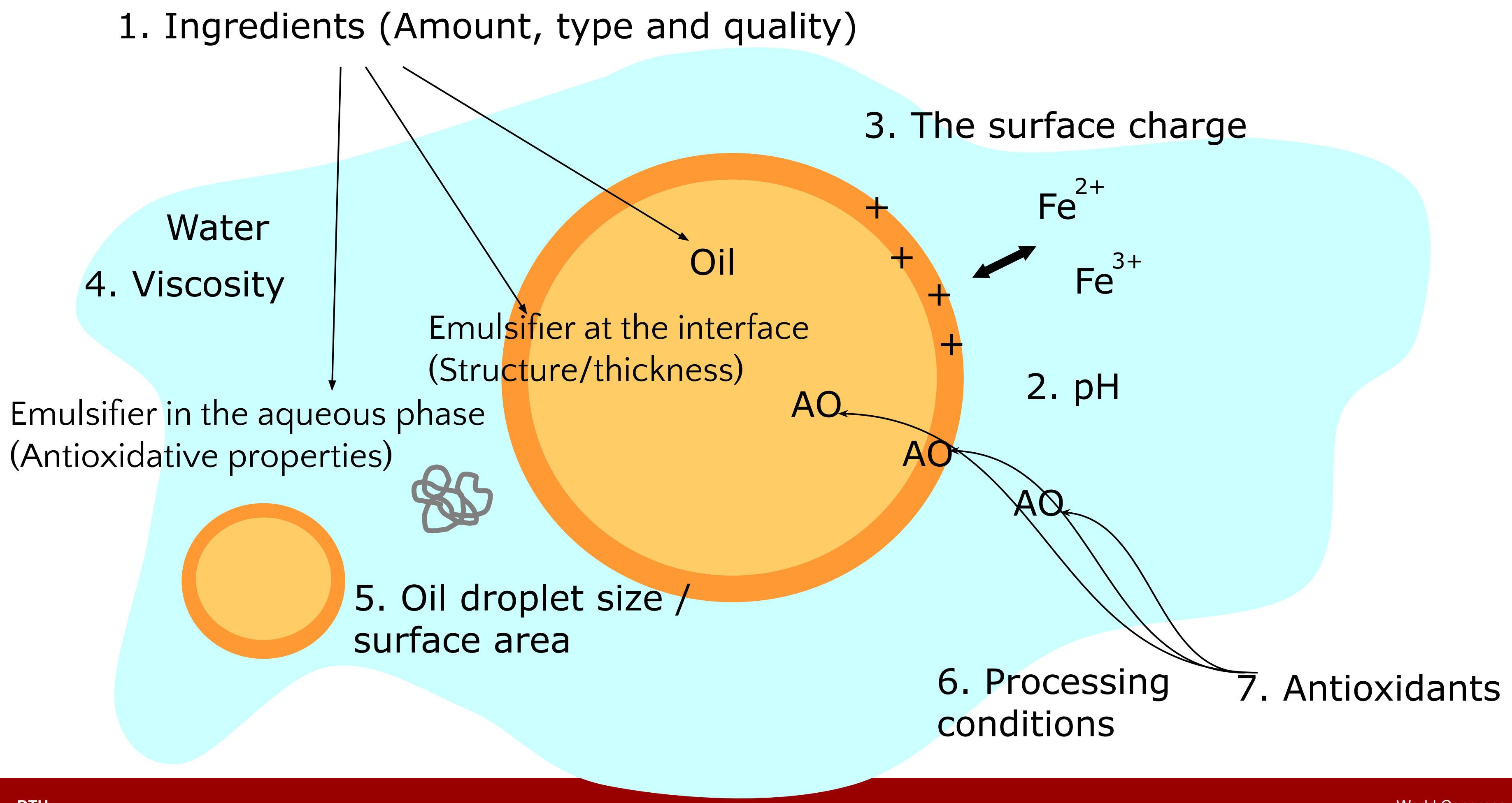
MASS TRANSPORT PATHWAYS



Adapted from Laguerre et al., Annual Rev. Food Sci. Technol., 2017

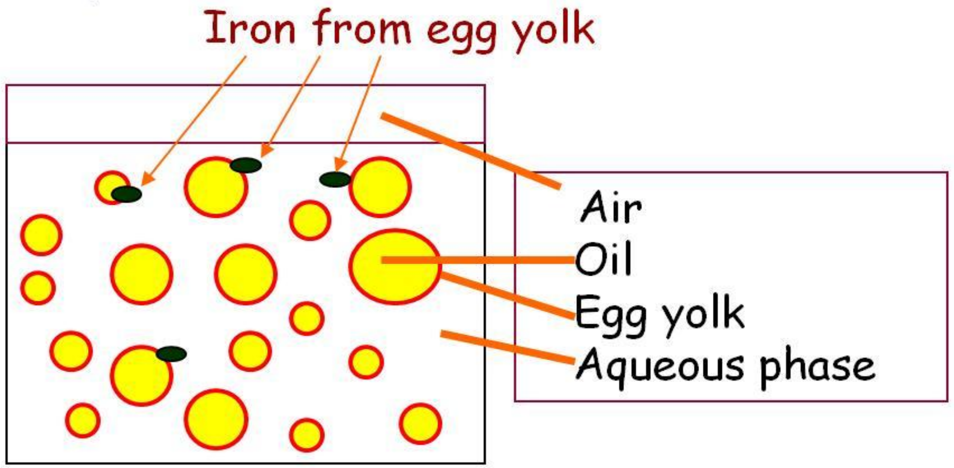
With courtesy of P. Villeneuve, CIRAD

Factors that can affect lipid oxidation in emulsified foods:



Oxidation mechanisms in mayonnaise

Mayonnaise with pH < 4



Fe in egg yolk is bound to phosvitin and lipovitilin

- Low pH and iron from egg yolk cause oxidation in mayonnaise

EDTA is a strong antioxidant in mayonnaise because it can chelate metals and prevent oxidation from being initiated at the **interface**

Jacobsen, PhD thesis 1999

Further on the oxidation mechanism of mayonnaise

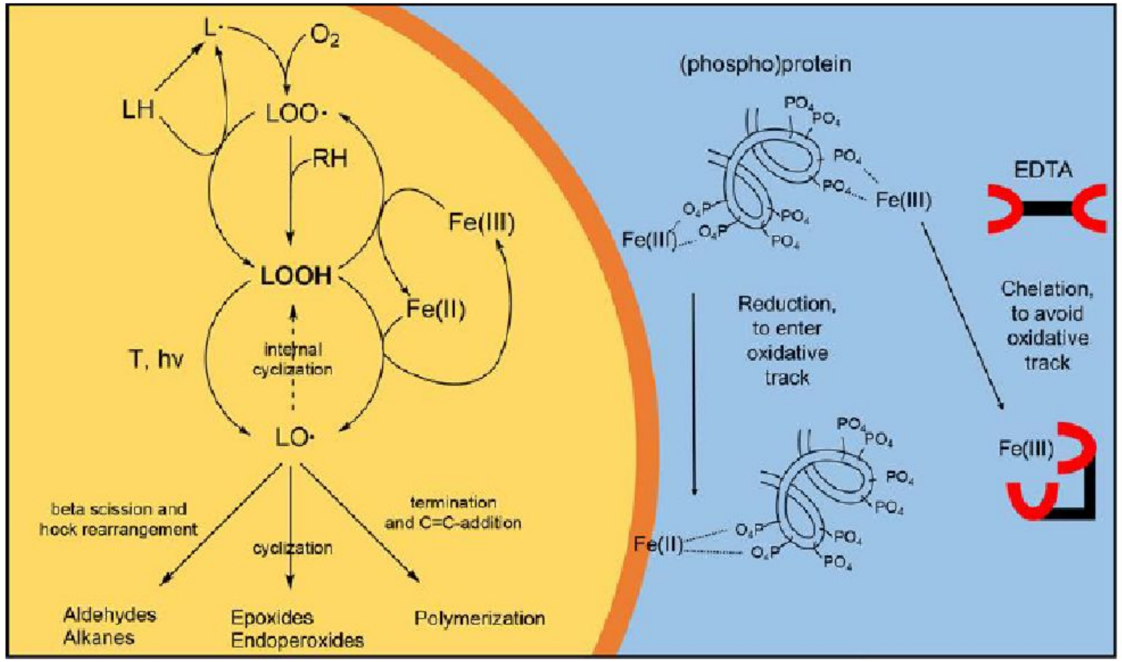


Figure 7.2. Schematic description of the lipid oxidation pathways in oil (left), emphasizing the central role of the hydroperoxides (LOOH) and the catalytic function of iron, a transition metal. On the right, the interplay between iron and a phosphoprotein and a chelator (EDTA) is schematically depicted. Here, unchelated iron is able to enter the oxidative track, whereas chelated iron stays mainly in the water layer, away from the oxidative interface.

Donny Merkx, PhD thesis, Wageningen University, 2021

Peptides and protein hydrolysates as antioxidants – a new approach

DTU

Provide

Predicted antioxidant peptides

- AnOxPePred tool
  - Method for predicting both the **free radical scavenging** and **chelating properties** of peptides based on the collected data from previous studies
    - uses **deep learning to predict**
    - trained on a **curated dataset consisting of experimentally-tested antioxidant and non-antioxidant peptides**
  - The predicted antioxidant activity is based on the peptide amino acid sequence,
    - inherent properties (e.g. size, local structure and charge)

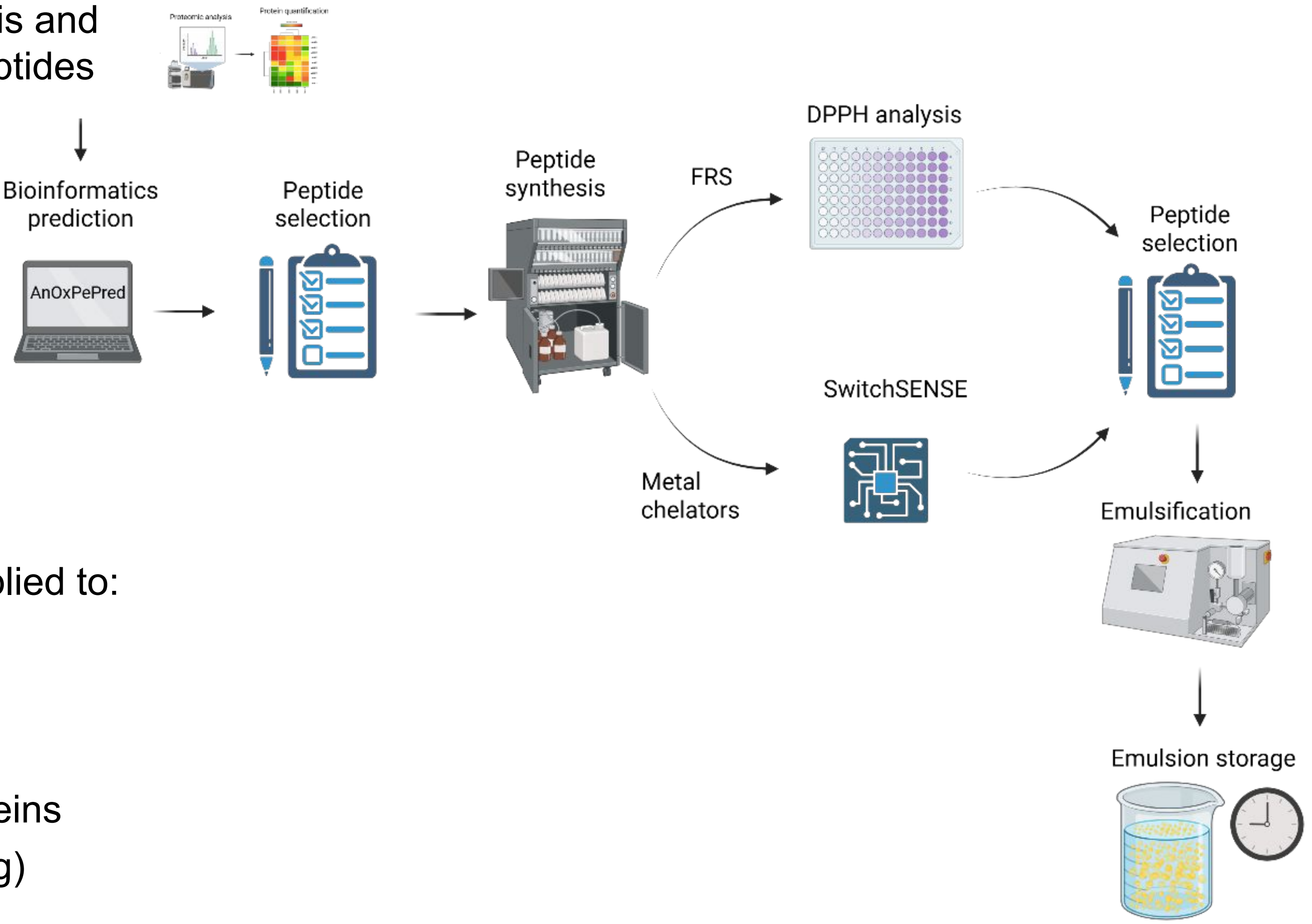
OPEN **AnOxPePred: using deep learning for the prediction of antioxidative properties of peptides**

Tobias Hegelund Olsen[1], Betül Yesiltas[2], Frederikke Isa Marin[1], Margarita Pertseva[1], Pedro J. García-Moreno[2], Simon Gregersen[3], Michael Toft Overgaard[3], Charlotte Jacobsen[2], Ole Lund[2], Egon Bech Hansen[2] & Paolo Marcatili[1]

Olsen, T.H., Yesiltas, B., Marin, F.I. *et al. Sci Rep* 10, 21471 (2020).
https://doi.org/10.1038/s41598-020-78319-w

Workflow Antioxidants peptides

Proteomics analysis and identification of peptides



This approach has been applied to:

- Potato proteins
- Seaweed proteins
- Single cell proteins
- Brewers spent grain proteins
- Insect proteins (upcoming)

Peptide sources

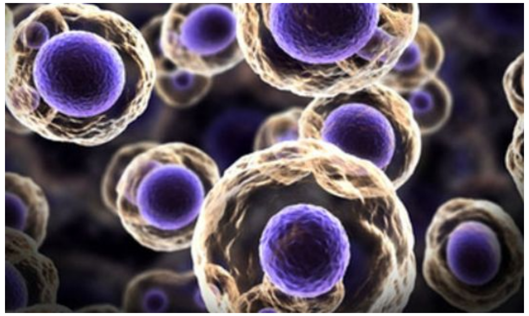
- Potato proteins
  - Obtained from potato juice, by-product in the potato starch industry
  - Protein fraction of juice at 1-2%

- Seaweed proteins from carrageenan production
  - Global annual production at 6.5 x 10^6 tons
  - Protein faction at 10-47% of the dry weight

- Single cell proteins
  - Edible unicellular microorganisms can be obtained from fermentation of methane
  - High production control and protein yield (70%)

potatoes

Red seaweed



single cell

Simplified brewing process

Barley → Malting

Malting → Mashing → Lautering → Wort boiling → Fermentation

Lautering → Brewers' spent grain — 20 kg wet

Hops → Wort boiling

Yeast → Fermentation

Fermentation →

100 L

https://brewerywastewater.com/spent-grain/

Peptides evaluated in emulsions at pH7

Selected antioxidant peptides

E1 – control

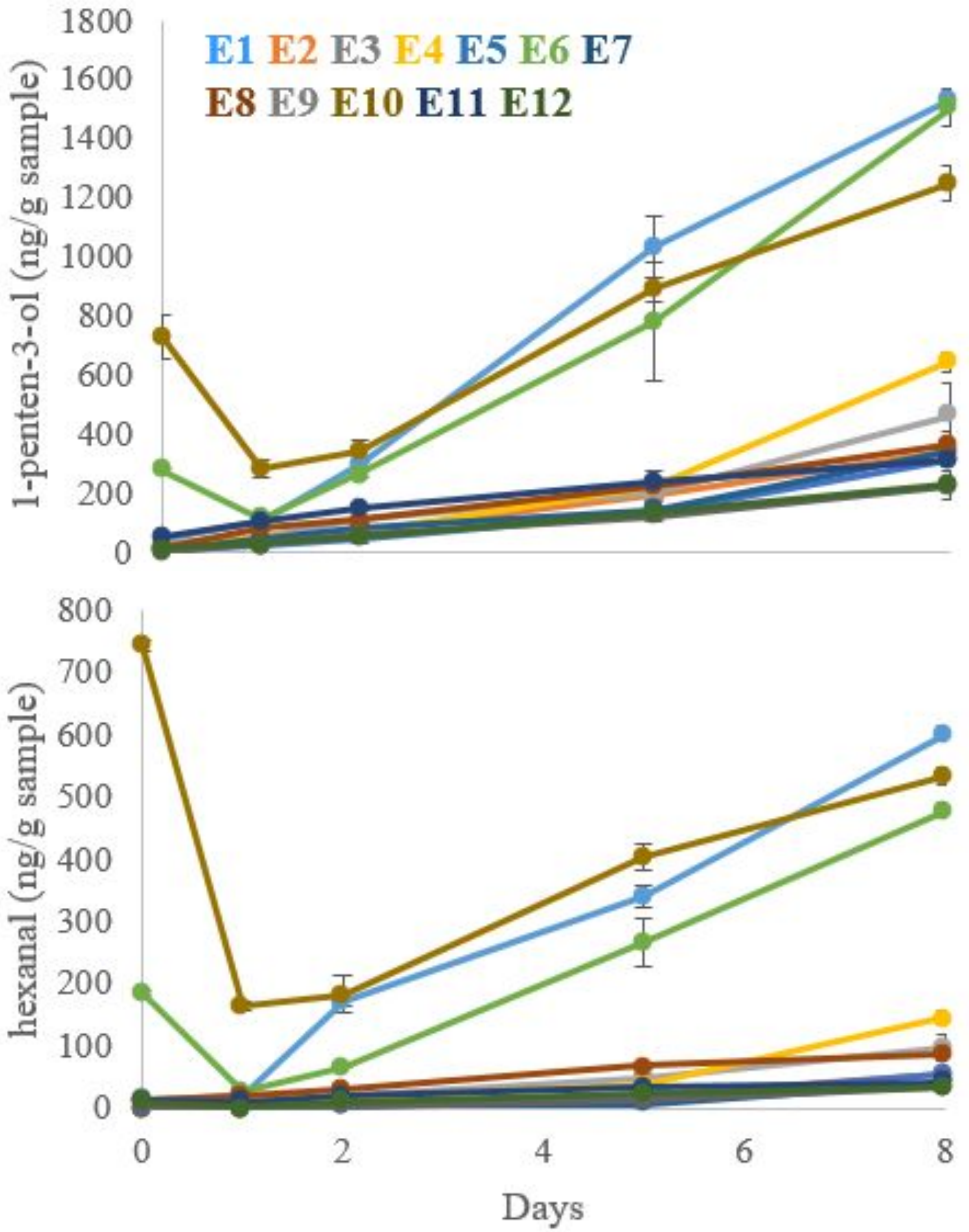
Peptide No	Amino acid sequence	Length	pI	Net charge at pH7	PRS score	PMC score	DPPH IC_{50} (mg/ml)	Relative abundance	Tryptic match	Emulsion code
111-P-SCA	KWGPLRW	7	11.4	2	0.46	0.22	6±2	11.2% / 14.1%	Moderate	E2
113-P-SCA	VPFYFEHGPHI	11	6.1	-0.8	0.64	0.22	16±2	23.2% / 18.6%	Terrible	E3
123-S-SCA	DFPVR	5	6.7	0	0.38	0.26	4±1	27.1% / 0.7%	Good/Perfect	E4
124-S-SCA	AGDWLIGDR	9	3.7	-1	0.43	0.20	3±0	2.7% / 1.9%	Perfect	E5
125-U-SCA	HWYD	4	4.9	-0.9	0.59	0.23	2±0	7.5% / 4.1%	Moderate	E6
128-U-SCA	MLWQYKPK	8	10.2	2	0.54	0.20	7±2	7.3% / 8.4%	Good/Perfect	E7
132-R-SCA	NNKWVPCLEFETEHGFVYREHH	22	5.8	-1.8	0.50	0.23	5±2	18.2%	Moderate	E8
133-R-SCA	YWTMWK	6	9.5	1	0.53	0.20	14±1	18.2%	Perfect	E9
135-P-CHE	HCPSH	5	7.2	0.1	0.51	0.30		17.7% / 11.9%	Moderate	E10
139-P-CHE	YKLLHCPSHLQCKN	14	8.7	2.1	0.47	0.26		17.7% / 11.9%	Good	E11
144-P-CHE	DDDNLVLPEVYDQD	14	0.5	-6	0.45	0.30		17.7% / 11.9%	Bad/Terrible	E12

Antioxidant peptides (0.05 wt%) were added in **5% fish oil-in-water emulsions** stabilized with 1 wt% Tween 20

-Microfluidizer □ homogenization; 50 µl Fe^{2+} □ initiate oxidation

-Emulsions stored for 8 days in darkness

Yesiltas et al. Food Chem, 2022



Volatile compounds

- Similar trend was observed for the control E1 and E6 and E10
  - oxidized more than the others
  - in line with PV and toco. results
- Most stable emulsions were observed for E12, E11, and E9, followed by E2, E5 and E7

Yesiltas et al. Food Chem, 2022

Evaluation of the results

Peptide No	Amino acid sequence	Length	pI	Net charge at pH7	PRS score	PMC score	DPPH IC_{50} (mg/ml)	Emulsion code
111-P-SCA	KWGPLRW	7	11.4	2	0.46	0.22	6±2	E2
113-P-SCA	VPFYFEHGPHI	11	6.1	-0.8	0.64	0.22	16±2	E3
123-S-SCA	DFPVR	5	6.7	0	0.38	0.26	4±1	E4
124-S-SCA	AGDWLIGDR	9	3.7	-1	0.43	0.20	3±0	E5
125-U-SCA	HWYD	4	4.9	-0.9	0.59	0.23	2±0	E6
128-U-SCA	MLWQYKPK	8	10.2	2	0.54	0.20	7±2	E7
132-R-SCA	NNKWVPCLEFETEHGFVYREHH	22	5.8	-1.8	0.50	0.23	5±2	E8
133-R-SCA	YWTMWK	6	9.5	1	0.53	0.20	14±1	E9
135-P-CHE	HCPSH	5	7.2	0.1	0.51	0.30	-	E10
139-P-CHE	YKLLHCPSHLQCKN	14	8.7	2.1	0.47	0.26	-	E11
144-P-CHE	DDDNLVLPEVYDQD	14	0.5	-6	0.45	0.30	-	E12

- Better performing peptides are rich in antioxidant and hydrophobic AAs
  - **antioxidant:** W (tryptophan), Y (tyrosine), D (aspartic acid), M (methionine), R (arginine), H (histidine) and K (lysine)
  - **hydrophobic:** A (alanine), F, (phenylalanine, G (glycine), V (valine), P (proline) and L (leucine)

Peptides selected for evaluation in emulsions and mayonnaise based on AnOxPePred and *in vitro* radical scavenging assay

Study A

5% fish o/w emulsion at pH4

- Physical and oxidative stability
- 11 peptides selected from radical scavenger assay

Study B

Mayonnaise

- Oxidative stability of peptides
- Best performing 4 peptides in Study A

Study C

Mayonnaise

- Concentration effect on oxidative stability
- Best performing peptide in Study B

Study A - 5% fish o/w emulsion at pH4

Emulsion code	Peptide No	Amino acid sequence	pI	PRS score*	PC score*
[redacted]					
E2	111-P-SCA	KWGPLRW	11.39	0.46	0.22
[redacted]					
E4	123-S-SCA	DFPVR	6.68	0.38	0.26
[redacted]					
E6	125-U-SCA	HWYD	4.87	0.59	0.23
[redacted]					
E8	132-R-SCA	NNKWVPCLEFETEHGFVYREHH	5.79	0.50	0.23
[redacted]					

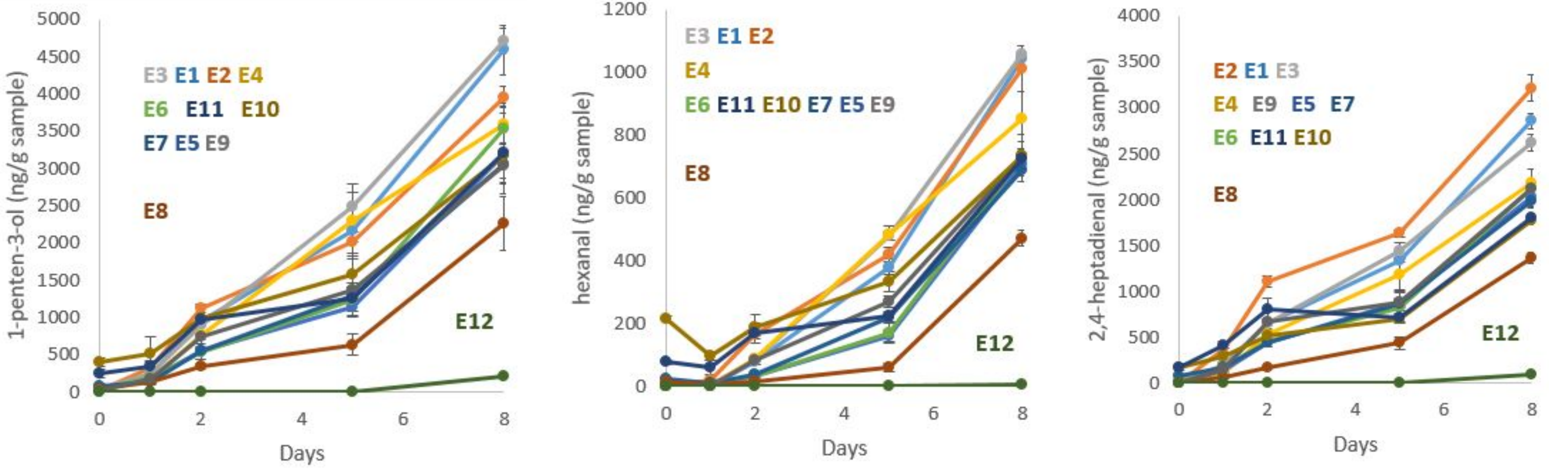
*Predicted Radical Scavenger (PRS) and Predicted Chelator (PC) score

Antioxidant peptides (0.05 wt%) were added in 5% fish oil-in-water emulsions stabilized with 1 wt% Tween 20 at pH 4

- Microfluidizer □ homogenization; 50 µl Fe^{2+} □ initiate oxidation
-
-

- **pH ~4**
- **D[3,2] □ 131 - 136 nm**
- **D[4,3] □ 227 - 244 nm**
- **ζ-potential □ (-) 4.7 – (-) 2.0 mV**

Study A - 5% fish o/w emulsion at pH4
Oxidative stability results – volatile compounds



- Three times higher oxidation levels compared to pH7 emulsions
- **E12** had the lowest development of volatiles during storage, followed by **E8. At pH 7 E8 was poor.**
- Control and **E2** was the worst and results were mostly in line with the PV and tocopherol results. **At pH7 E2 was good**

Varona et al. Food Chem, 2023

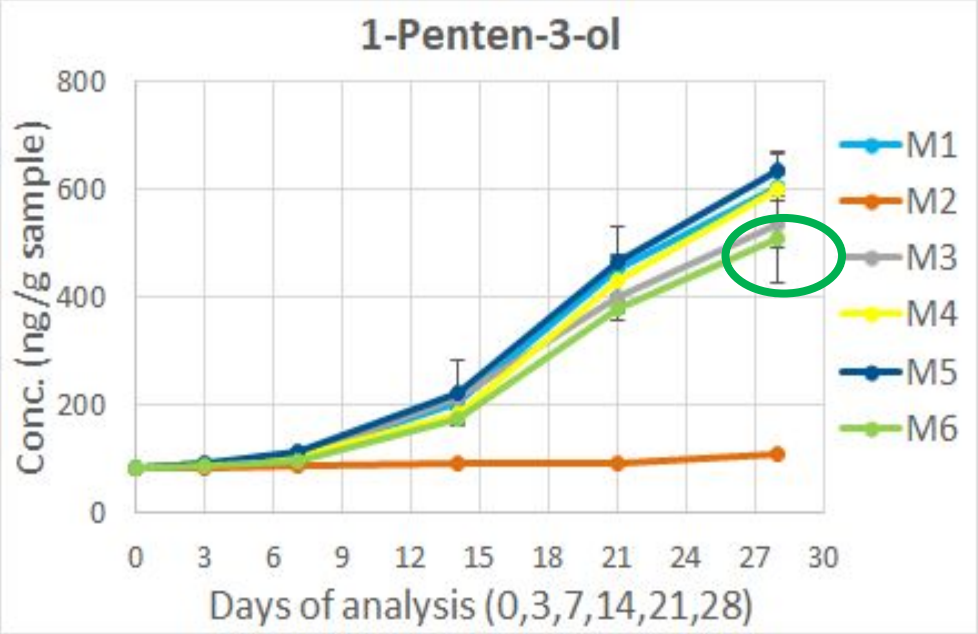
Study B – Mayonnaise with antioxidant peptides Experimental set up and storage

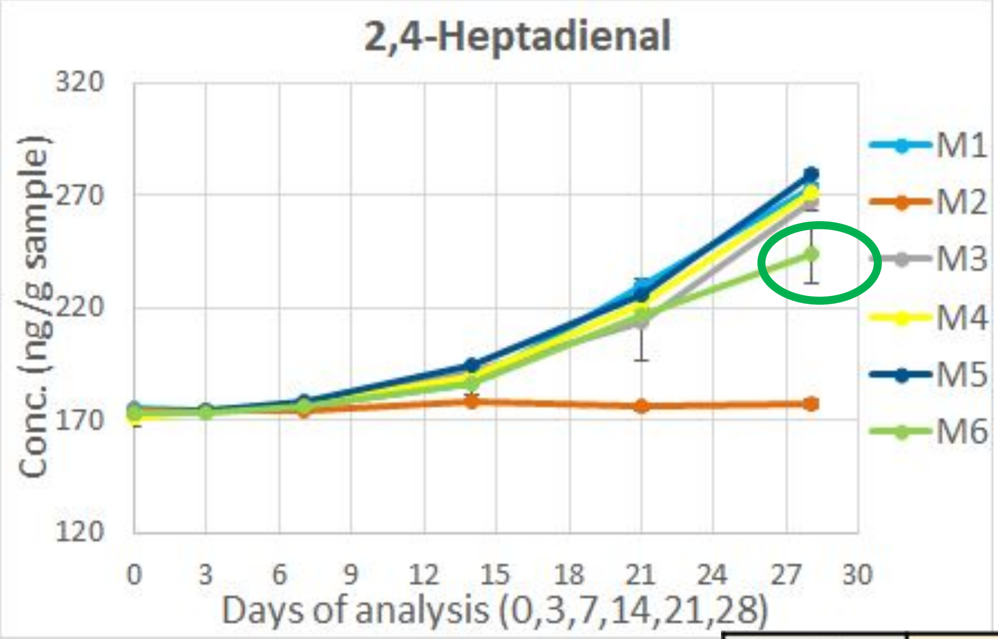
Emulsion code	Antioxidant
M1	-
M2	EDTA
M3	124-S-SCA
M4	128-U-SCA
M5	132-R-SCA
M6	144-P-CHE

- Fish oil enriched mayonnaise (20% of the total oil replaced with fish oil)
- 4 peptides (75 ppm) tested, one from each protein source
  - Seaweed - 124-S-SCA (E4)
  - Single cell - 128-U-SCA (E7)
  - Spinach - 132-R-SCA (E8)
  - Potato - 144-P-CHE (E12)
  - EDTA (commonly used synthetic antioxidant)
- Control mayonnaise without any antioxidant added
- 4 weeks of storage at RT, in darkness

PEPTIDES	Sequence	pI
124-S-SCA	AGDWLIGDR	3.71
128-U-SCA	MLWQYKPK	10.21
132-R-SCA	NNKWVPCLEFETEHGFVYREHH	5.79
144-P-CHE	DDDNLVLPEVYDQD	0.52

Study B – Mayonnaise with antioxidant peptides
Oxidative stability – volatile compounds





- **EDTA** had a very low volatile compound formation
- **Potato** peptide had the lowest levels among other peptides (E12)

- **Potato** peptide (144-CHE selected for concentration studies)

Note that it was predicted to be a metal chelator!

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Emulsion code	Antioxidant
M1	-
M2	EDTA
M3	124-S-SCA
M4	128-U-SCA
M5	132-R-SCA
M6	144-P-CHE

Study C – Concentration effect of peptide in mayonnaise Experimental set up and storage

Best performing antioxidant potato peptide

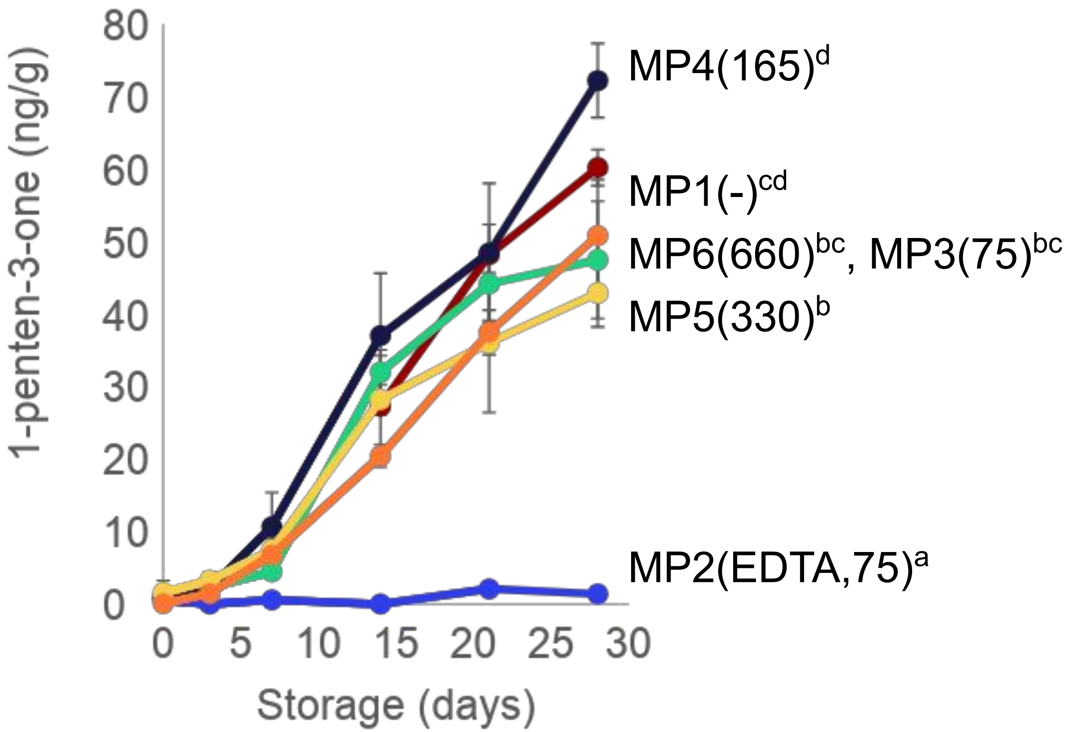
Name	Sequence	Length	pI
144-P-CHE	DDDNLVLPEVYDQD	14	0.52

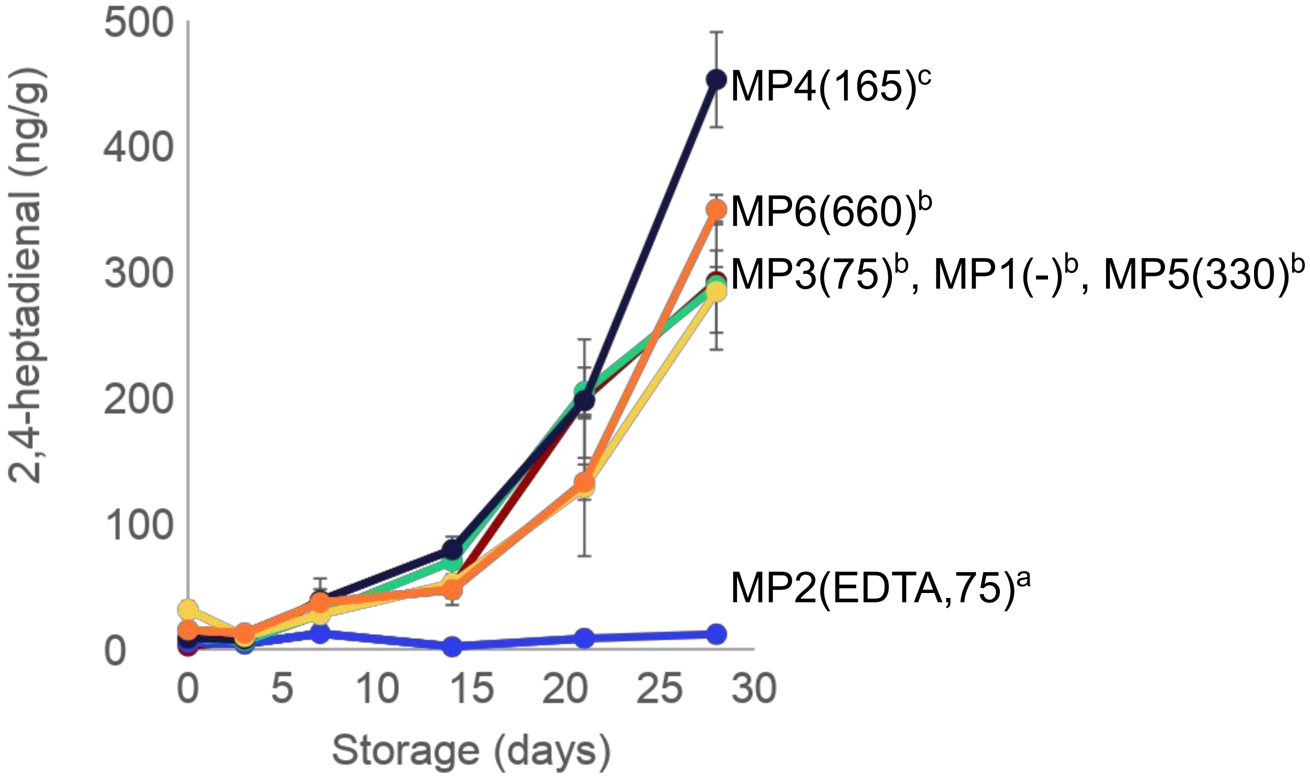
28-day fish oil enriched mayonnaise storage experiment at RT

Mayonnaise code	Antioxidant used	Antioxidant, ppm
MP1 (-)	-	-
MP2 (EDTA,37.5)	EDTA	75
MP3 (37.5)	144-P-CHE	75
MP4 (82.6)	144-P-CHE	165
MP5 (165.3)	144-P-CHE	**330**
MP6 (330.6)	144-P-CHE	660

- Control mayo without any antioxidants, **MP1**
- Mayo with EDTA, **MP2**
- Mayo with potato peptide, **MP3-6**
  - Peptide concentrations starting with the same mass concentration as for EDTA and increased up to the **equimolar concentration of EDTA** and twice as much

Study C – Concentration effect of peptide in mayonnaise
Oxidative stability – volatile compounds





- **EDTA** provided much better results compared to potato peptides in all concentrations
- Lowest oxidation values for potato peptide was added in the same molar concentration as EDTA

Varona et al. Food Chem, 2023

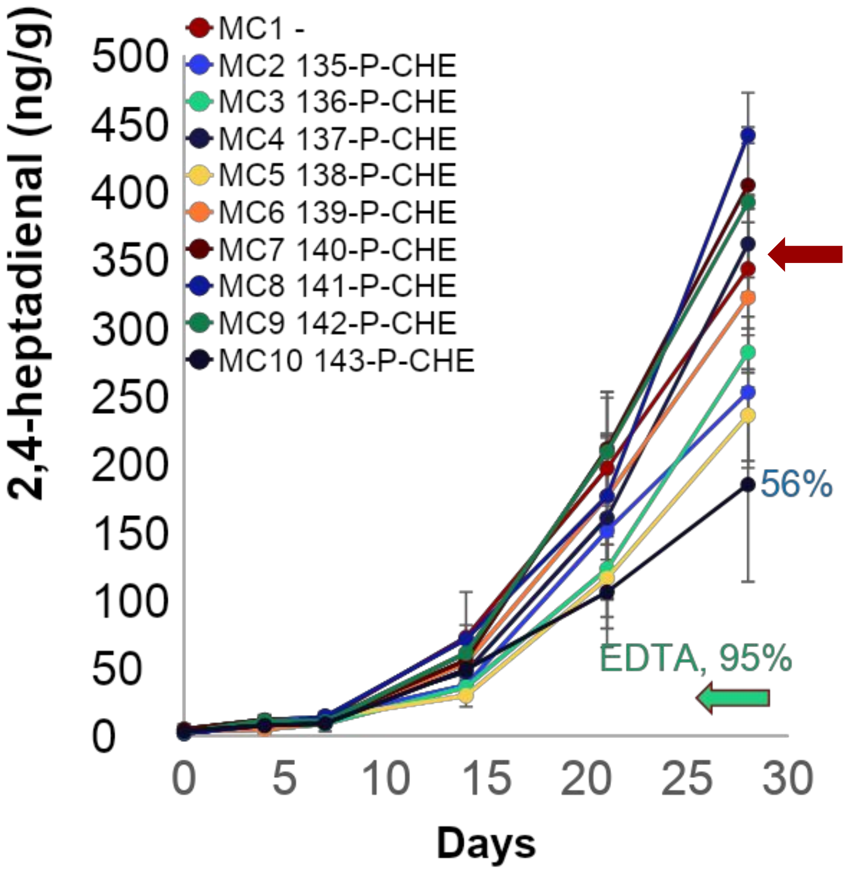
Peptides selected for evaluation in mayonnaise based on AnOxPePred scores for metal chelating activity and other parameters related to potential metal chelating activity

Selected peptides

Protein source	Sequence	Peptide	Length	pI	PFRS	PMCS	RA-A (%)	RA-B (%)	TM
potato	**HCPSH**	**135-P-CHE**	5	7.23	0.51	0.30	17.70%	11.90%	4
	MDDAS	**136-P-CHE**	5	0.64	0.31	0.23	13.23%	33.53%	1
	CEGES	**137-P-CHE**	5	0.86	0.36	0.23	1.10%	0.12%	5
	ALQWMLVI	**138-P-CHE**	8	3.72	0.44	0.20	16.34%	12.01%	1
	YKLLHCPSHLQCKN	**139-P-CHE**	14	4.00	0.47	0.26	17.70%	11.90%	6
	GMKNVYKLLHCPSHLQCKNIGSN	**140-P-CHE**	23	9.65	0.53	0.25	4.60%	2.00%	1
	LGYNLLYCPFSSDDQFCLKVGVVHQN	141-P-CHE	26	5.03	0.29	0.15	6.76%	3.37%	4
	ENPIVLPSTCHDDDNLVLPEVYDQDGHPL	**142-P-CHE**	29	3.42	0.35	0.26	7.81%	2.19%	6
	DFNLVDGDVAAVDPSLLSISV	**143-P-CHE**	21	0.42	0.30	0.23	2.70%	4.80%	1
	DDDNLVLPEVYDQD	144-P-CHE	14	0.52	0.45	0.30	17.70%	11.90%	2
seaweed	**DLSDLSQP**	**145-S-CHE**	8	0.73	0.32	0.29	2.76%		4
	QLVQLLPPFHV	**146-S-CHE**	11	7.52	0.45	0.24	5.59%	0.06%	4
	IDLPDLKQSDVDPVVAKCCEV	147-S-CHE	21	3.61	0.37	0.14	2.76%		1
	QSDVDPVVAK	**148-S-CHE**	10	3.71	0.31	0.23	2.76%		9
	EAEMR	**149-S-CHE**	5	4.15	0.37	0.23	2.76%		7
single cell	**PLHVPV**	**150-U-CHE**	6	8.26	0.50	0.27	8.89%	32.36%	1
	PSLGQQAQAPA	**151-U-CHE**	11	4.07	0.39	0.24	1.21%	0.03%	3
	MVDALQFHEEHGEVCPAGWNK	152-U-CHE	21	4.51	0.59	0.22	2.68%	1.41%	9
	CVQSHR	**153-U-CHE**	6	9.21	0.39	0.24	1.26%	0.96%	9
	MEWEVPEK	**154-U-CHE**	8	3.85	0.47	0.23	7.26%	8.35%	9

Oxidative stability – Chelator potato peptides

Volatile compounds (DHS/GC-MS)



Control

% inhibition at day 28 for best performing peptide

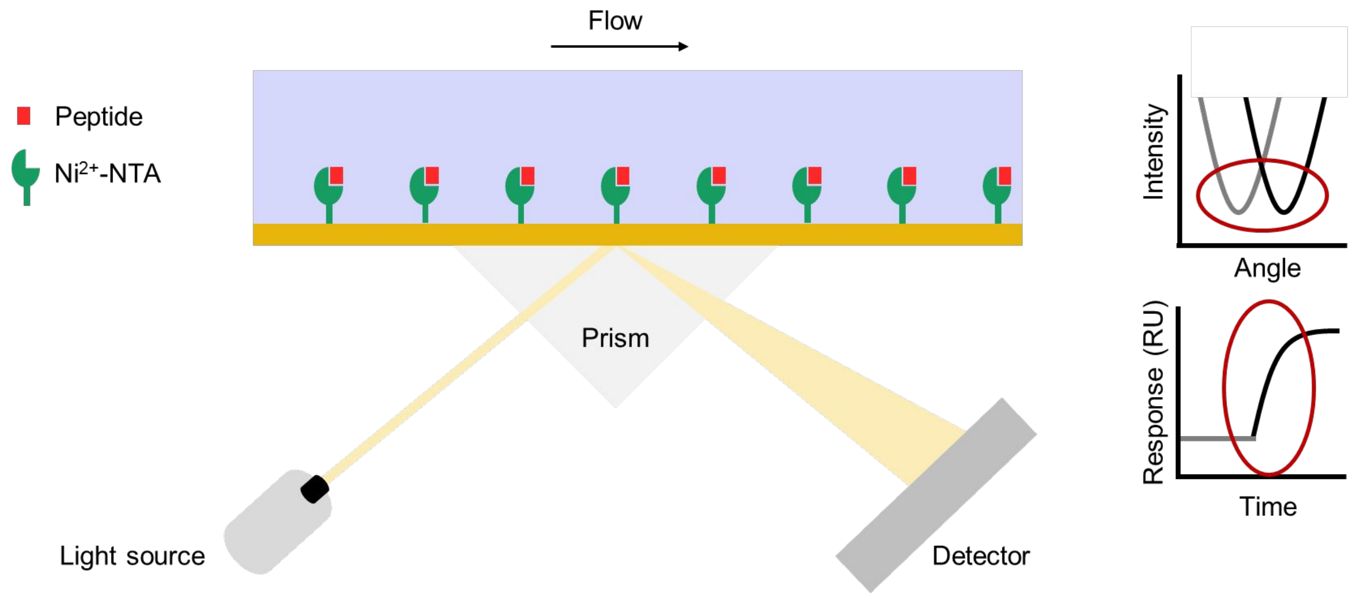
% inhibition at day 28 for mayo with EDTA

From previous studies

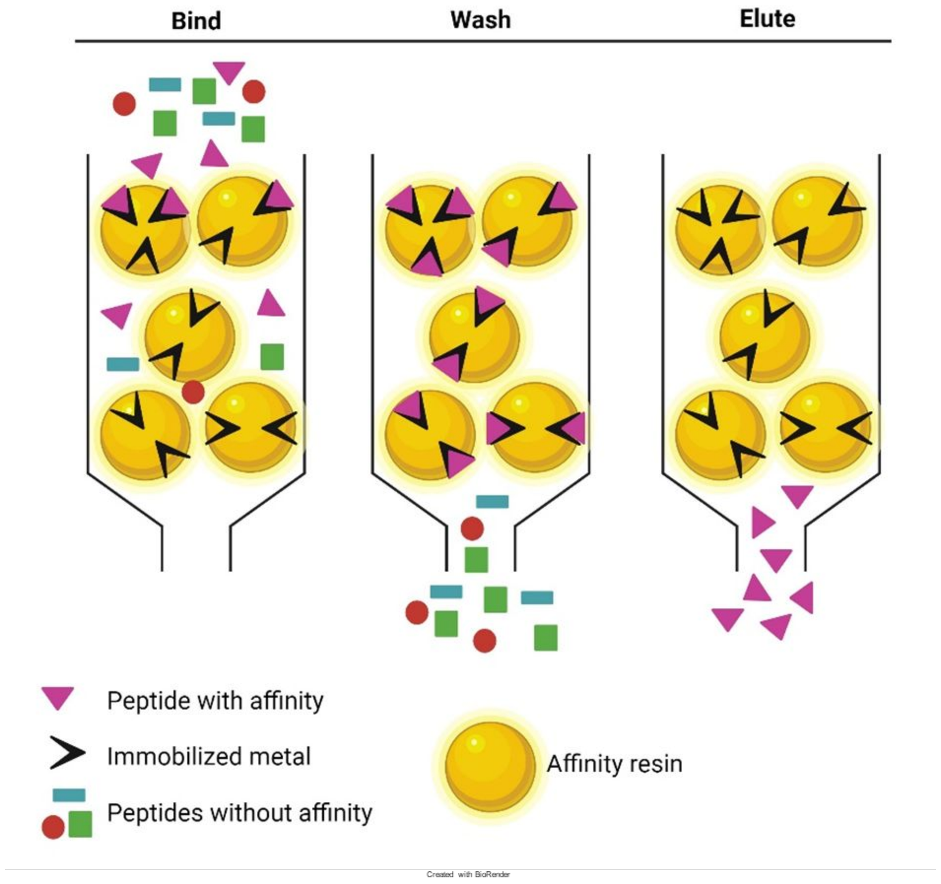
Screening of metal chelating peptides

Ferrozine assay does not work, so what to do?

Can we screen for metal chelating peptides?
Examples of potential techniques



Surface plasmon resonance (SPR)

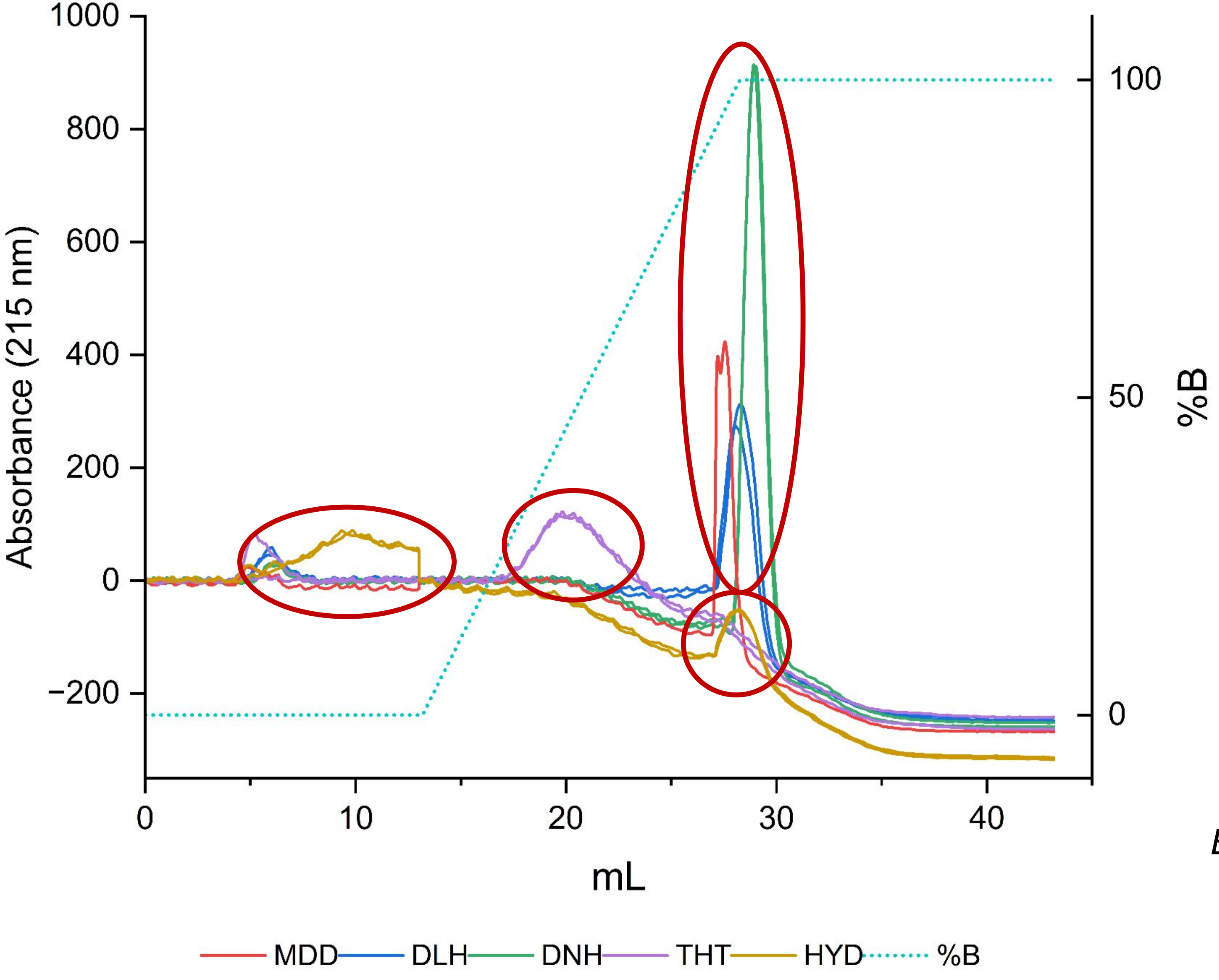


Immobilized metal affinity chromatography (IMAC)

SPR works at pH7, but work by M Bjørlie showed that more Development is necessary before it can be used at pH 4

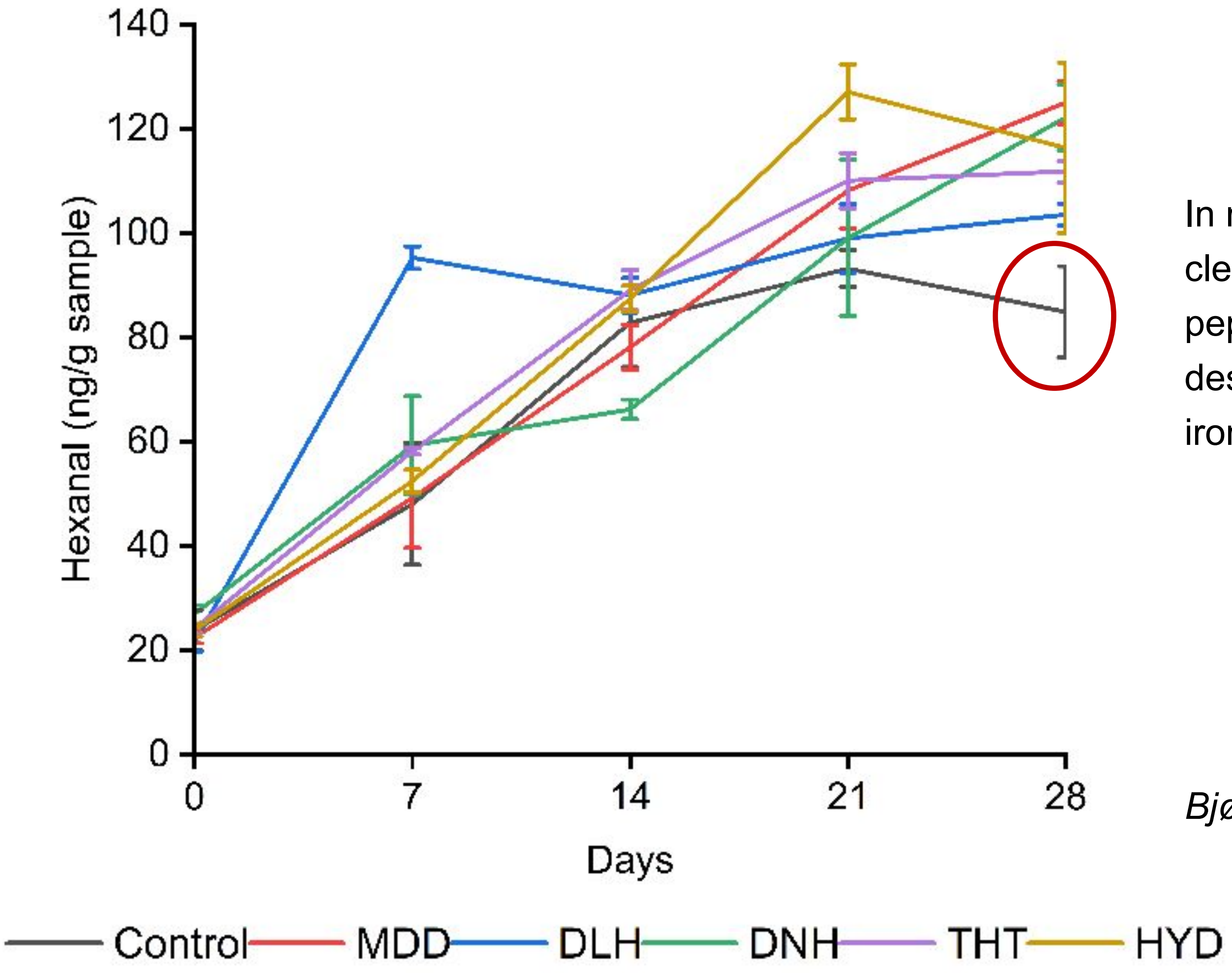
Separation of peptides and a hydrolysate by IMAC at pH 4

Fe^{3+}



Bjørlie, M. PhD thesis 2024

Effect of peptides and hydrolysates in mayonnaise



In mayonnaise there was no clear antioxidant effect of peptides and hydrolysates despite differences in iron binding capacity

Bjørlie et al. Food Chem, 2025

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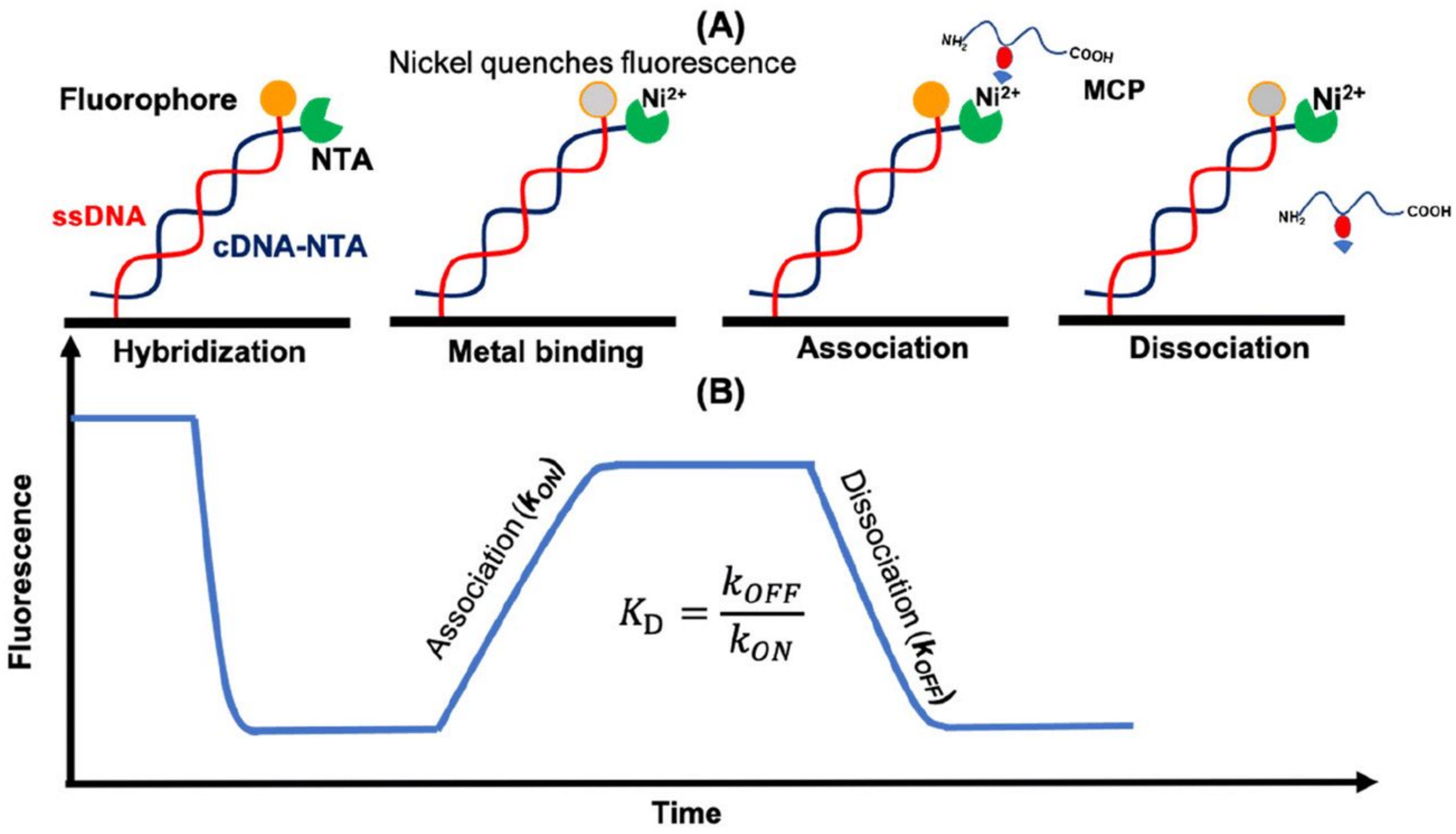
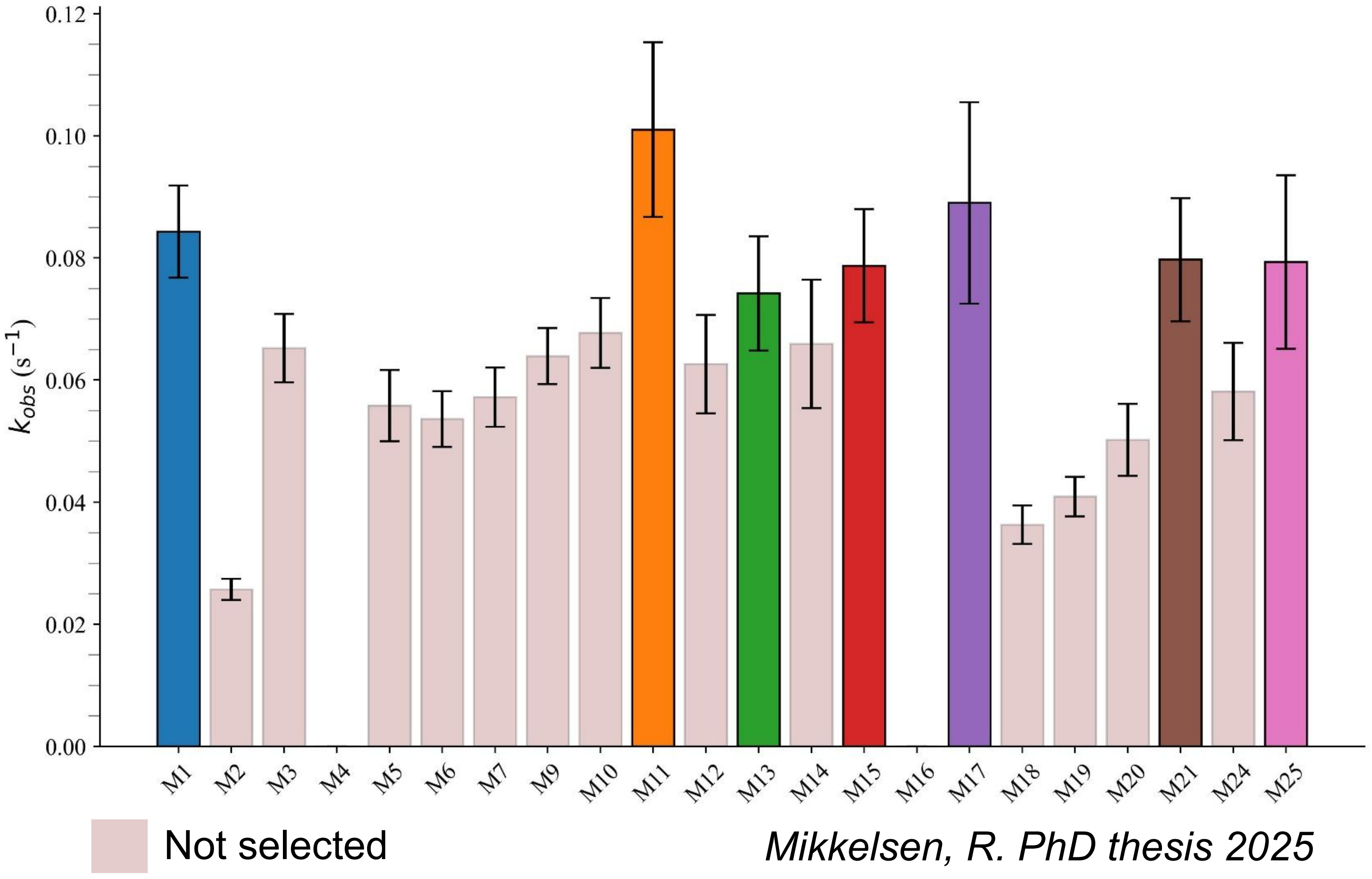


Screening of metal chelating peptides from brewers spent grain

	Metal chelating peptides	AnOxPePred score
M1	PQQPQPFPQQPFPSQQPFPQQPPFWQQQP	0.422
M2	QPQPFPQQPFPSQQPFPQQPPFWQQ	0.394
M3	TQDQTPLLQEQQDQM	0.377
M4	QVGQCSFQQPQPQQGQQQQ	0.361
M5	TFTSSIHFLAKTHQHM	0.343
M6	SLQLVQGVSQPQQQSQQQQ	0.342
M7	PHP	0.339
M8	ESFGFSSTLRAATSGQAFPQCVFDHWD	0.367
M9	FGGGLTTEQPQGGKQPFHCQQTT	0.362
M10	RDLHNQAQ	0.361
M11	GQQGYPSTTSPQQSGQGQQLGQGQQPGQGQ	0.358
M12	PGSTFPQQPGQGQQPGQRQPW	0.354
M13	PSTTSPQQSGQGQQLGQGQQ	0.353
M14	PYPIATSPHQPGQWQQPGQGQ	0.353
M15	LHPN	0.350
M16	PFLLLLHRLRPNQLRPLPTT	0.349
M17	DTPSSEFNHRFQELGL	0.349
M18	PQQPQPFPQRPFPSQQPFPQQPPFWQQQP	0.429
M19	TPQQPPLPQGQQDQM	0.396
M20	FPQRPFPSQQPFPQQ	0.373
M21	PPP	0.340
M22	EELVQQVLSAGWREGLNVACENALPRYDP	0.110
M23	VKLFNAVVVEDLVVKENRVAGVITNWALAS	0.080
M24	TRRYMT	0.168
M25	MTD	0.237

Ni association constant determined by switchSENSE®



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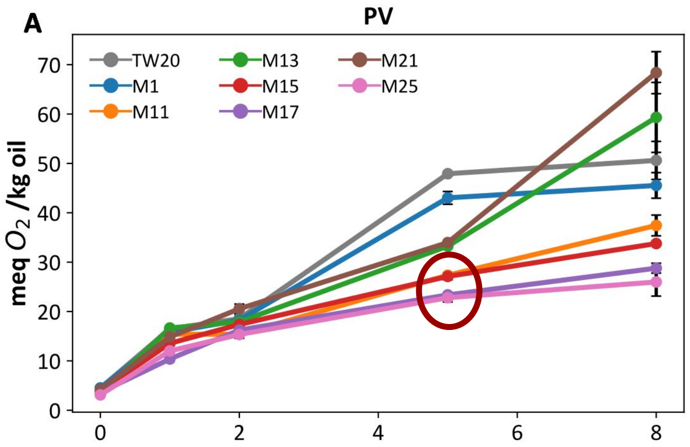
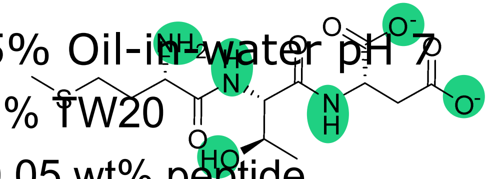
Emulsion storage experiment

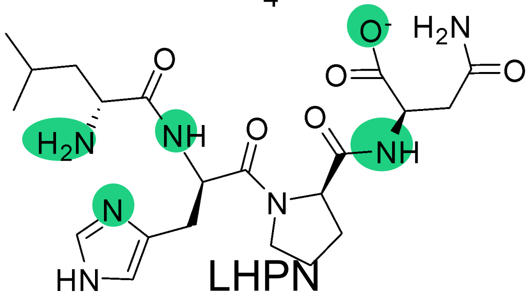
Peptide code	Peptide Sequence
TW20	-
M1	PQQPQPFPQQPFPSQQPFPQQPPFWQQQP
M11	GQQGYPSTTSPQQSGQGQQLGQGQQPGQGQ
M13	PSTTSPQQSGQGQQLGQGQQ
M15	LHPN
M17	DTPSSEFNHRFQELGL
M21	PPP
M25	MTD



- 5% Oil-in-water pH 7
- 1% TW20
- 0.05 wt% peptide
- Adition of $FeSO_4$



MTD



LHPN

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Conclusions

- Lipid oxidation mechanisms in emulsions are complex and affected by many different factors
- Interface composition and quality of emulsifier very important for oxidative stability
- Mass transfer phenomenon not fully understood but reverse micelles from LOOH could play a role
- EDTA is still the strongest antioxidant in mayonnaise

Conclusions

- Peptides are promising natural alternatives to EDTA especially in emulsions at neutral pH
- New screening techniques for selecting metal chelating compounds are needed
- SPR and IMAC are promising techniques but need improvement
- Switchsense has also shown promising results

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Benefitting people and society

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