The *in vitro* hydrolysis of isoeugenyl acetate and eugenyl acetate

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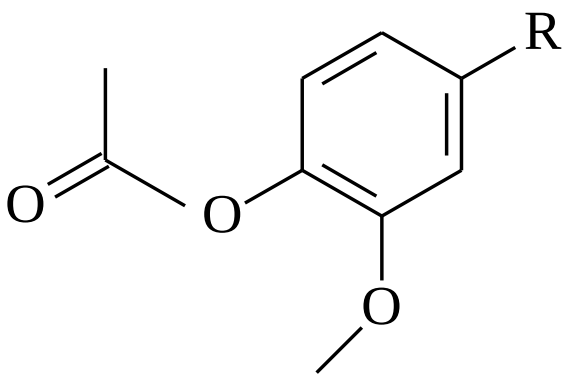
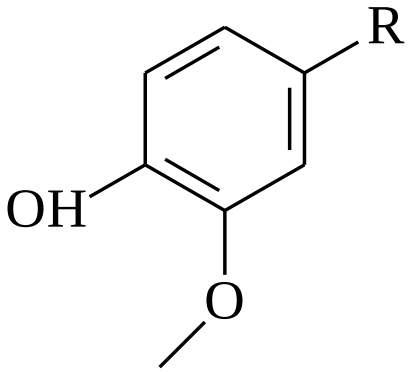
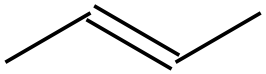
Objective

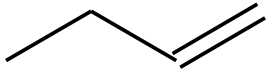
Determine the extent of hydrolysis for isoeugenyl acetate and eugenyl acetate in various biological matrices

Hydrolytic Reaction





IA, R= 

EA, R= 

ester

M.W 206.24

alcohol

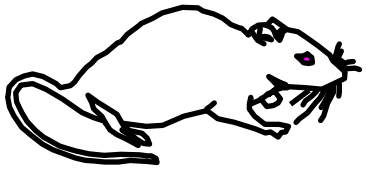
M.W 164.20

Specific Aims

- Determine if skin, blood, and liver possess hydrolytic activity towards isoeugenyl acetate and eugenyl acetate
- Establish stoichiometric relationship for the disappearance of the esters and the formation of the alcohols over time
- Determine kinetic constants for the hydrolytic conversion to the corresponding alcohols in the respective tissues.
- Assess the integrated metabolism of the esters in monolayers of SD rat hepatocytes, an intact cellular system

Isolation and Treatment

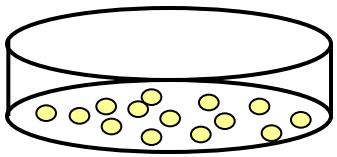
Male SD rat

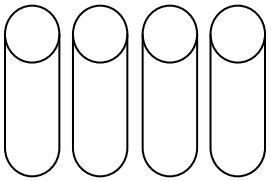


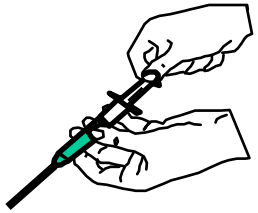
Liver Skin Blood

Hepatocyte Isolation

Subcellular Fractionation







IA or EA
~500 μM

Incubate 37°C → sample prep → HPLC

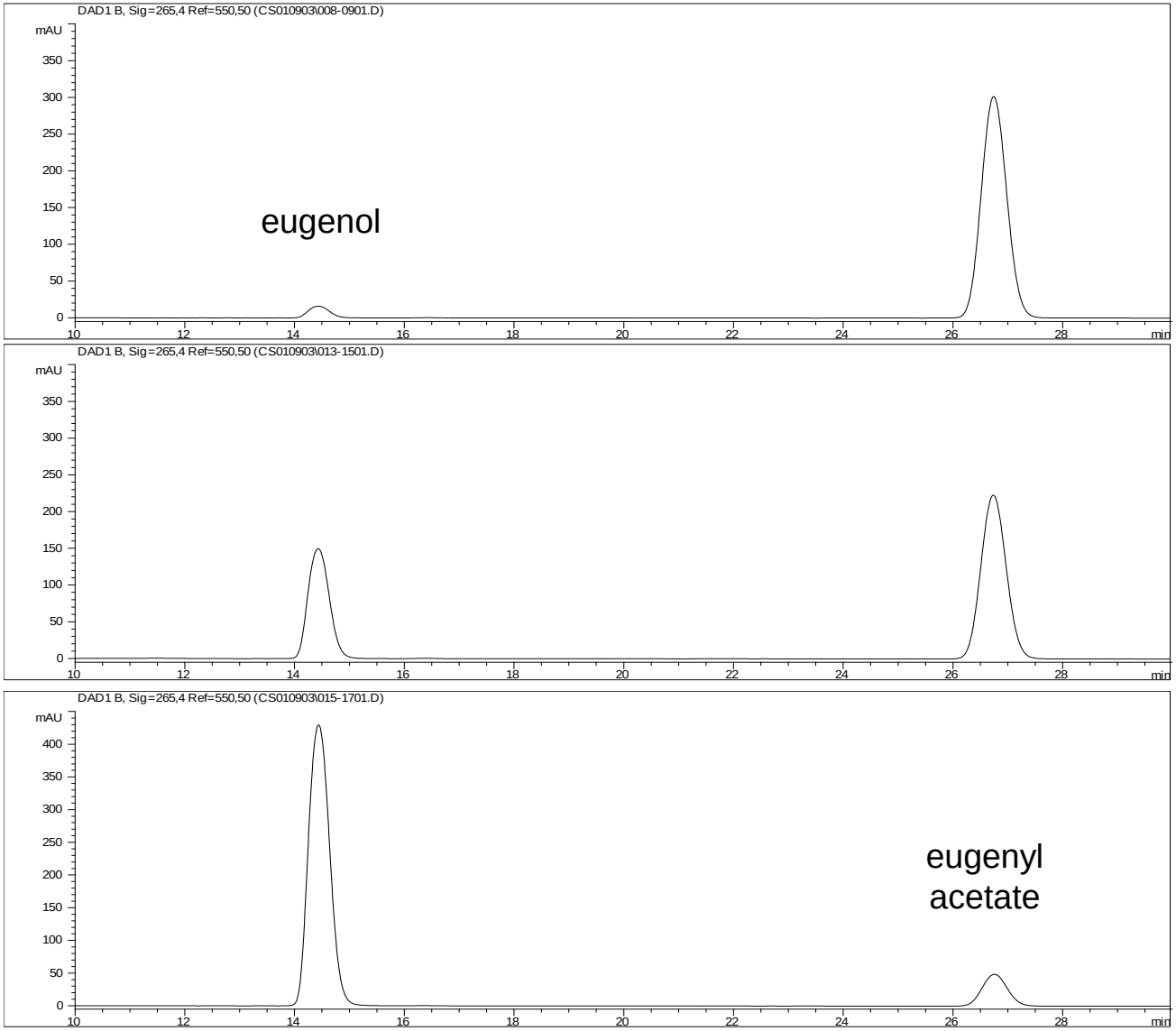
- Reversed-phase alpha bond C18 column
- Mobile phase: acidified water and acetonitrile
- Monitoring wavelengths of 254 nm and 265 nm

Hydrolysis of EA by rat hepatic microsomes - (HPLC Profile)

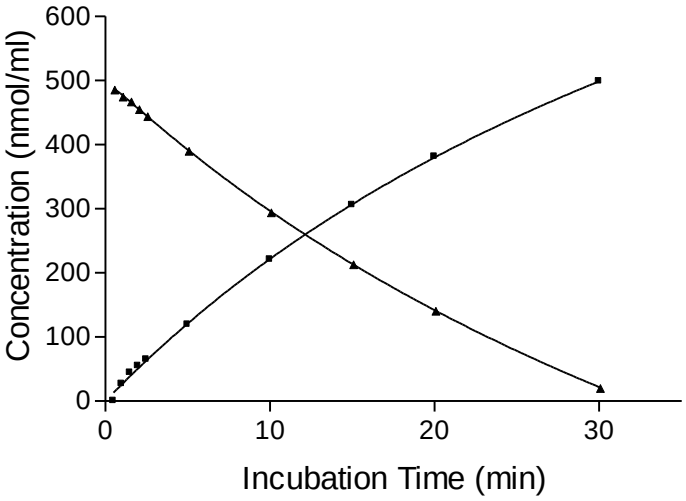
A. 0.5 min

B. 5 min

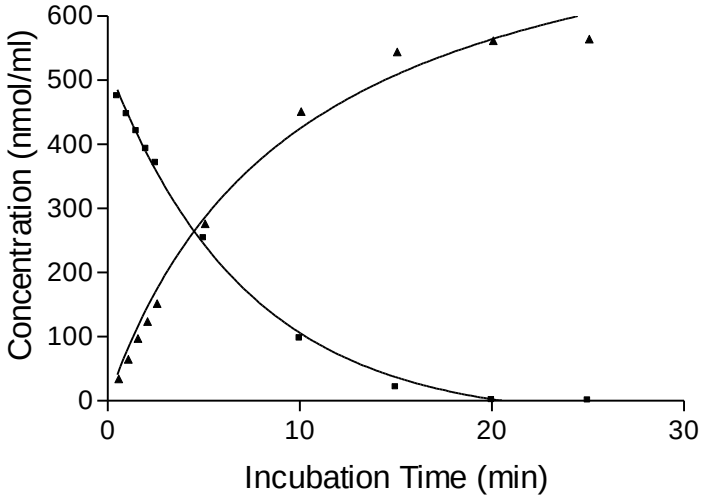
C. 20 min



Ester Hydrolysis in hepatic microsomal preparations



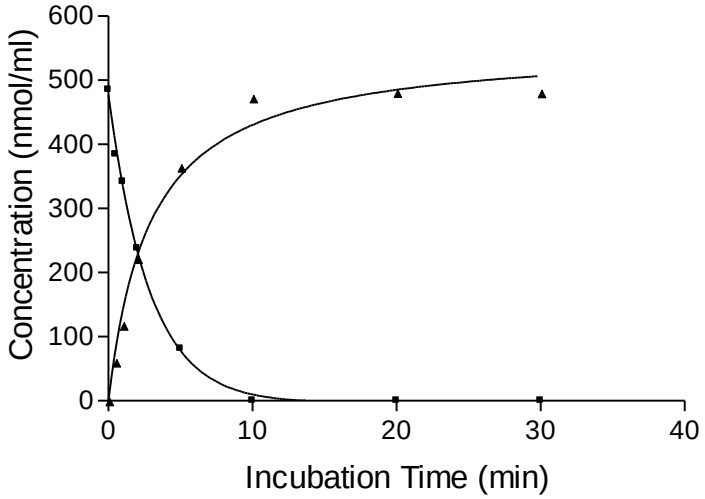
A. Human Male



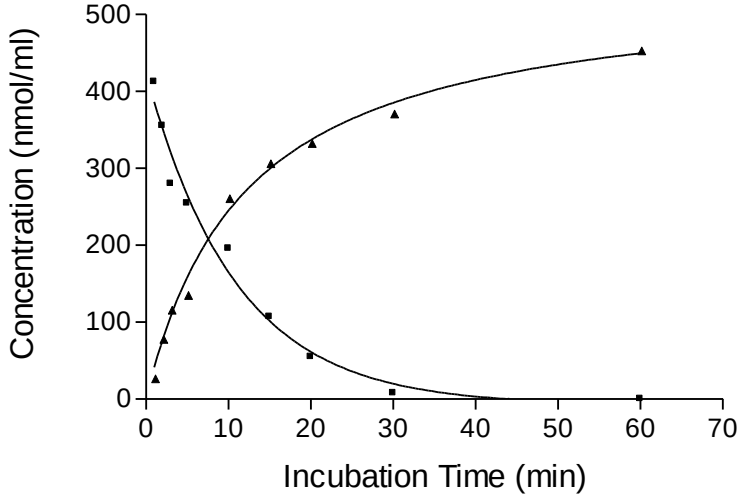
B. SD Male Rat

- Isoeugenyl-acetate concentration : 532 nmol/ml, (0.11 mg/ml) N=3
- Human female hepatic tissue also hydrolyzed IA to isoeugenol within 30 min
- Hydrolytic activity towards eugenyl-acetate is comparable for the respective tissues

Ester Hydrolysis in human blood and plasma preparations



A. Blood:PBS (1:3)



B. Plasma:PBS (1:10)

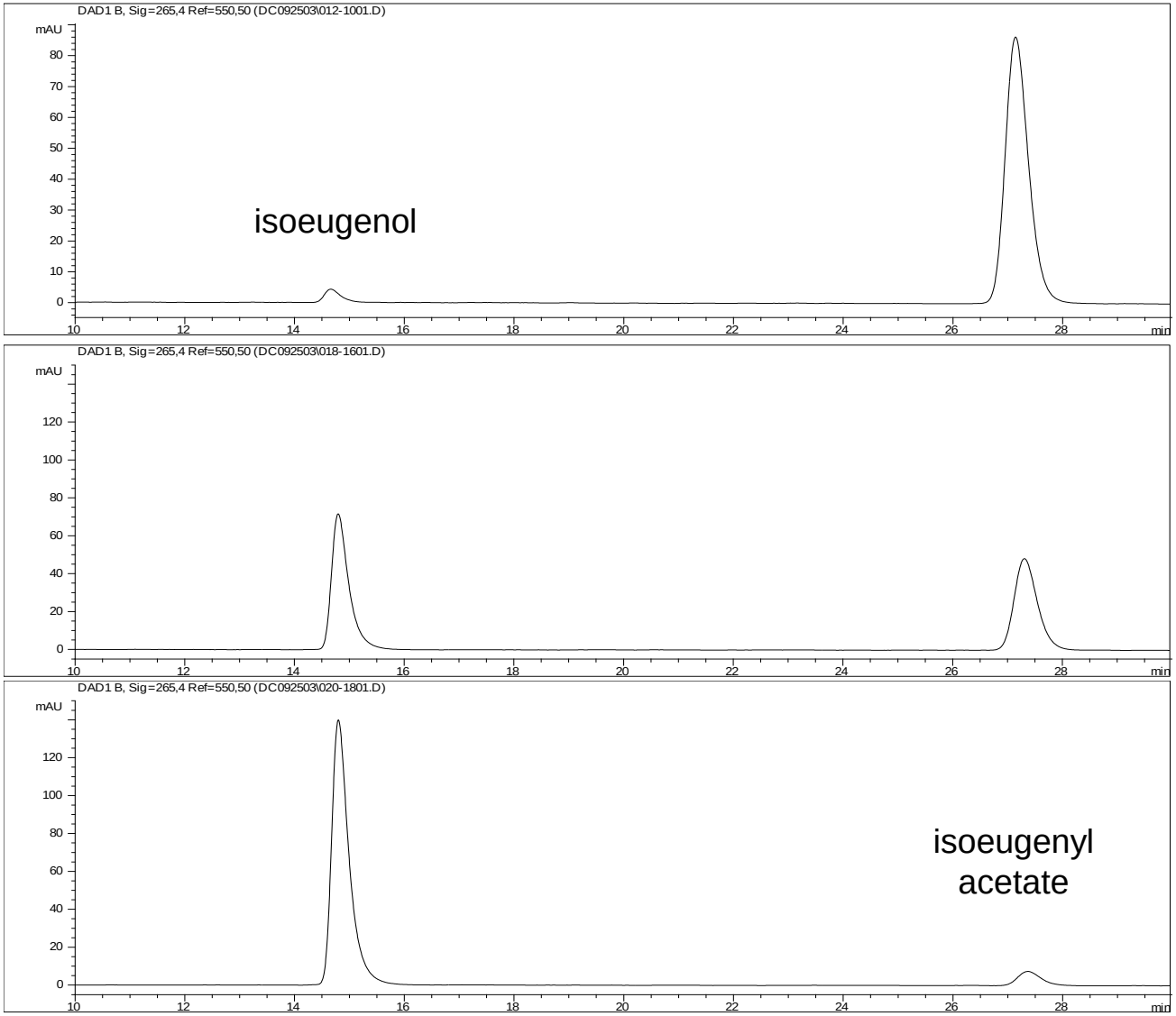
- Isoeugenyl-acetate concentration : 488 nmol/ml, (0.10 mg/ml) N=2
- Blood and plasma from male SD rats also hydrolyzed both IA and EA.
- Studies indicate increased carboxylesterase activity in plasma preparations

Hydrolysis of IA by rat skin microsomes - (HPLC Profile)

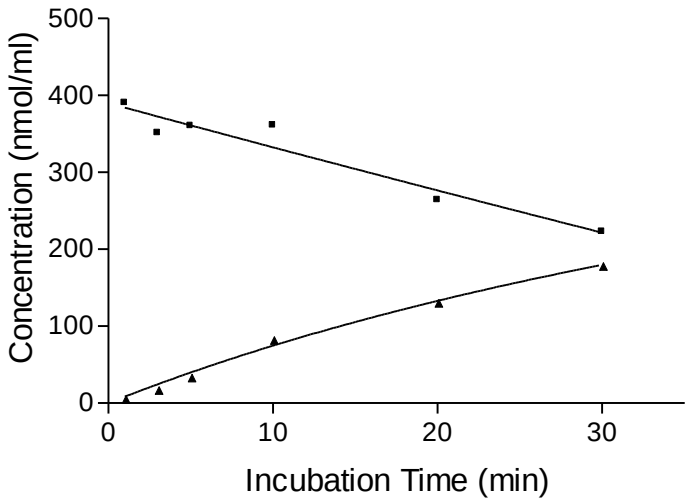
A. 1 min

B. 20 min

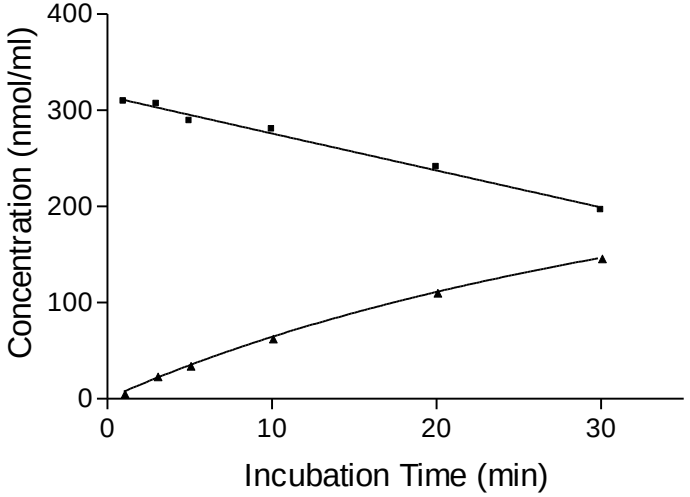
C. 60 min



Ester Hydrolysis by rat skin microsomes and cytosol



A. Microsomes



A. Cytosol

- Isoeugenyl-acetate concentration : 488 nmol/ml, (0.10 mg/ml) N=4
- Microsomal Incubations consisted of 0.0375 mg/ml protein
- Cytosol Incubation consisted of 0.1 mg/ml protein
- Studies with eugenyl-acetate are comparable

Parameters for Kinetic Analysis

- Formation of isoeugenol and eugenol was measured in skin and hepatic tissue preparations

- Skin Protein Concentrations: 0.0375 (M) & 0.1 mg/ml (C)

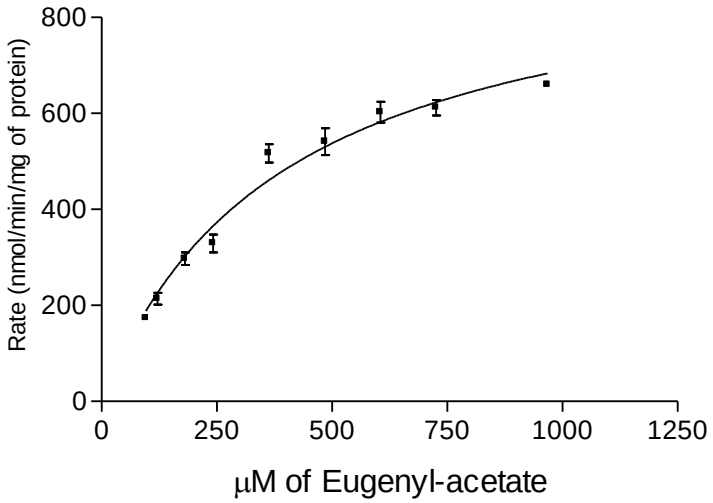
- Hepatic Protein Concentrations: 0.0125 (M) & 0.1 mg/ml (S-9)

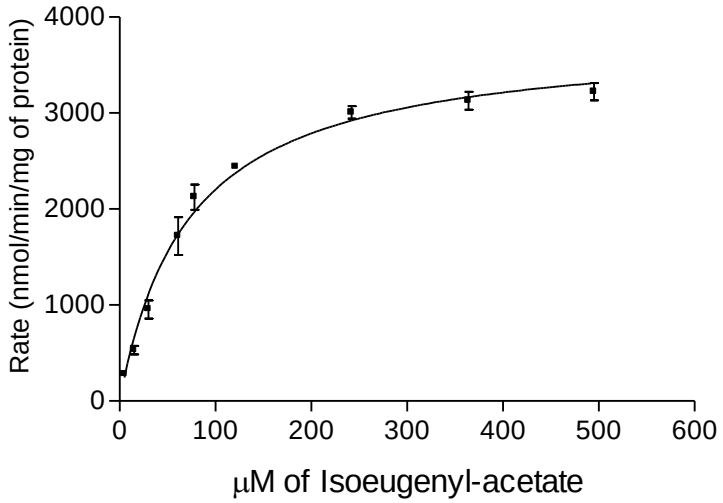
- Dosing Range: IA [5 to 495 μM], EA [39 to 970 μM]

- Duration of incubation: skin (15 min) and hepatic (3 min)

- Kinetic Data was normalized to nmol / min / mg of protein

Concentration Dependent Ester Hydrolysis





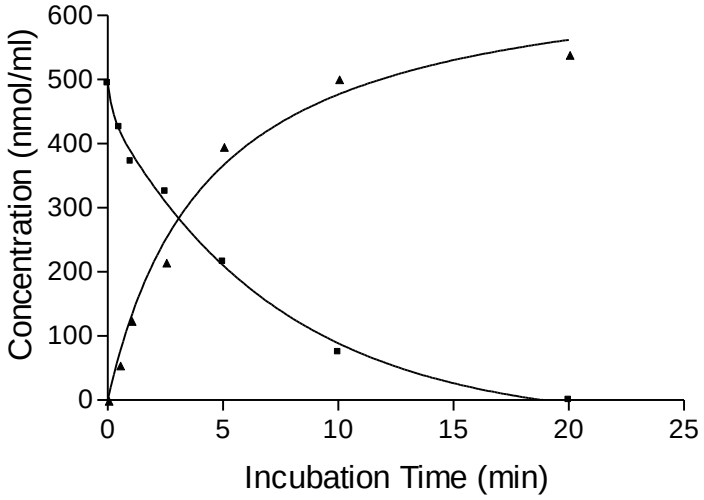
- Formation of eugenol by male SD rat skin microsomes (left) N=2
- Formation of isoeugenol by human male hepatic microsomes (right) N=3

Kinetic Constants for Alcohol Formation

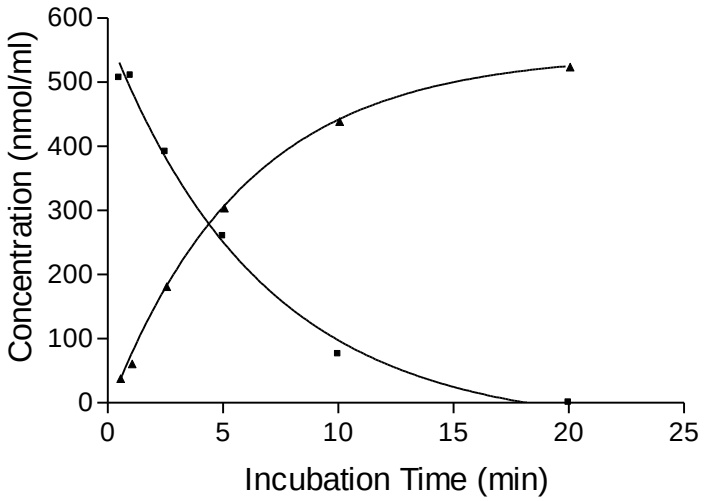
Subcellular Fraction	V_{max} (nmol/min/mg protein)		K_m (μM)		CL_{int} (ml/min)	
	IA	EA	IA	EA	IA	EA
Rat Skin Cytosol	97	114	137	216	0.7	0.5
Rat Skin Microsomes	505	638	190	223	2.7	2.9
Rat Hepatic S-9	52	60	110	173	0.5	0.4
Rat Hepatic Microsomes	3822	3829	91	97	42.0	**39.6**
Human Male Microsomes	3795	3656	72	89	52.5	41.3
Human Female Microsomes	3072	2748	51	52	60.1	52.9

- CL_{int} = intrinsic metabolic clearance (Vmax / Km)
- Note the similarities between human and rat hepatic microsomes with respect to the intrinsic metabolic clearance of EA and IA

Integrated Metabolism of the esters in an intact cellular system



A.



B.

- Isoeugenyl-acetate (A) concentration: 538 nmol/ml (0.11 mg/ml)
- Eugenyl-acetate (B) concentration: 483 nmol/ml (0.10 mg/ml)
- Within approximately 20 minutes hydrolytic conversion was complete

Summary

- IA and EA are rapidly hydrolyzed by tissue esterases to the corresponding alcohols, isoeugenol and eugenol.
- Stoichiometric conversion to the alcohols was observed in hepatic, blood, and skin preparations when incubated with IA and EA.
- When the localization of esterases was evaluated, the most extensive activity was observed in the hepatic microsomal fraction.
- Human hepatic and blood preparations displayed similar rates of hydrolysis to that of rodents when incubated under the same conditions.

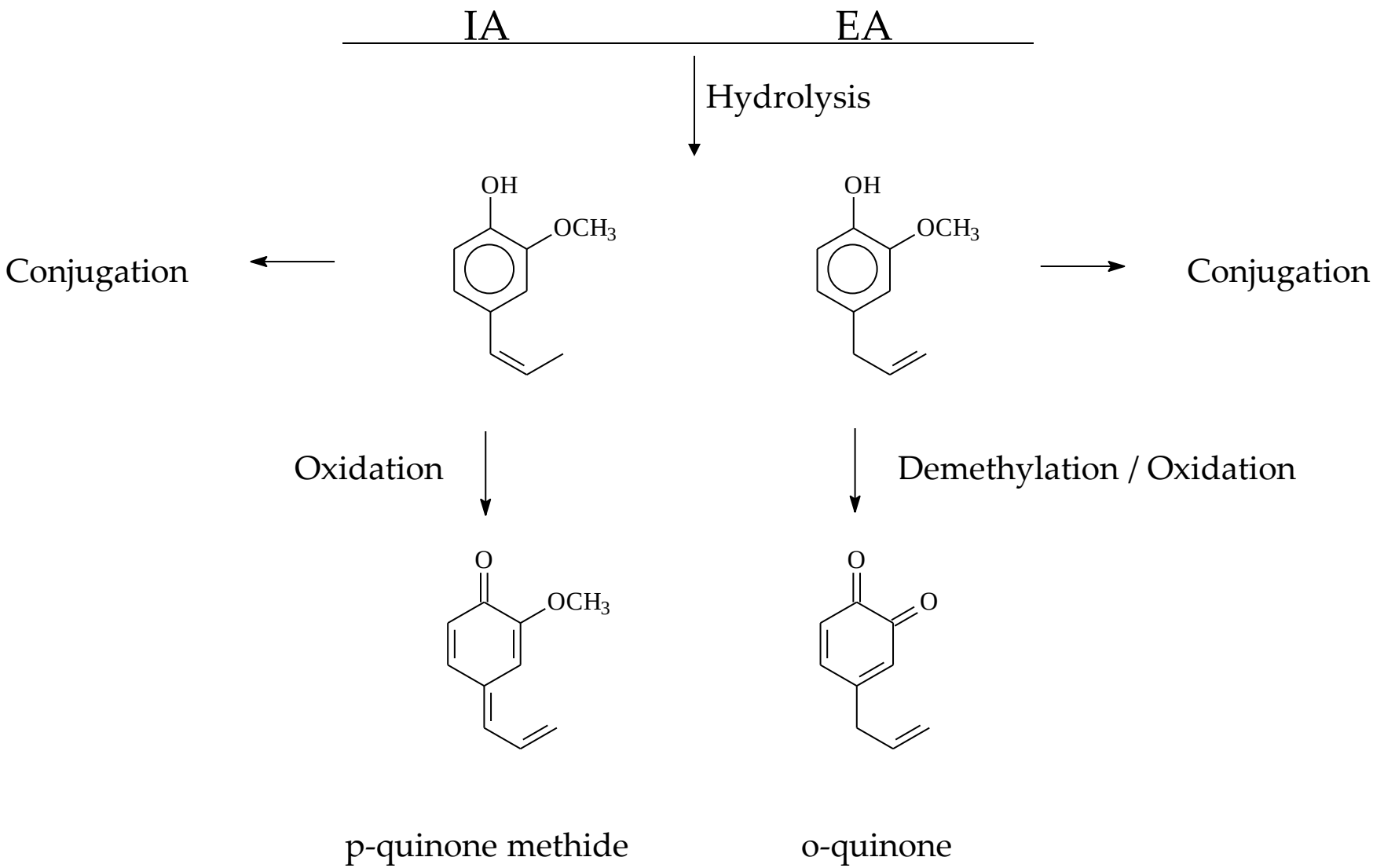
Conclusions

- Absorption of IA and EA into the systemic circulation would be minimal due to hydrolytic activity in skin, blood as well as the liver
- Toxicological data presently available for isoeugenol and eugenol can be used for the safety assessment of the esters
- Animal data can be extrapolated to humans for rational safety evaluation for the use of esters in fragrance and food products
- Slow rate of hydrolysis of esters in skin correlates with their decreased sensitization potential

NOELs based on HRIPT

Compound	NOEL
Isoeugenyl-acetate	2 %
isoeugenol	0.2-0.5 %
eugenyl-acetate	8 %
eugenol	~ 5 %

Potential reaction pathways for skin sensitization



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