ISOLATION OF BIOACTIVE COMPOUNDS FROM USEFUL FOOD AND PHARMACEUTICAL MATRICES USING TWEEZING ADSORPTIVE BUBBLE SEPARATION

M. Coelhan[1], P. Yılmaz[2], B. M. Gerken[2], S. Berner[2], A. Nicolai[2], M. Backleh-Sohrt[2], L. Thompson[2], H. Parlar[1,2], H. Zorn[3], D. Linke[4], R. G. Berger[4]

1) Research Center Weihenstephan for Brewing and Food Quality, Technische Universität München, Germany

2) Department for Chemical-Technical Analysis and Chemical Food Technology, Technische Universität München, Germany

3) Institute of Food Chemistry and Food Biotechnology, University of Giessen, Germany

4) Institute of Food Chemistry, Leibniz Universität Hannover, Hannover, Germany

1st International Congress on Food Technology, Antalya, November 3-6, 2010

Separation Techniques for Biological Active Compounds from Plant Materials

Methods	Disadvantages
Distillation, Centrifugation, Crystallization	Not always applicable, especially for unstable compounds
Extraction with different solvents or solvent mixtures	Residues of solvents and pesticides in the extract Degradation of biological active substances
Extraction with supercritical Gases	Costly and demanding, technically complicated Undesirable residues
Membrane filtration	Mainly for macro-molecular substances, not very efficient
Gelchromatography	Used mainly for analytical purposes

History of Adsorptive Bubble Separation

1920-1932	Development at the Royal Academy of Science in Leipzig/Germany by Ostwald
1950 - 1970	Further Development by Schildknecht and Maas, University of Heidelberg
1970 - 2000	Only few activities
Since 2000	Systematic Research on ABS at the Technical University of Munich, Department for Chemical-Technical Analysis; Research Center for Brewing and Food Quality

- 1920 – Patent announcement, Ostwald W., „Process for the Vaporization of Fluids with Purpose of Enrichment. …. Through Foam Fractionation“
- Applications in sewage cleaning and protein enrichment in recent decades

Adsorptive Foam Fractionation

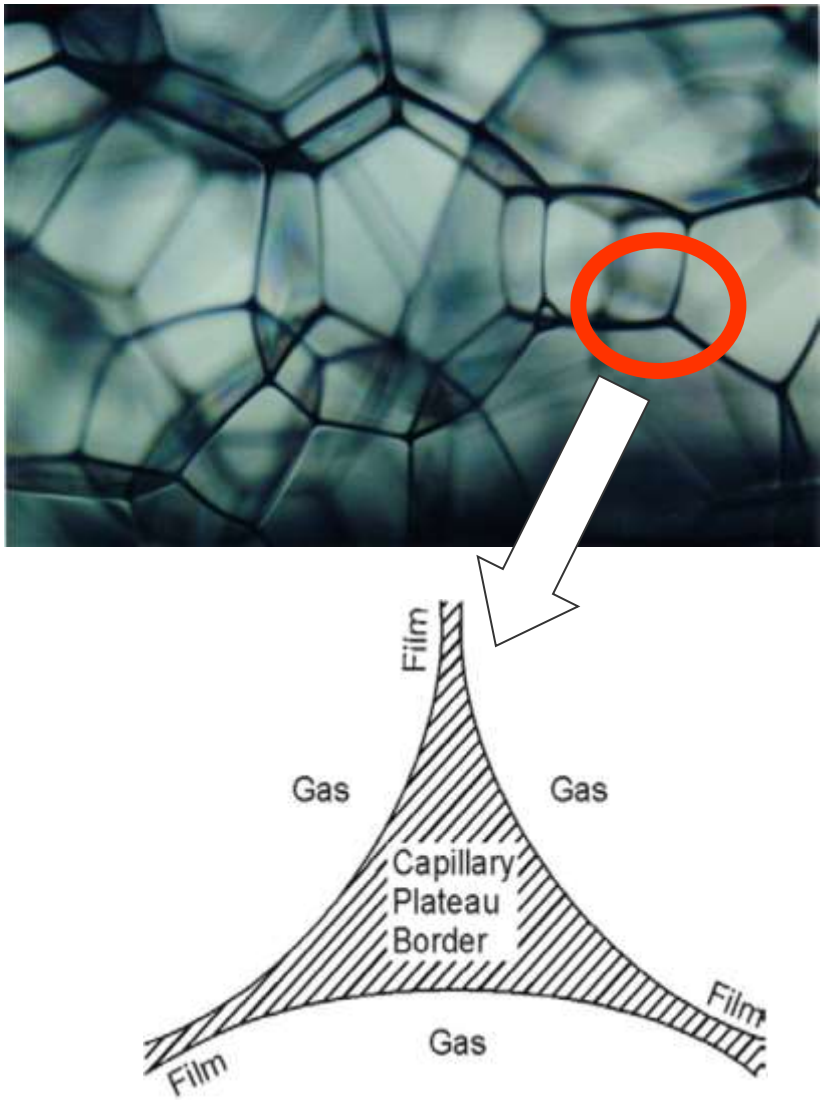
Based on the tendency of certain molecules (or colloids) present in highly dilute aqueous solutions, to preferentially adsorb at the gas-liquid interface of foams

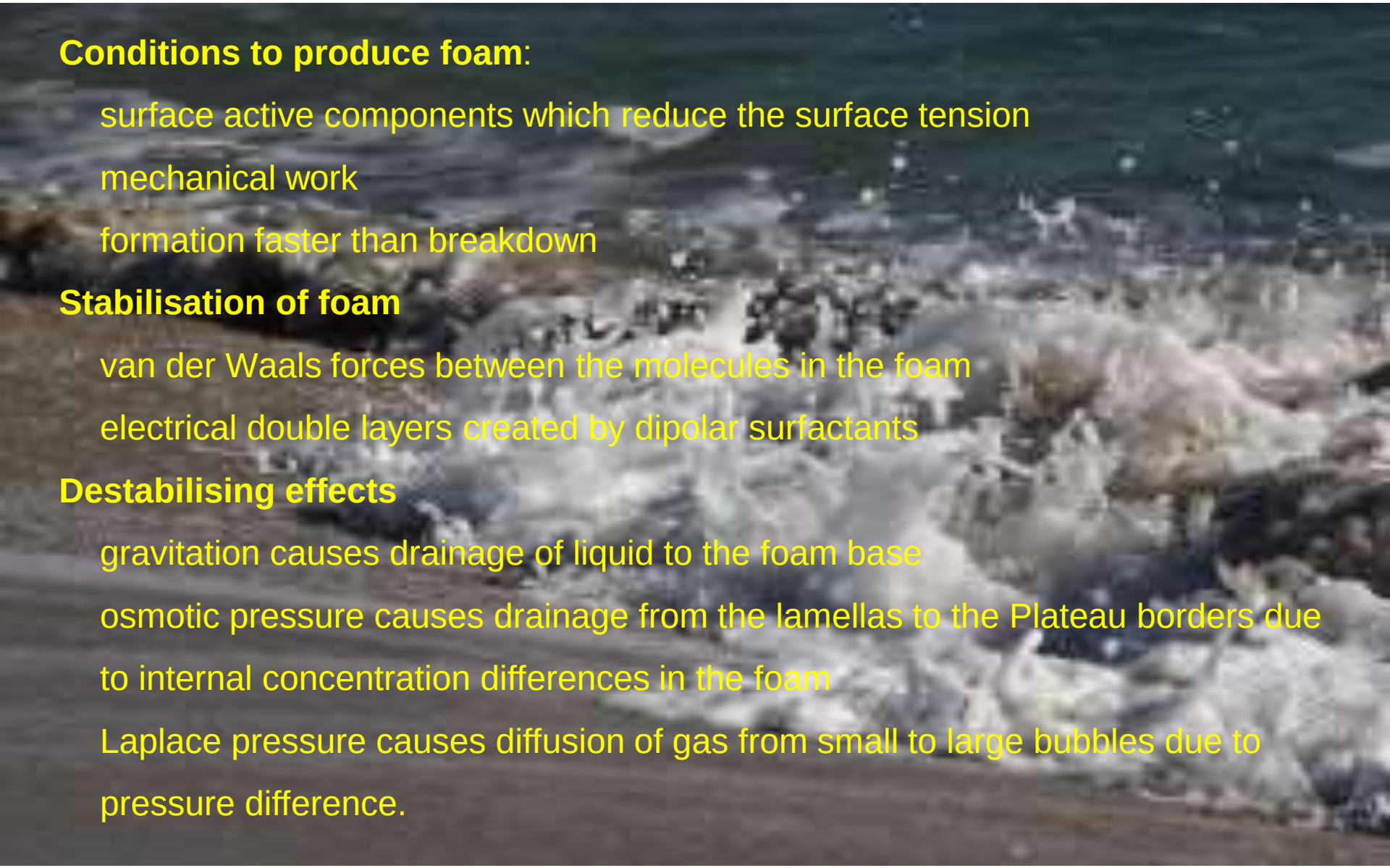
Two basic assumptions must be made:

a) Ability of the molecules in question to selectively adsorb at the gas-liquid interface
b) Existence of a large gas-liquid interface for the Adsorptive Foam Fractionation

Foam

- Formed by trapping many gaseous bubbles in a liquid (or solid)
- Extremely complex system consisting of polydisperse gas bubbles separated by draining films
- Typically disordered and have a variety of bubble sizes
- Ideally, the lamellaes are connected by three and radiate 120° outward from the connection points, known as Plateau borders





Conditions to produce foam:

surface active components which reduce the surface tension

mechanical work

formation faster than breakdown

Stabilisation of foam

van der Waals forces between the molecules in the foam

electrical double layers created by dipolar surfactants

Destabilising effects

gravitation causes drainage of liquid to the foam base

osmotic pressure causes drainage from the lamellas to the Plateau borders due to internal concentration differences in the foam

Laplace pressure causes diffusion of gas from small to large bubbles due to pressure difference.

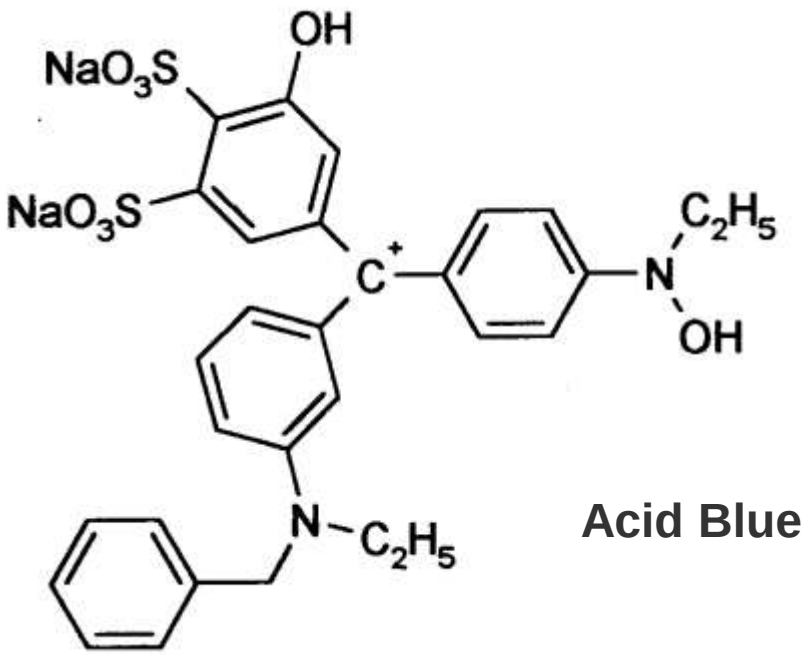
Foaming parameters

- Are the variables of foaming process
- Must be optimized and controlled for the highest reproducibility and efficiency (considering drainage effects, agglomeration, solution characteristics, etc.)

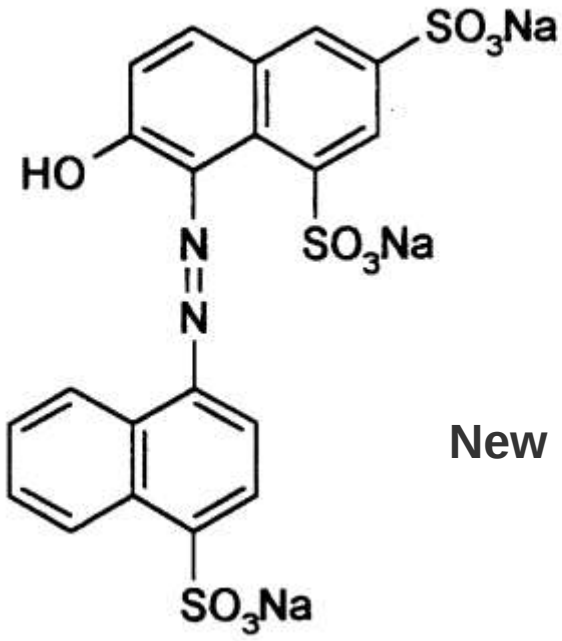
- Technical & Physico-chemical

Technical	Physico-chemical
➢ Column dimension	➢ pH-value
➢ Column temperature	➢ Ion strength
➢ Gas flow	➢ Concentration in initial solution
➢ Glass frit	➢ Additives

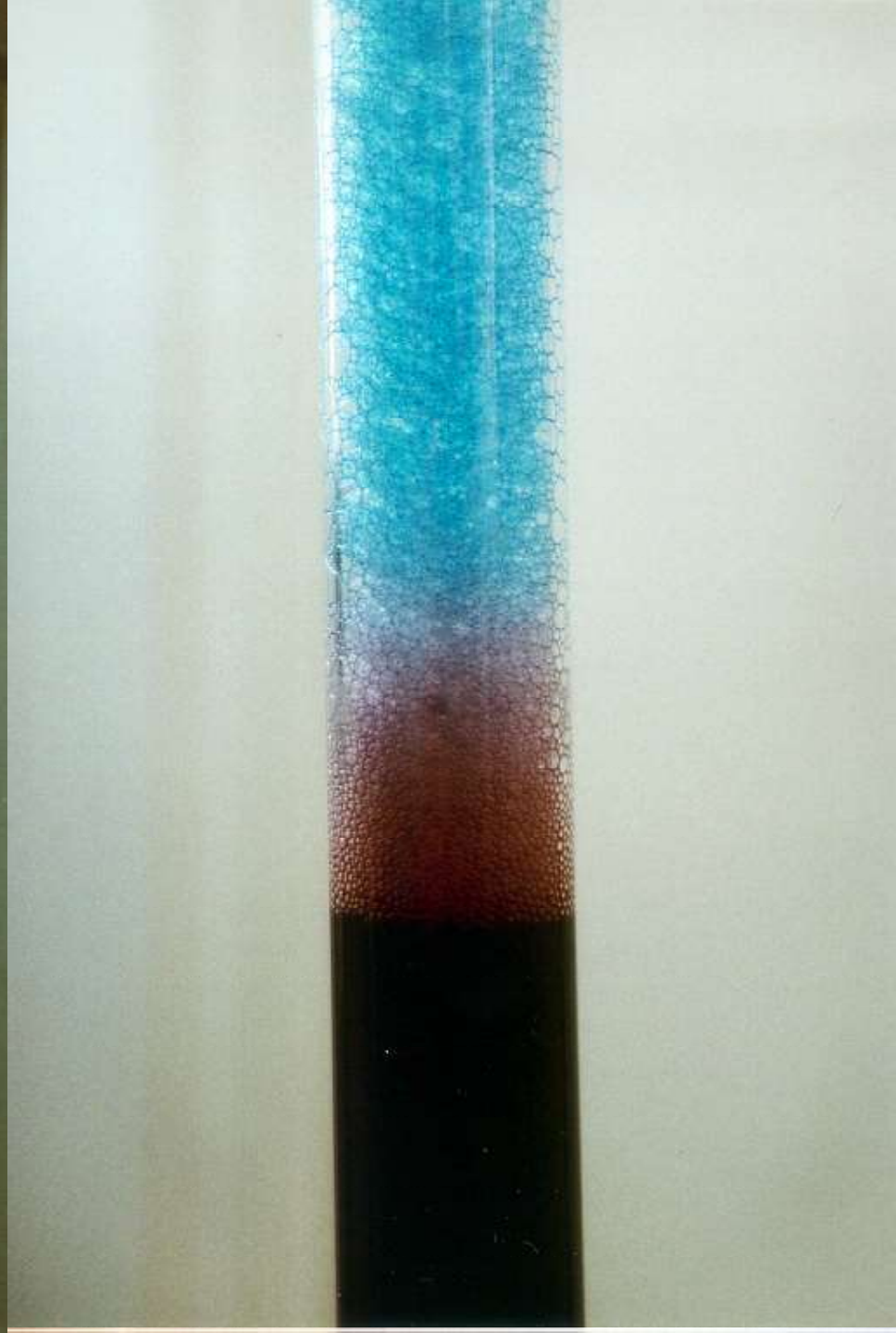
ABS – Example



Acid Blue

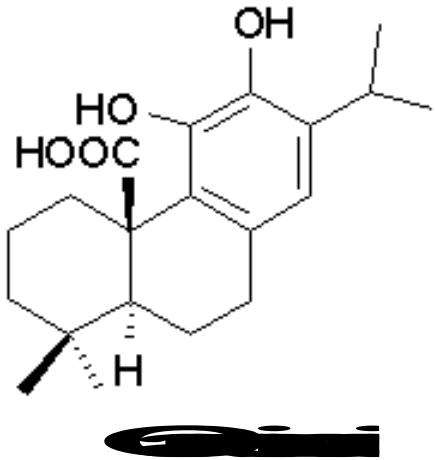


New Coccine

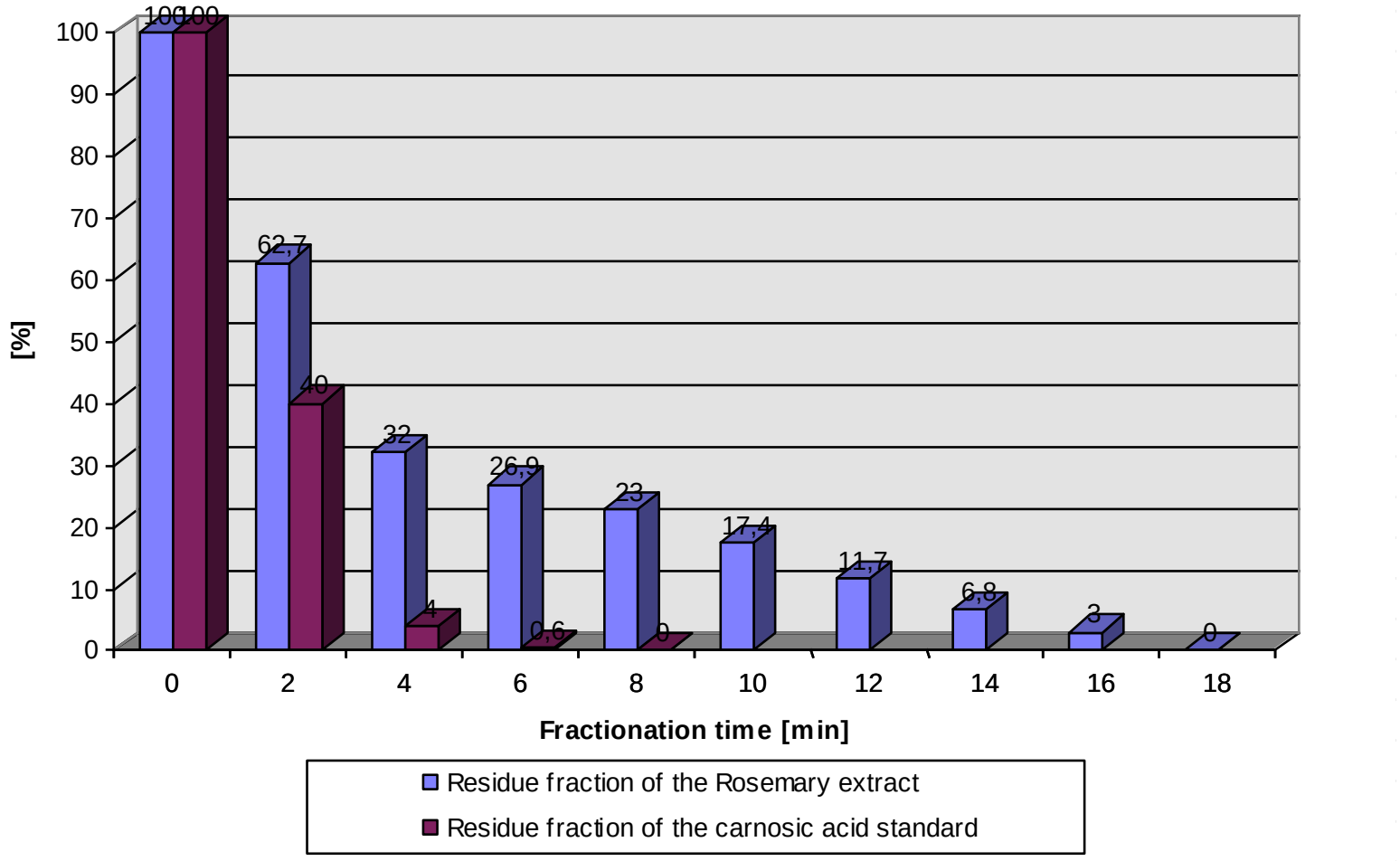


Rosemary (*Rosmarinus officinalis*)

- Native to the Mediterranean region.
- The most effective antioxidant in rosemary: carnosic (carnosolic) acid.
- Carnosic acid may shield the brain from free radicals, lowering the risk of strokes
- Carnosic acid is labile at higher pH values but stable in acid medium up to 110 °C.
- Rosemary extracts have a sufficiently high protein content of ca. 5% *to enable an effective foam* separation of nonpolar or medium-polar components.



Loss of Carnosic Acid in the Start Solution During ABS (at pH 4)



Backleh M, et. Al. Rapid quantitative enrichment of carnosic acid from rosemary (*Rosmarinus officinalis* L.) by isoelectric focused adsorptive bubble chromatography. J. Agric. Food Chem. 51 (5): 1297-1301, 2003.

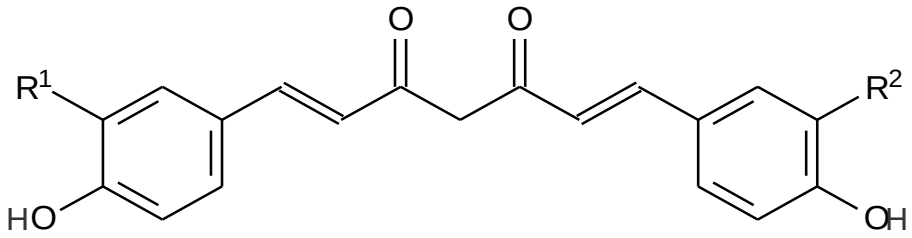
Curcuma (*Curcuma longa*), Indian Saffron, Turmeric

- Plant of the ginger family, native to tropical south Asia
- The rhizomes are boiled for several hours and then dried in hot ovens, after which they are ground into a deep orange-yellow powder

- Canned beverages and baked products, dairy products, ice cream, yogurt, yellow cakes, orange juice, biscuits, popcorn color, sweets, cake icings, cereals, sauces, gelatins, etc. Curcuma is a significant ingredient in most commercial curry powders

- Currently investigations for possible benefits in Alzheimer`s disease, cancer, arthritis, and other clinical disorders.
- Curcumin was identified as responsible for most of the biological effects of curcuma.
- Curcuma contains up to 5% curcumin. Natural Yellow 3.
- Curcumin acts as a free radical scavenger and antioxidant, inhibiting lipid peroxidation and oxidative DNA damage.
- **Its antioxidant effect is eight times more powerful than vitamin E** and it is significantly more effective in preventing lipid peroxide formation than the synthetic antioxidant BHT.
- The powerful anti-oxidation property of curcumin has an important role in keeping curry for a long time without it turning rancid.

Curcuma longa L. (Curcuma, Turmeric)



(1*E*,6*E*)-1,7-bis(4-hydroxy-3 methoxyphenyl)-1,6-heptadiene-3,5-dione.

		C_{init} mg/L	*R (%)*	*ER*	*Log Pow*
Curcumin	R1: OCH_3 R2 OCH_3	4.42	92 ± 7	19.5 ± 1.3	1.15
Demethoxycurcumin	R1: OCH_3 R2: H	3.12	91 ± 5	17.6 ± 1.1	1.00
Bis-demethoxycurcumin	R1: H R2: H	5.89	90 ± 5	15.4 ± 1.1	0.85

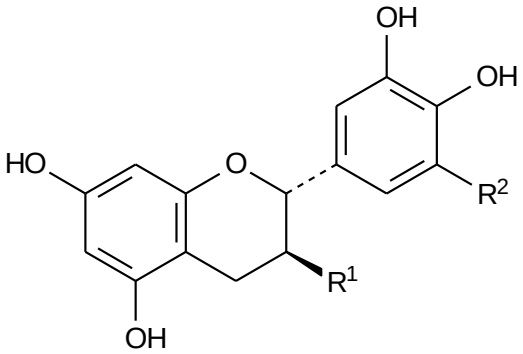
Initial volume: 100 mL; flow rate N_2: 15 mL/min, pH 6.0; foaming time: 100 min

Backleh-Sohrt et.al. Efficiency of foam fractionation for the enrichment of nonpolar compounds from aqueous extracts of plant materials. J. Nat. Prod. 68 (9), 1386-1389, 2005.

- ***Camellia sinensis*** (tea plant): cultivated commercially in more than 20 countries, mainly in Asia, Africa and South America
- Green tea (unfermented), black tea (fermented), oo-long tea (semi-fermented)
- Polyphenols (30%, mostly catechins), alkaloids, polysaccharides (40%), proteins + amino acids (4.5-6%), lipids (4%) and small quantities of organic acids, saponins, vitamins and minerals

The quantities of catechins, the ratio between components vary greatly

Caffeine, the main alkaloid present in *Camellia sinensis;* up to 5% in green tea



Camellia sinensis (L.) O. Kuntze, Theaceae, Green Tea

		C_{init} mg/L	*R (%)*	*ER*	*Log Pow*
(+)-Catechin	R1: exo-OH R2: H	4.3	19 ± 2	14 ± 2.0	0.53
(–)-Gallocatechin gallate	R1: Exo-gallate R2: OH	63.4	n.d.	1.0	0.12
(–)-Epigallocatechin gallate	R1: Endo-gallate R2: OH	96.5	n.d.	1.0	0.09
(–)-Epicatechin	R1: Endo-OH R2: H	23.8	39 ± 4	34 ± 3.0	0.67
(–)-Epicatechin gallate	R1: Endo-gallate R2: H	23.7	n.d.	1.0	0.07
(–)-Epigallocatechin	R1: Endo-OH R2: OH	13.0	n.d.	1.0	0.13

Initial volume: 70 mL; flow rate N_2: 15 mL/min; pH 6.5; 30 mg saponin; foaming time: 100 min, n.d. not determined

Glycoalkaloid poisons α-solanine and α-chaconine in potato

- Naturally produced as a defense mechanism against insects, disease, and predators.
- Together appr. 95% of total potato glycoalkaloids
- Solanine content in commercial varieties of potatoes: <200 mg/kg.
- Under stress conditions (i.e. mechanical injury, fungus, insect attack) concentrations of 1 g/kg or more
- Deep frying potatoes at 170°C effectively lower glycoalkaloid levels, but boiling has no effect.

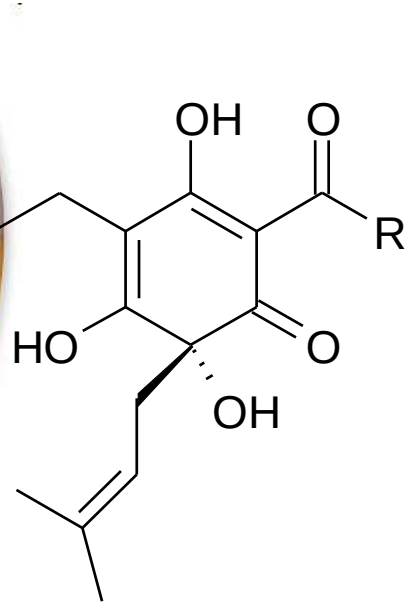
Time and pH dependent Enrichment of Solanidine Alkaloids – α-Solanine (1), α-Chaconine (2)

Start solution | *Foam fractions* | *Foam*

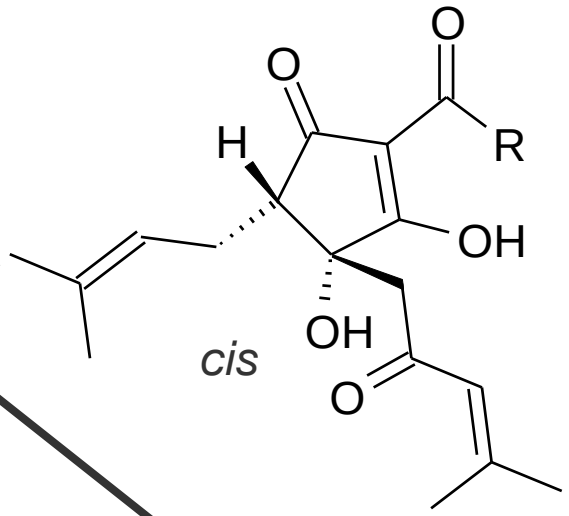
pH	1+2 (µg)	t = 10 min (µg)	t = 20 min (µg)	t = 30 min (µg)	∑ (µg)	Recovery (%)
5.0	1700	637 ± 3	223 ± 2	74 ± 1	934	55.0
6.0	1700	1020 ± 5	501 ± 3	170 ± 2	1691	99.5
7.0	1700	814 ± 4	274 ± 2	75 ± 2	1185	69.7
8.0	1700	640 ± 3	257 ± 2	45 ± 1	992	58.3
6.0	100 (1)	60 ± 2	33 ± 2	6 ± 1	99	99
6.0	1600 (2)	960 ± 3	507 ± 3	84 ± 1	1551	97

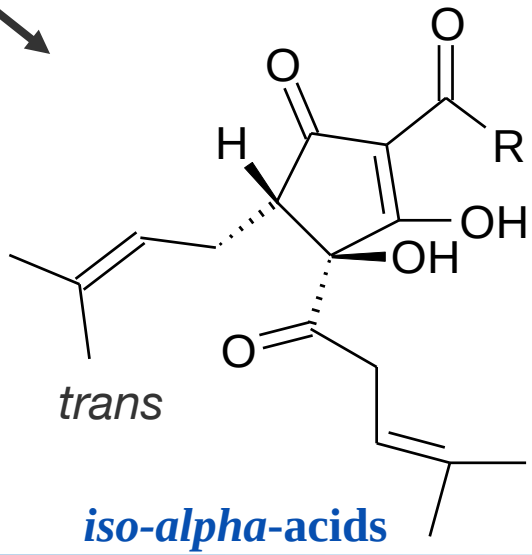
Backleh et. Al. Enrichment of the glycoalkaloids alpha-solanine and alpha-chaconine from potato juice by Adsorptive Bubble Separation using a pH gradient. J. Sep. Sci. 27 (12), 1042-1044, 2004.

Isolation of bitter substances from beer



heat





$R = CH(CH_3)_2$ *Co-Humulon*

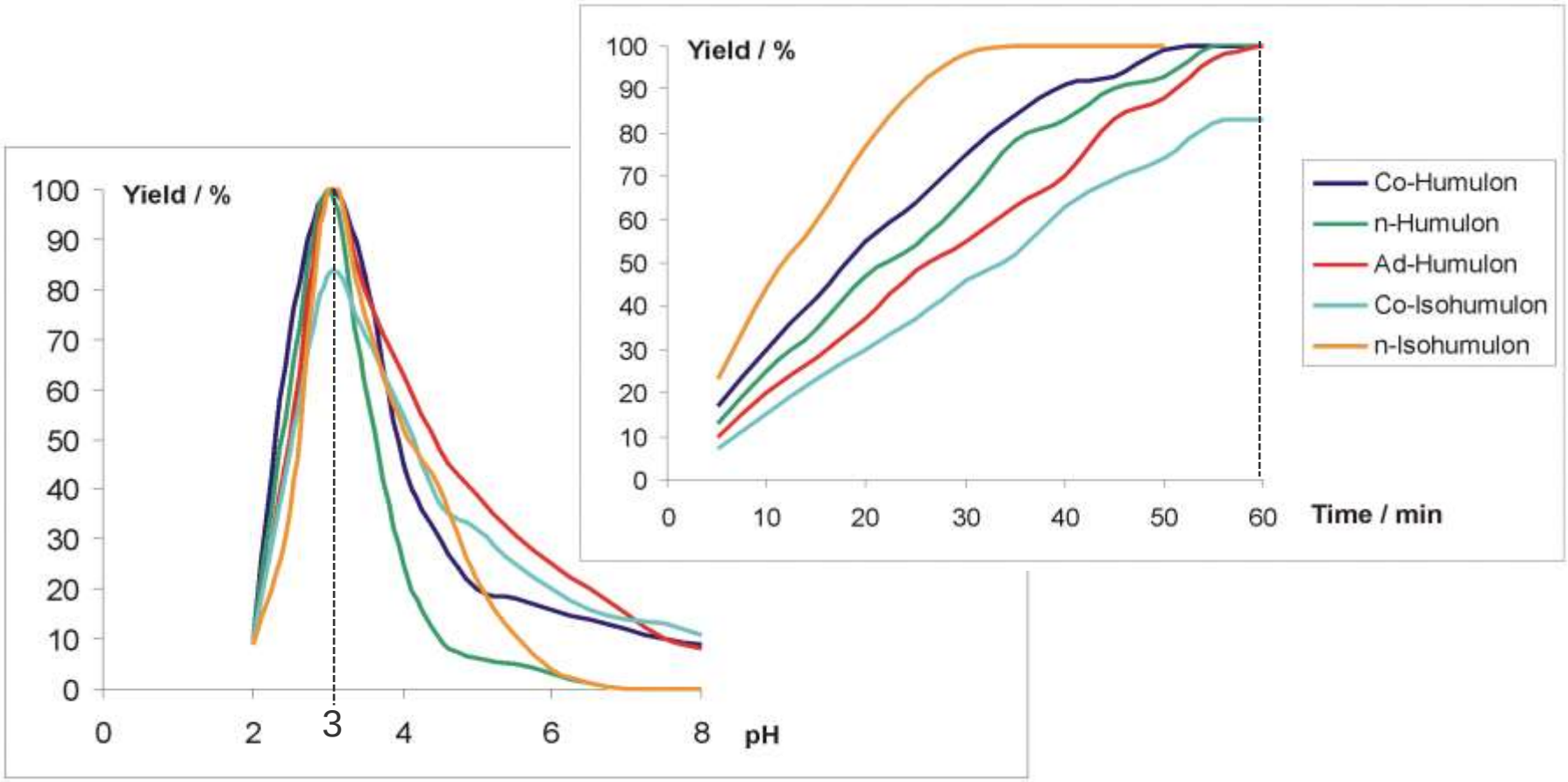
$CH_2CH(CH_3)_2$ *N-Humulon*

$CH(CH_3)C_2H_5$ *Ad-Humulon*

***alpha*-acids**

***iso-alpha*-acids**

Yield of Humulones in Dependence of Time and pH During ABS of Pilsener Beer



Backleh-Sohrt et. al. Efficiency of foam fractionation for the enrichment of nonpolar compounds from aqueous extracts of plant materials. J. Nat. Prod. 68 (9), 1386-1389, 2005.

Cannabis sativa

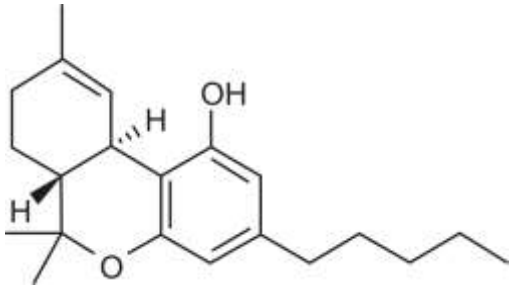
Cannabis sativa L. spp. *Sativa* for fiber and oil production

Cannabis sativa L. spp. *indica* legal cultivation for medical purposes

(marihuana, hemp, henf, granja and pot)

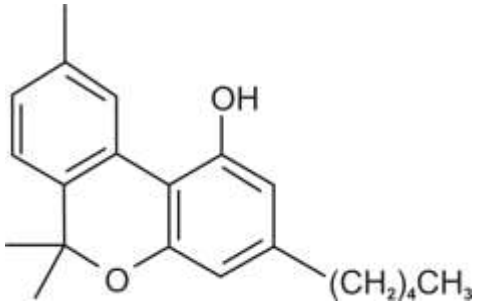
- 500 components identified and characterised
- Cannabinoids with biological and pharmacological activities
- Major cannabinoids: Δ^9– tetrahydrocannabinol, cannabinol and cannabidiol, etc.
- The main active principle is Δ^9– tetrahydrocannabinol (Δ^9– THC), 0.3-20%, psychoactive
- Cannabinol (CBN) and cannabidiol (CBD), not psychoactive

Cannabis sativa L.



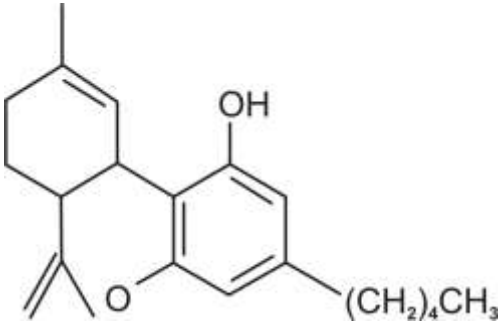
	C_{init} mg/L	C_{Foam} mg/L	*R (%)*	*ER*	*Log Pow*
THC	16.22	99.8	73.81	6.15	0.51

Δ^9-Tetrahydrocannabinol



CBN	17.75	117.67	79.55	6.63	-

Cannabinol

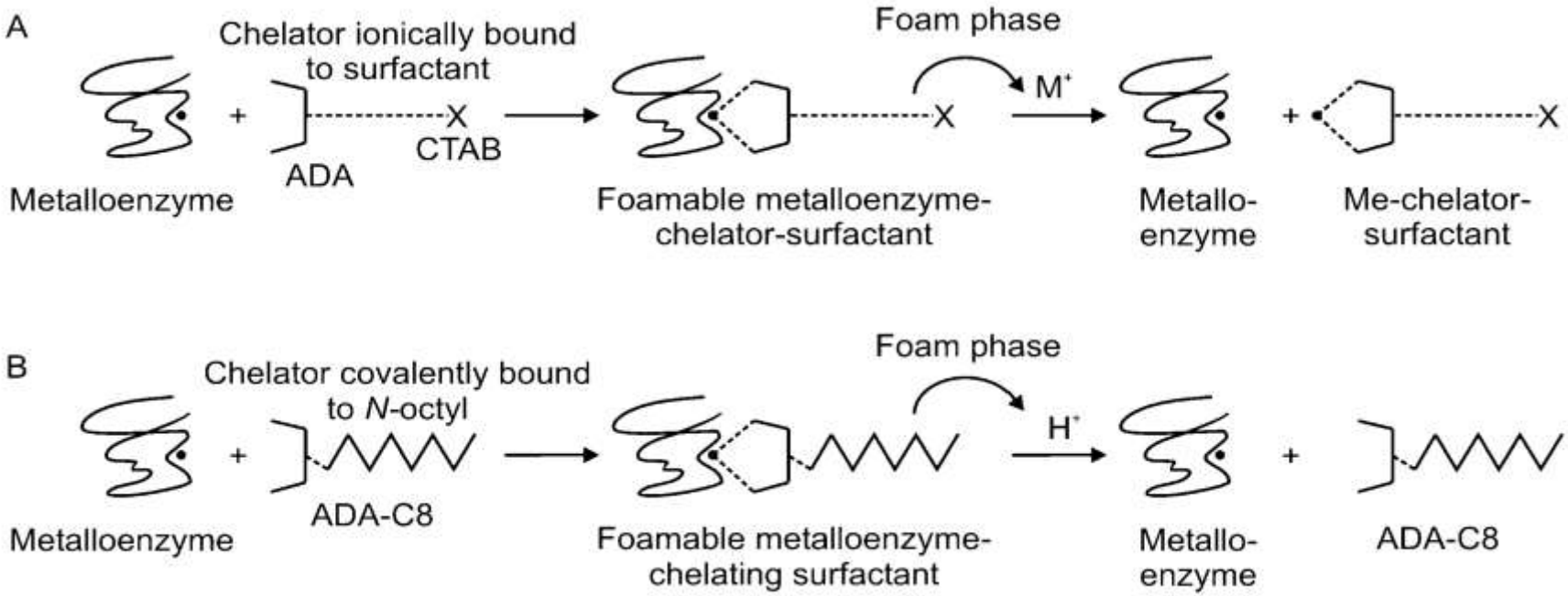


CBD	28.43	180.32	76.10	7.11	-

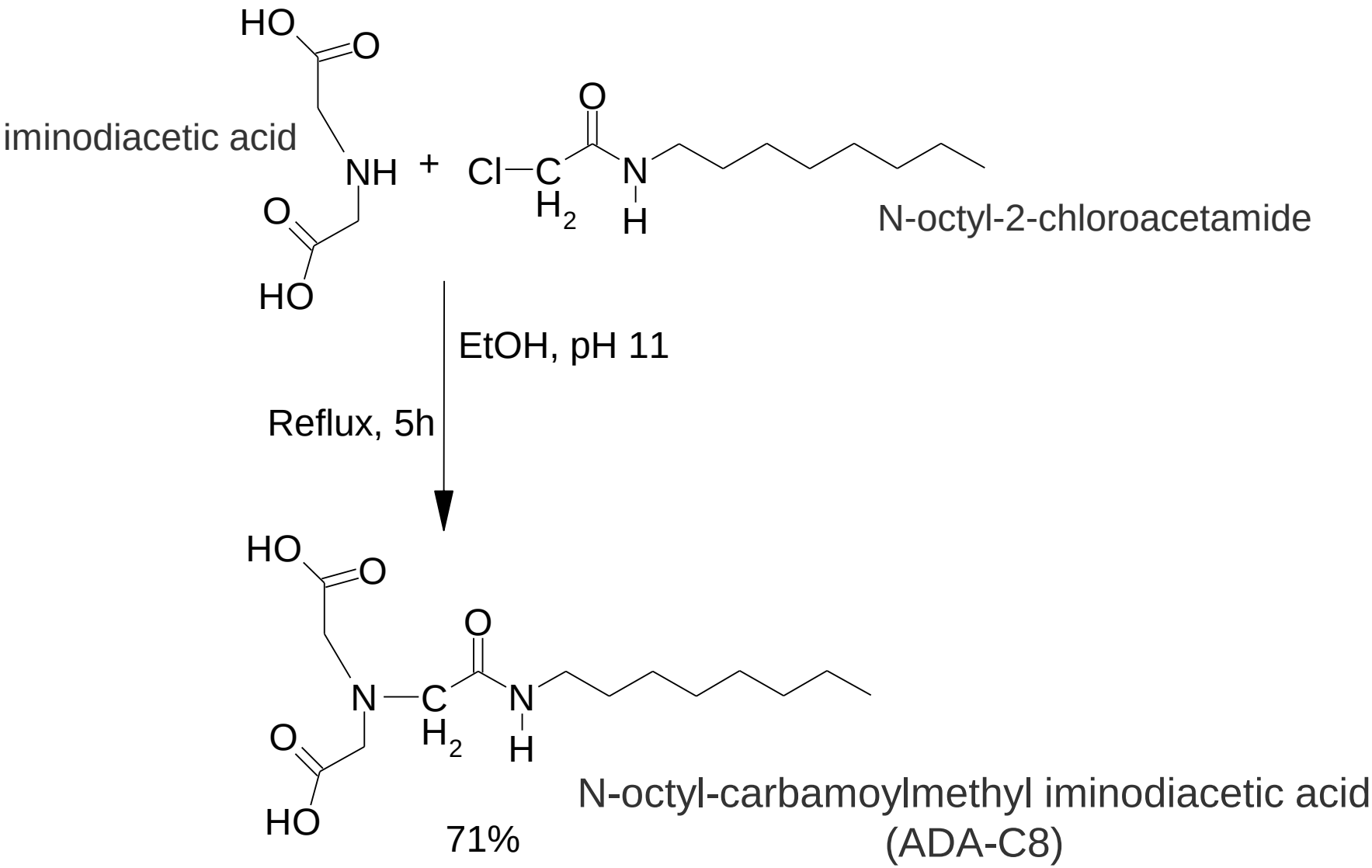
Cannabidiol

Initial volume: 100 mL, foaming time: 90 min, pH 10, Saponin: 50 mg

Application of ABS to Metalloenzymes



Application of ABS in combination with a selective surface-active chelator is termed tweezing-adsorptive bubble separation (Tweezing-ABS)


iminodiacetic acid
N-octyl-2-chloroacetamide
EtOH, pH 11
Reflux, 5h
71%
N-octyl-carbamoylmethyl iminodiacetic acid
(ADA-C8)

Tweezing Adsorptive Bubble Separation of Enzymes

Experiments with cetyltrimethylammonium bromide (CTAB); CTAB-ADA; ADA-C8; or the metal-ADA-C8 chelate.

- The enrichment (ER) and recovery (R) values were calculated using the following equations

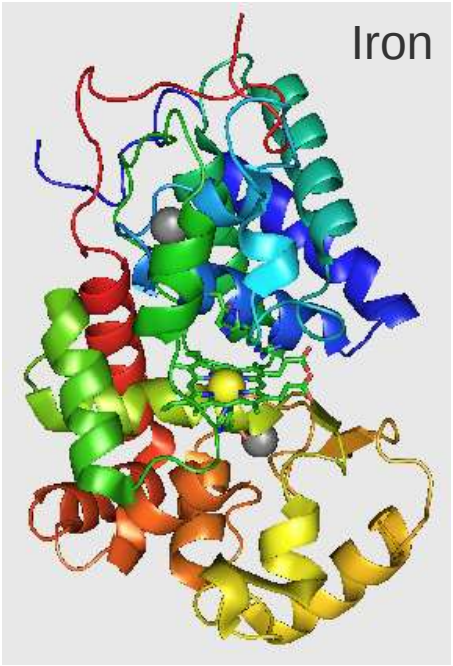
$$ER = \frac{E_{Af}}{E_{As}} \qquad R = \frac{E_{Af} \cdot V_f}{E_{As} \cdot V_s} \cdot 100$$

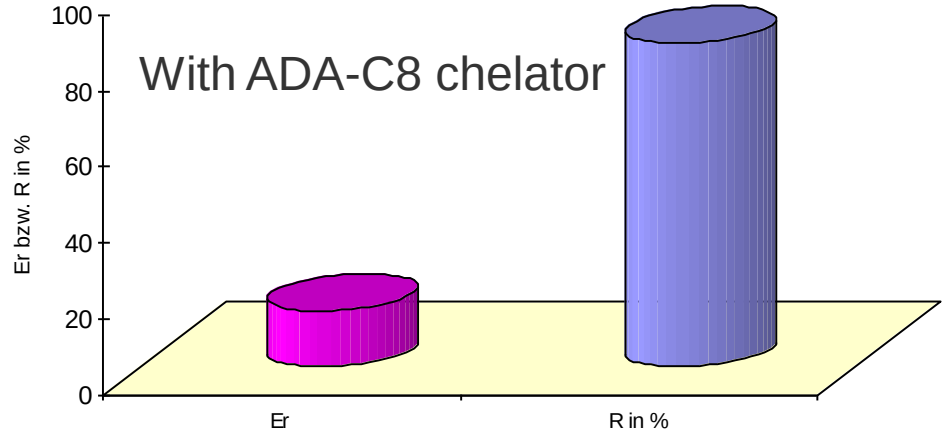
E_{Af} is the enzymatic activity in foam (units L^{-1})

E_{As} the enzymatic activity in the starting solution (units L^{-1}), V_f the volume of the liquefied foam (L), and V_s the volume of the starting solution (L).

Horseradish peroxidase

- Widely used as a label for immunoglobulins in many immunochemistry tests including ELISA, immunoblotting and immunohistochemistry.
- The most desired label for antibodies since it is the smallest and most stable of the three most popular enzyme labels (HRP, alkaline phosphatase, and B–galactosidase)



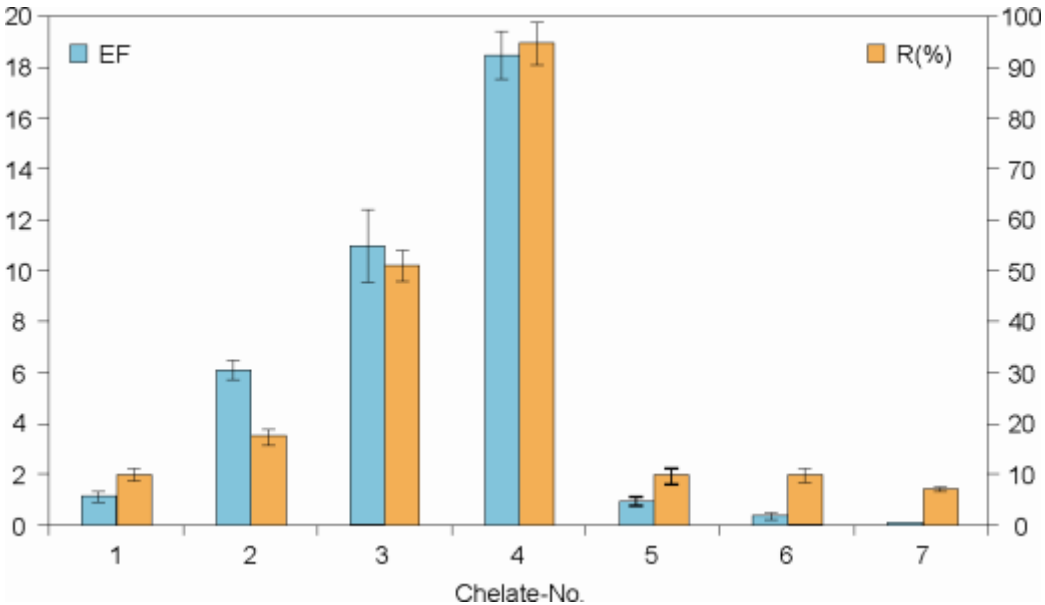


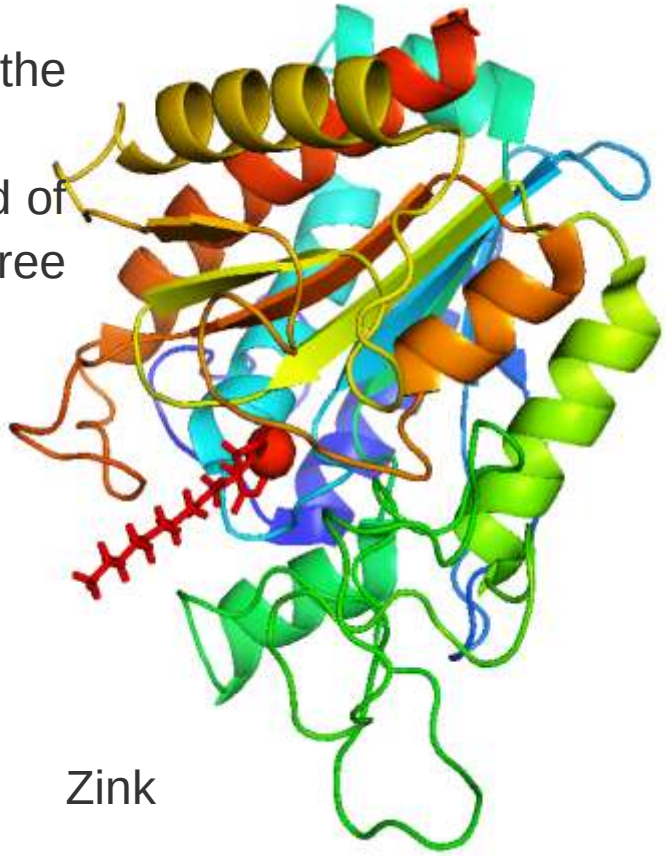
	Horseradish peroxidase
ER	17.7 ± 1.5
R (%)	85.3 ± 5.5

Gerken et al. Tweezing-adsorptive bubble separation. Analytical method for the selective and high enrichment of metalloenzymes. Anal. Chem. 77 (19), 6113-6117, 2005.

Carboxypeptidase A

- Digestive group of enzymes having primarily the function to break down peptides in the gut
- catalyzes the hydrolysis of the terminal amino acid of a polypeptide from the end that contains a free carboxyl group.





Zink

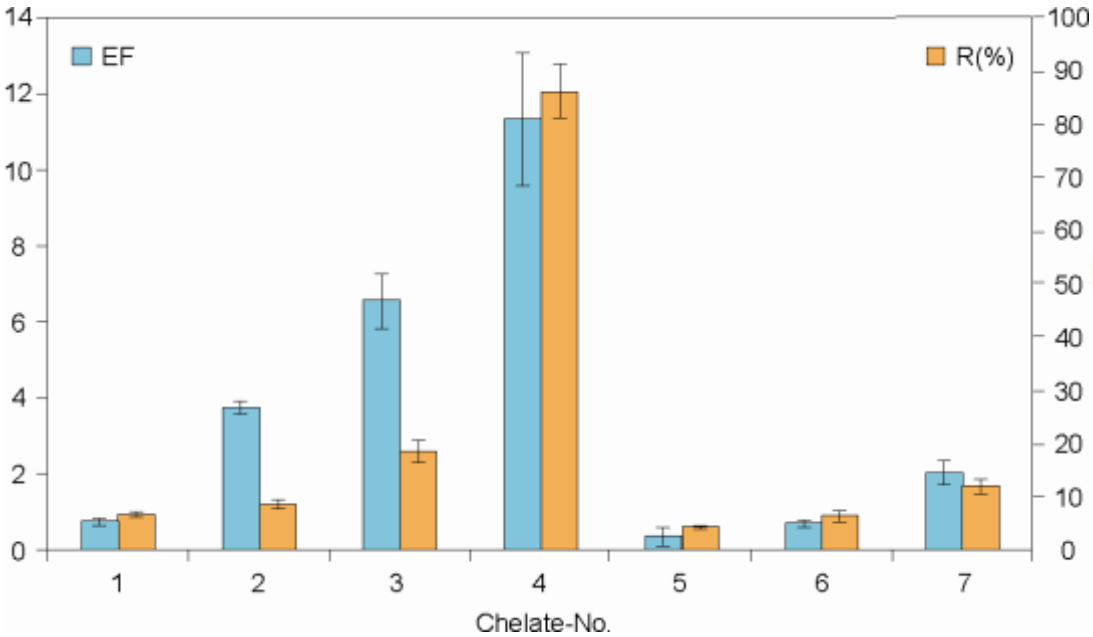
Enrichment factor (EF) and Recovery (R%) of Carboxypeptidase A in complex with ADA (1), ADA-C8 (2 to 4) and with Zn^{2+} - ADA-C8 (5-7) for verification.

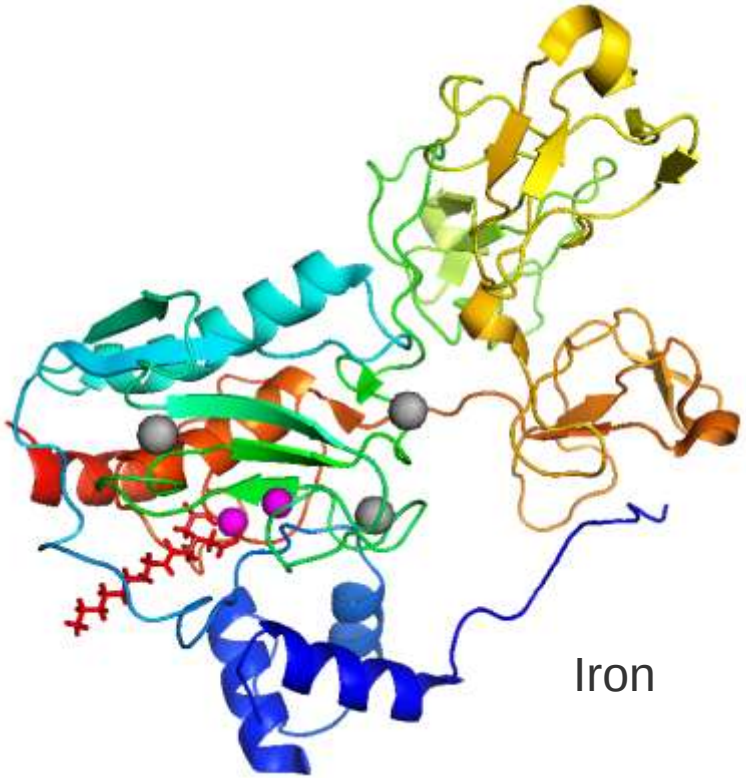
Haller et al. High enrichment of MMP9 and Carboxypeptidase A by tweezing adsorptive bubble separation (TABS). Appl Biochem Biotechnol, 2010, 162, 1547-1557

Matrix-Metalloproteinase; MMP9

- Central role in the breakdown of extracellular matrix
- Primary function is degradation of proteins.
- Negatively involved in a variety of pathological conditions such as cancer, inflammation, infection, brain degeneration, and vascular diseases once its activity is deregulated
- MMP-9 was suggested to have the potential as a biomarker for acute coronary syndrome

Matrix-Metalloproteinase MMP9



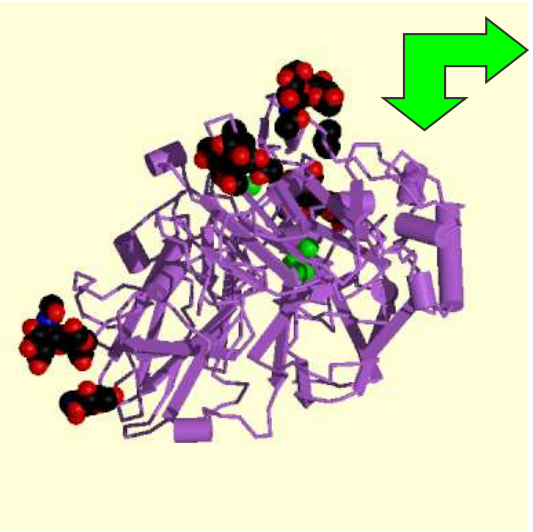


Enrichment factor (EF) and Recovery (R%) of MMP9 in complex with ADA (1), ADA-C8 (2-4) und with Zn^{2+} - ADA-C8 for verification (5-7)

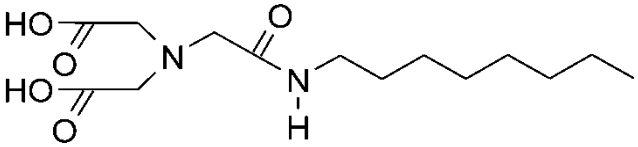
Haller et.al. High enrichment of MMP9 and Carboxypeptidase A by tweezing adsorptive bubble separation (TABS). Appl Biochem Biotechnol, 2010

Laccases (EC 1.10.3.2)

- multi copper-containing oxidase enzyms found in many plants, fungus and some bacteria
- Catalyse polymerization or depolymerization processes
- For pulp delignification, textile dyeing/textile finishing, cosmetics, oxidation of organic pollutants, diagnostic, and synthetic uses



ADA-C8



Chelator Spacer Surface active part

Enrichment

13-fold

Structure of Laccase C (● Cu-Ions); source: www.expasy.org. This enzyme contains a three-fold cored cupper cluster, consisting of a two-cored Cu^{II} unit (type 3), of a three-fold coordinated one-cored Cu^{II} center (type 2), and additionally, of a blue copper center (type 1).

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Biotechnology Advances 24 (2006) 500–513

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Research review paper

Industrial and biotechnological applications of laccases: A review

Susana Rodríguez Couto *, José Luis Toca Herrera *

Department of Chemical Engineering, Rovira i Virgili University, Av. Països Catalans 26, 43007 Tarragona, Spain

Received 20 January 2006; received in revised form 29 March 2006; accepted 1 April 2006
Available online 18 April 2006

Abstract

Laccases have received much attention from researchers in last decades due to their ability to oxidise both phenolic and non-phenolic lignin related compounds as well as highly recalcitrant environmental pollutants, which makes them very useful for their application to several biotechnological processes. Such applications include the detoxification of industrial effluents, mostly from the paper and pulp, textile and petrochemical industries, use as a tool for medical diagnostics and as a bioremediation agent to clean up herbicides, pesticides and certain explosives in soil. Laccases are also used as cleaning agents for certain water purification systems, as catalysts for the manufacture of anti-cancer drugs and even as ingredients in cosmetics. In addition, their capacity to remove xenobiotic substances and produce polymeric products makes them a useful tool for bioremediation purposes. This paper reviews the applications of laccases within different industrial fields as well as their potential extension to the nanobiotechnology area.

2.1. Food industry

Laccases can be applied to certain processes that enhance or modify the colour appearance of food or beverage. In this way, an interesting application of laccases involves the elimination of undesirable phenolics, responsible for the browning, haze formation and turbidity development in clear fruit juice, beer and wine.

Laccases are currently of interest in baking due to its ability to cross-link biopolymers. Thus, Selinheimo et al. (2006) showed that a laccase from the white-rot fungus *Trametes hirsuta* increased the maximum resistance of dough and decreased the dough extensibility in both flour and gluten dough.

Recently, Minussi et al. (2002) have described the potential applications of laccase in different aspects of the food industry such as bioremediation, beverage processing, ascorbic acid determination, sugar beet pectin gelation, baking and as a biosensor. However, they suggested that more studies of laccase production and immobilisation techniques at lower costs are needed to improve the industrial application of this enzyme.

Advantages of ABS

- ABS is a powerful technique for the removal or enrichment of surface-active and -inactive substances.
  - either soluble or insoluble
  - from aquatic solutions
  - from suspensions
  - from high diluted solutions
  - at room temperature
  - with different gases
- ABS is cost efficient and sustainable.
  - It uses basic materials such as glass ware, stainless steel, etc., therefore, development and maintenance costs are low.
  - The use of e.g. solvents can be omitted – ABS is an eco-friendly method
- Obstacle:
  - Still empirical research on ABS is needed; numerous parameters have to be investigated for achieving the highest efficiency of enrichment possible

We gratefully acknowledge the support of the following institutions:

- European Union (EU-ISTC-G-777)
- Federal Ministry for Education & Research (BMBF), Berlin, Germany (0339819/2)
- Research Circle of the Nutrition Industry (FEI), Bonn, Germany (498/02)
- Adalbert Raps Research Center, Kulmbach, Germany (2-2004)

THANK YOU FOR YOUR ATTENTION!