LIFE ENV/GR/000223, DIONYSOS project

Development of an economically viable process for the integrated management via utility of winemaking industry wastes:

i. production of high added value natural products, and

ii. organic fertilizer

SERKOS A. HAROUTOUNIAN

Professor of Chemistry

AGRICULTURAL UNIVERSITY OF ATHENS

Department of Sciences - Chemistry Laboratory

The vinification process produces large quantities of solid organic waste, mainly composed by sugars, polyphenols, lipids, tannins etc.

In Greece, annually the 450 wineries process about **550.000** tones of grapes producing Solid Organic Waste (SOW) that accounts about **13-15 % of the processed biomass**

Most wineries are small enterprises -spread around Greece- that cannot afford to manage their wastes by environmentally acceptable practices.

Thus, they usually discard them to the environment in order to be transformed to fertilizer by aerobic degradation.

The large volume and the organic components of the waste constitute serious environmental pollution factors, because they

possess significant antimicrobial and phytotoxic properties which limit the spectrum and activity of microorganisms involved and slower (or stop) their bio-degradation process

This uncontrolled disposition of waste to local environment results in the appearance of intense phytotoxic phenomena which

a. affects the plant growth,

b. contaminates the water,

c. results in the degradation of the quality of the drinking water and/or

the death of fragile aquatic animal species

Main objective of the project was the development of an economically viable process for the integrated management of winemaking industry wastes and the recovery of the high added value natural polyphenols

The technology used is friendly for the environment since utilizes as solvents only water and alcohol (both are fully recyclable)

and has the advantage to

1. require small capital and space investments,

2. be adaptable by all wineries, since refers to the compilation of simple sub-units

3. be used for the management of other agricultural wastes

The process refers to the extraction of solid waste, the subsequent adsorption of polyphenols by resins and the polyphenol recovery.

The recovery of high added value polyphenols is of considerable financial and market interest since they exhibit significant biological activities, mainly as antioxidants. Research results have indicated that they act as inhibitors of the oxidation of human low density lipoprotein (LDL) cholesterol retarding the appearance of athirogenesis and the development of Coronary Heart Diseases (CHD).

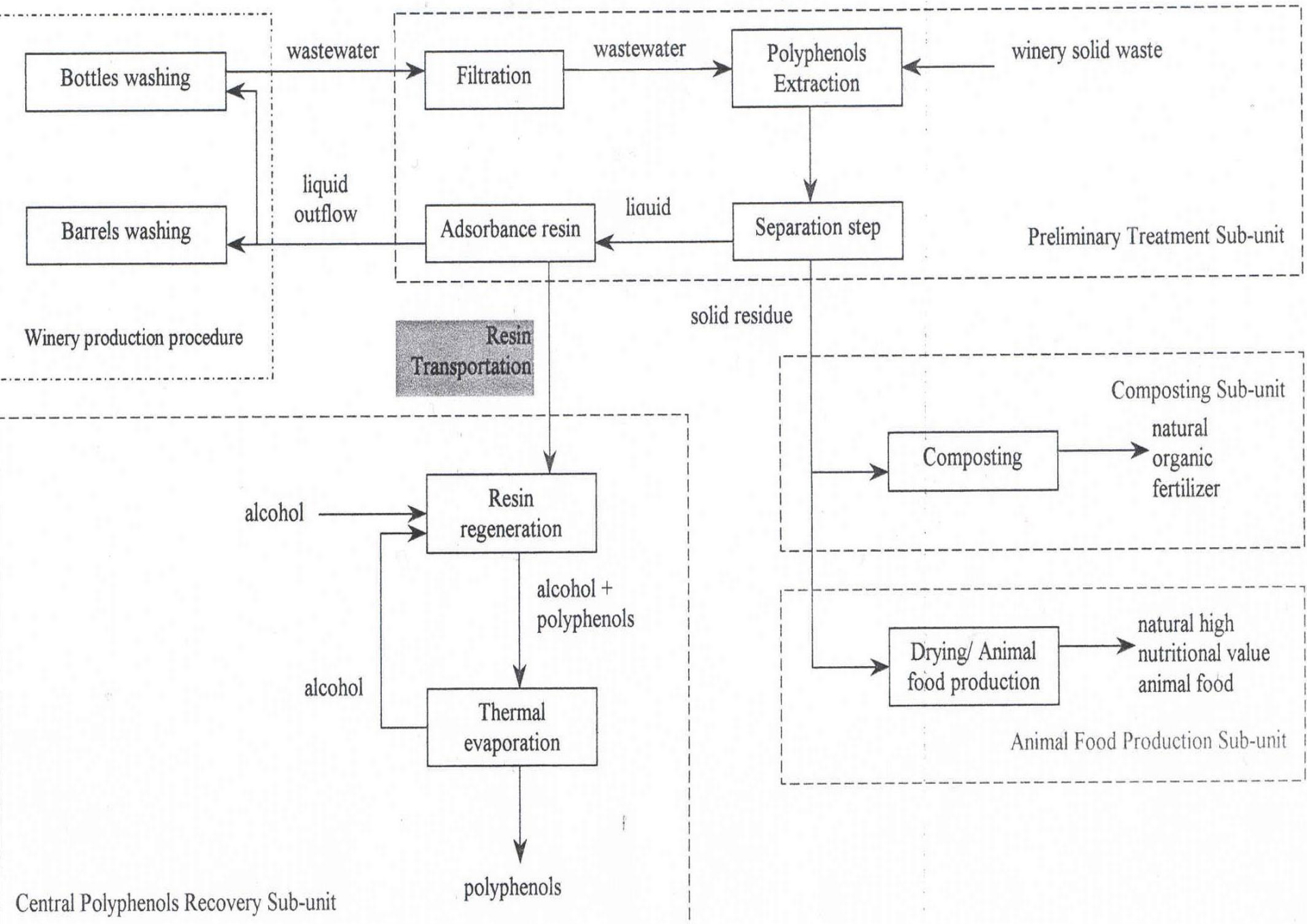
In this regard, polyphenols find wide applications and uses either as food supplements and/or row materials for food, cosmetic and drug industries.

The integrated management system of winemaking industry wastes also includes the use of the remaining (after the polyphenol recovery process) solid for the production

of high nutritional value animal food, since contains significant amounts of amino acids, proteins, sugars etc

natural organic fertilizer by composting – a controlled, non polluting process.

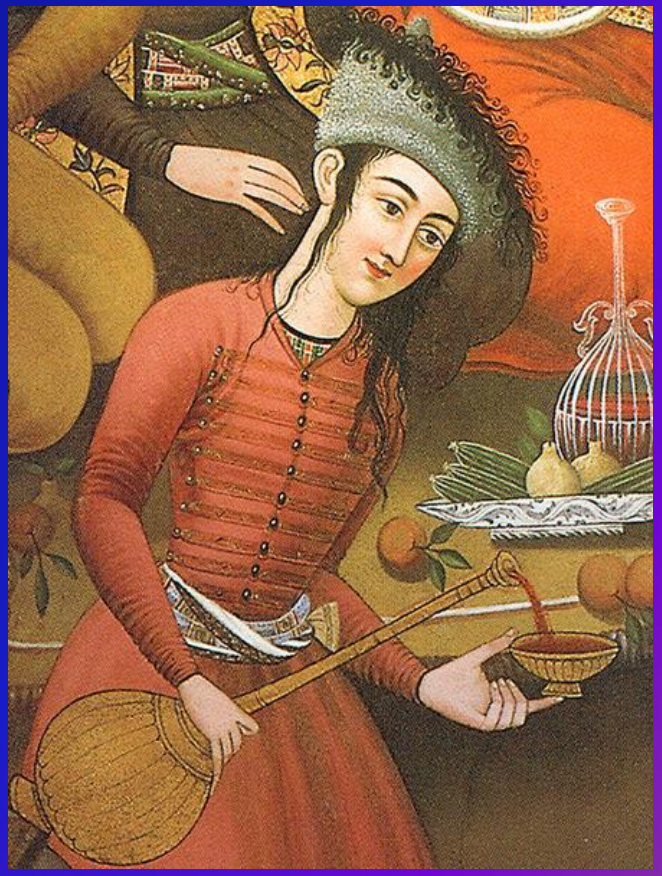
DIONYSOS - Pilot Plant Flow Diagram

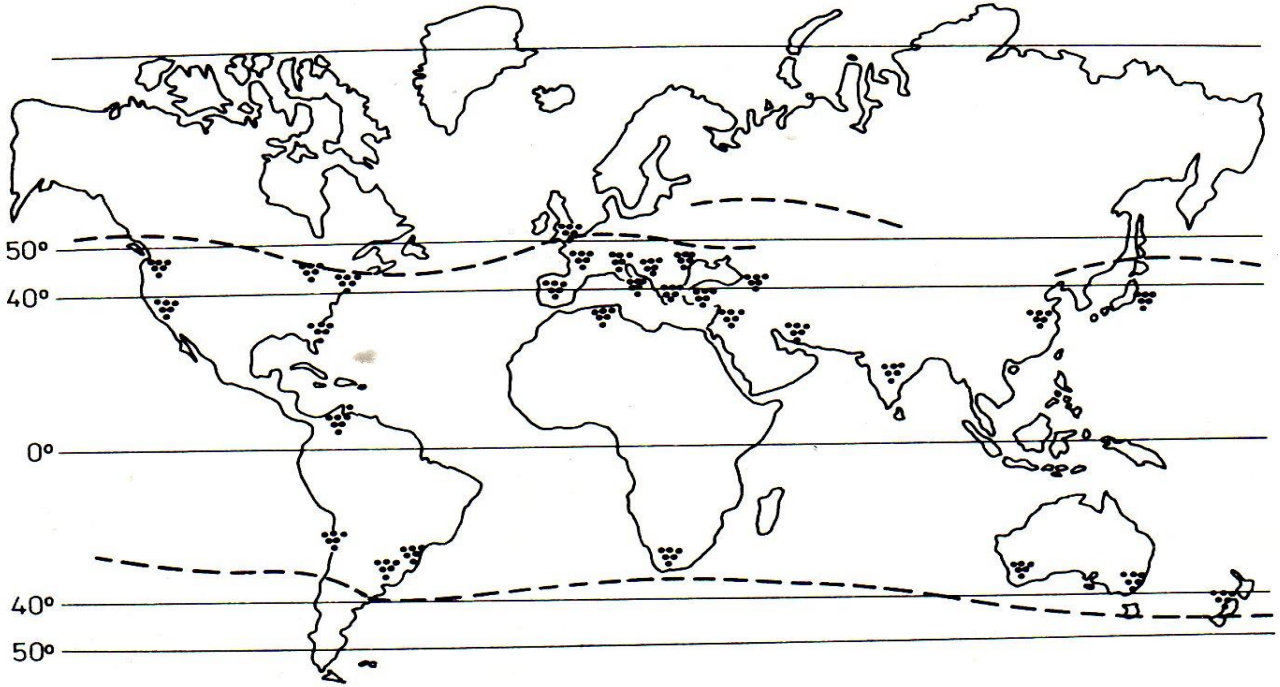


Wine is the product of yeast promoted alcoholic fermentation of grape juices

Viticulture and winemaking are practices known for many centuries. First recorded activity was in Asia minor at 7000 BC.

Wine has greatly contributed to human culture via dietary, medicinal and religious uses





Viticultural areas at the temperature zone of the world

Wine in Ancient Greece

The earliest evidence of wine production in Greece is a stone foot press at Vathipetro, a Minoan villa of Crete, dated at 1600 BC.

Wine in Ancient Greece

Wine was subject of great importance in Greek poetry, art and religion. Dionysus was the dedicated deity of this "elixir."

Greek islands were famous for their particular vintage flavor. Among them, the islands of Lesbos and Santorini were known to produce the most popular wines.

Wines from Santorini were exported to many parts of the known world, while Lesbos produced the very rare and sweet Essenczian wines.

Ancient Greeks mixed their wines with sea water and various spices.

The history of wine as a medicine

From ancient years, wine was considered as a powerful medicine and consumed by the privileged people

Some of traditional medical uses of wines

- Anticeptic
- Tranquillizer/sedative
- Hypnotic
- Anesthetic
- Antinauseant
- Treatment of anemia
- Mixed with other bioactive products (eg. honey)

Wine and health: contemporary trend

FRENCH PARADOX

↓

Moderate wine consumption

↓

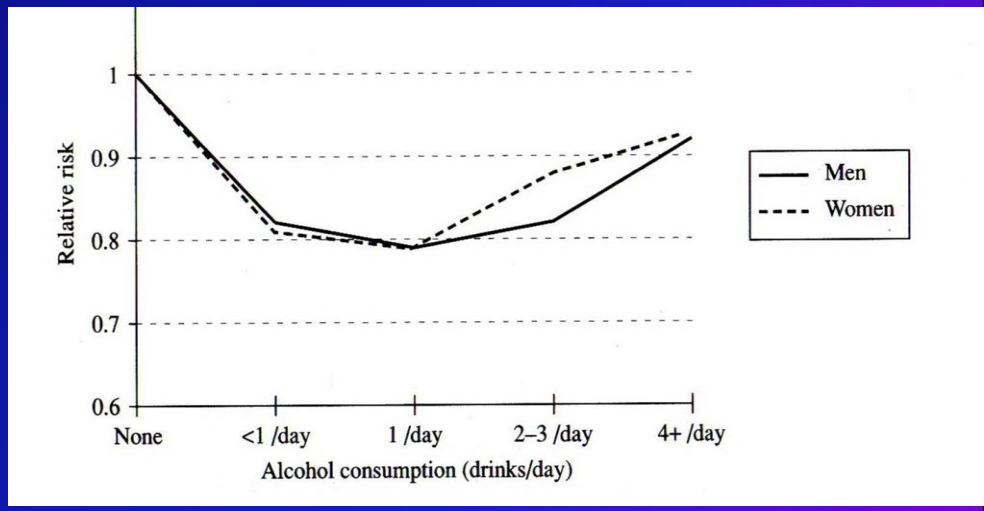
Low heart mortality rates

↓

Inhibition of LDL cholesterol oxidation

↓

Wine polyphenols



Grape berries content

- Water
- Carbohydrates
- Organic acids
- Lipids
- Vitamins
- Nitrogen containing compounds
- Minerals
- Phenolic compounds - POLYHENOLS

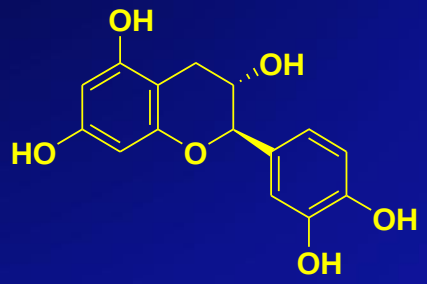
The polyphenol content of wines depends on:

- ✓ Grape variety
- ✓ Climate
- ✓ Soil
- ✓ Fungi infections
- ✓ Viticultural practices
- ✓ Grape ripening stage
- ✓ Vinification process

Polyphenols: group of secondary plant metabolites

Flavonoids

Flavan-3-ols

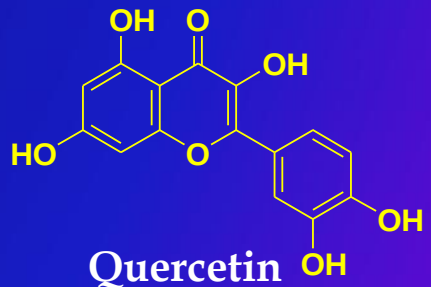


(+)-Catechin

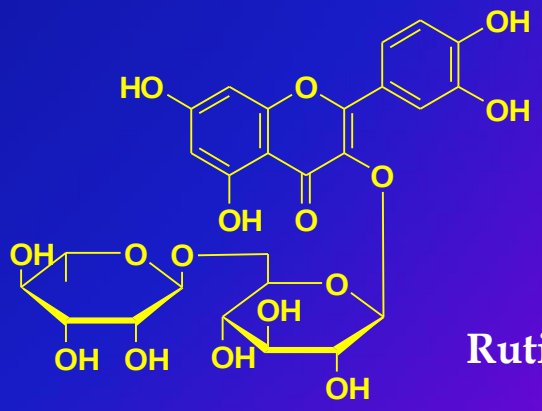
(-)-Epicatechin

Flavonols

Kaemferol



Quercetin

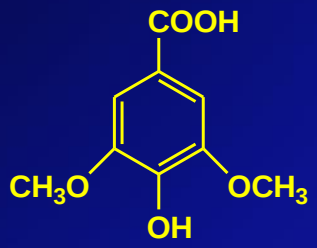


Rutin

Non-Flavonoids

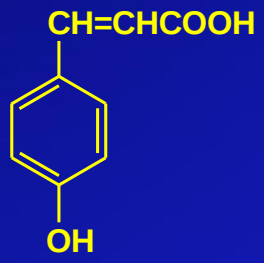
phenolic acids

Gallic acid



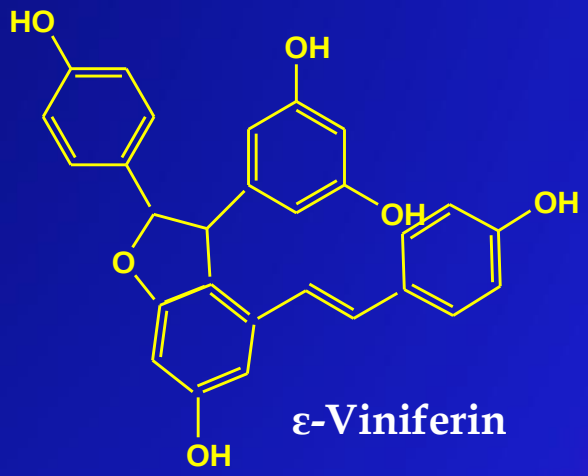
Syringic acid

Caffeic acid

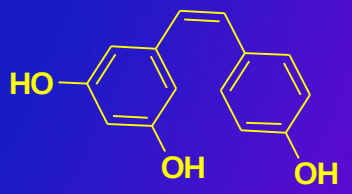


p-Coumaric acid

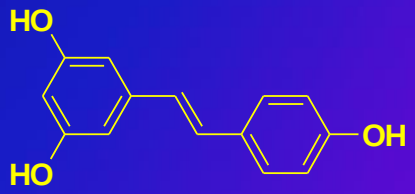
Stilbens



ε-Viniferin



Cis Resveratrol



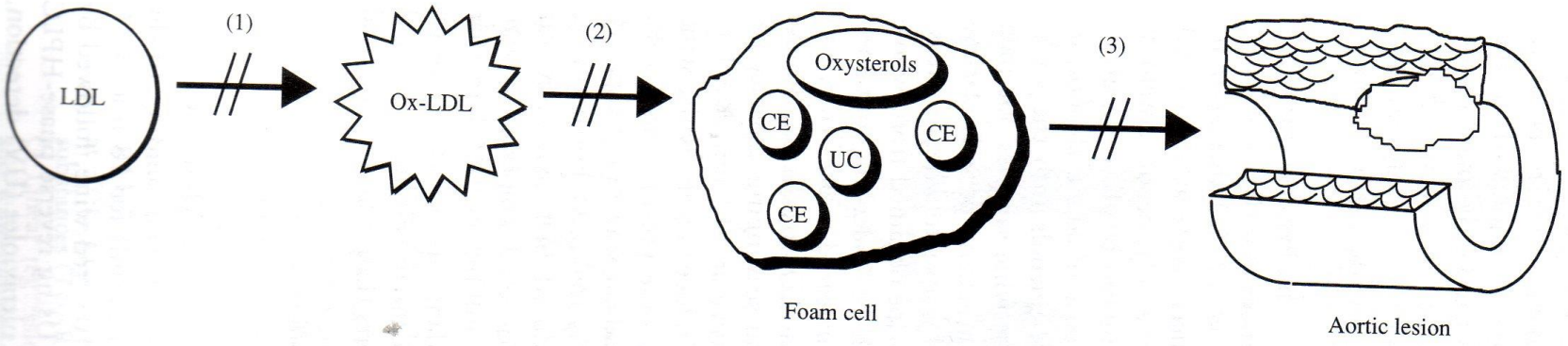
Trans- Resveratrol

MAIN BIOLOGICAL PRIOPERTIES OF POLYPHENOLS

- **Antioxidant**
- **Prevention of Coronary Heart Disease (CHD) Development**
- **Antimicrobial, Antiviral, Antiinflammatory**
- **Antitrhombotic, Antihypertensive, Vasodilatory**
- **Antimutagenic**
- **Anticancer**

The antioxidant activities of polyphenols are attributed on their ability to act as potent scavengers of:

- Hydroxy radicals
- Superoxide anions
- Lipid peroxy radicals



Schematic representation of Antiatherogenic effect of wine polyphenols
M.Aviram & B. Fubrman 2003

Solid Waste

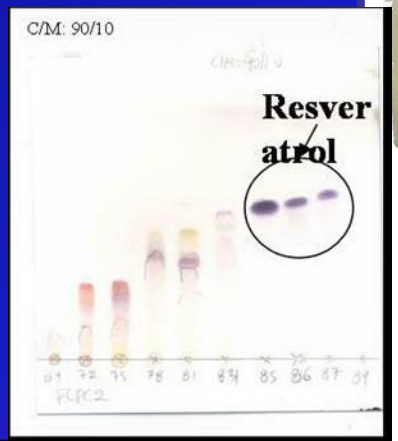
Pretreatment subunit

Production of organic fertilizer by composting

Production of animal food

Central unit for the recovery of high added value polyphenols

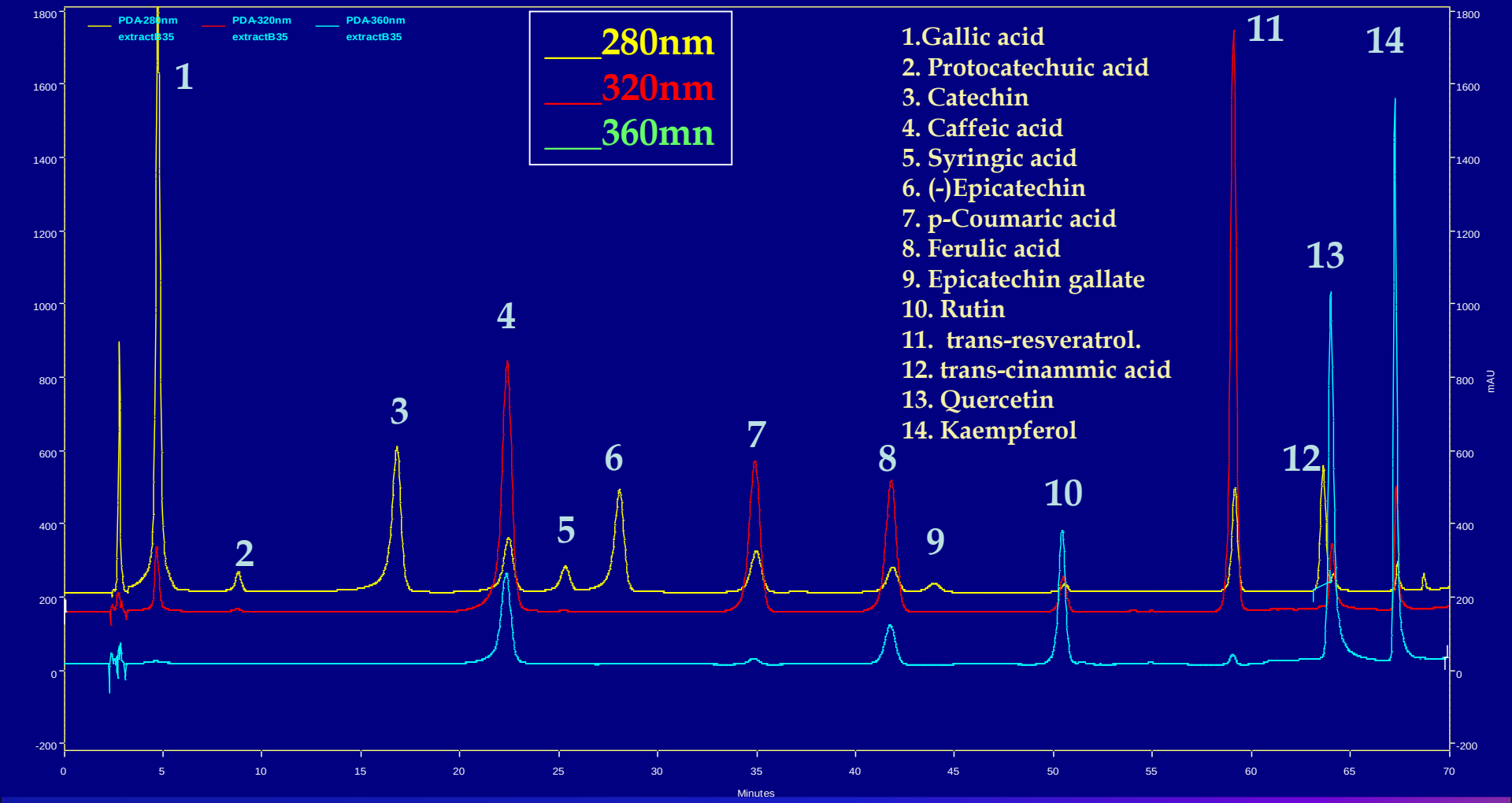
Recovery of high added value polyphenols



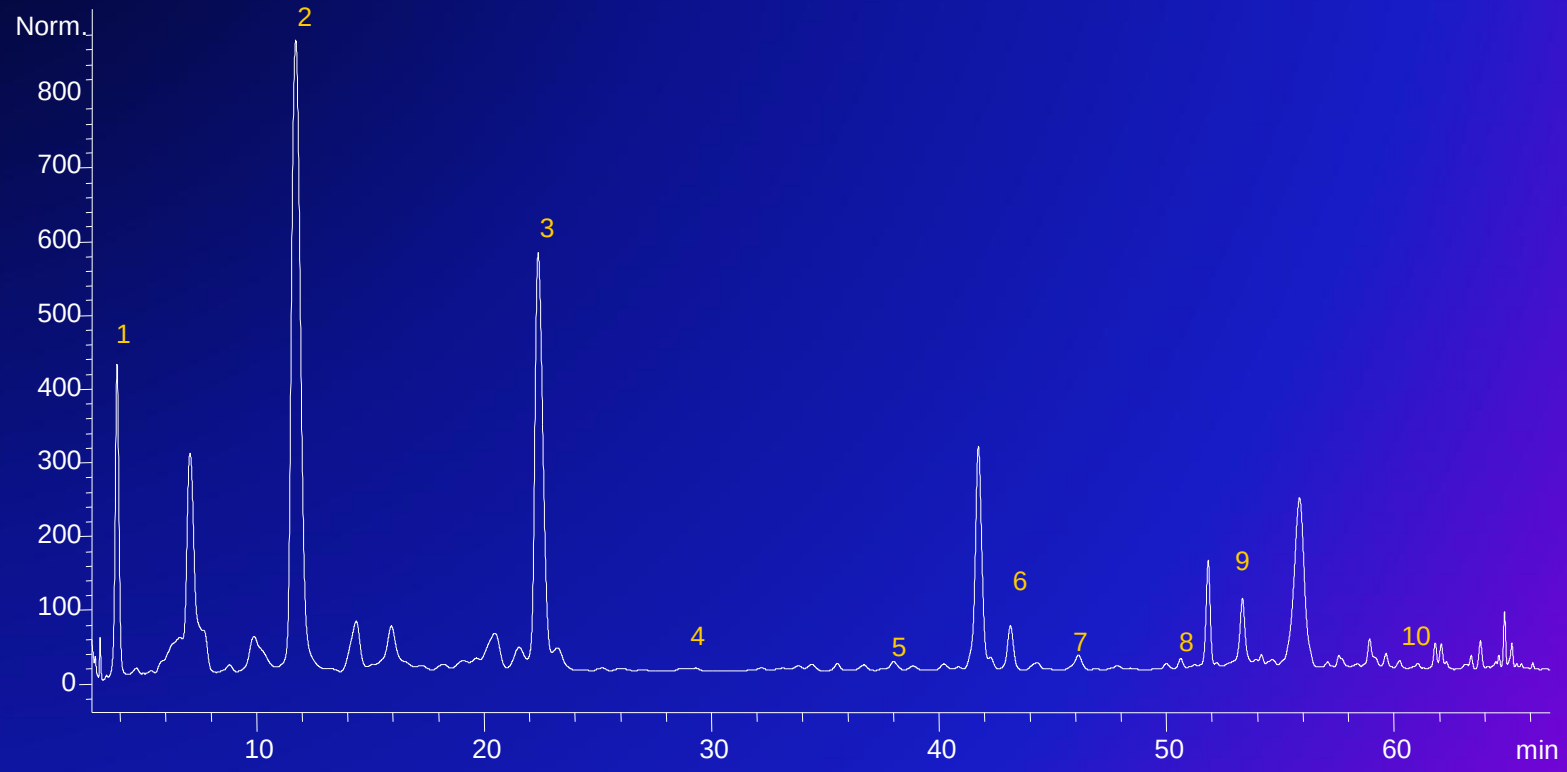
Polyphenols profile & quantitation assays:

- ✓ Photometric Methods (total phenols)
- ✓ Thin Layer Chromatography
- ✓ Gas Chromatography
- ✓ High Performance Liquid Chromatography

Polyphenol reference compounds



Qualitative and quantitative assays of bioactive compounds in vinification products using High Performance Liquid Chromatography (HPLC)



1 gallic acid, 2 catechin, 3 epicatechin, 4 p-coumaric acid, 5 ferulic acid
6 rutin, 7 tryptophol, 8 myricetin, 9 trans-resveratrol, 10 quercetin

GRAPE POMACE

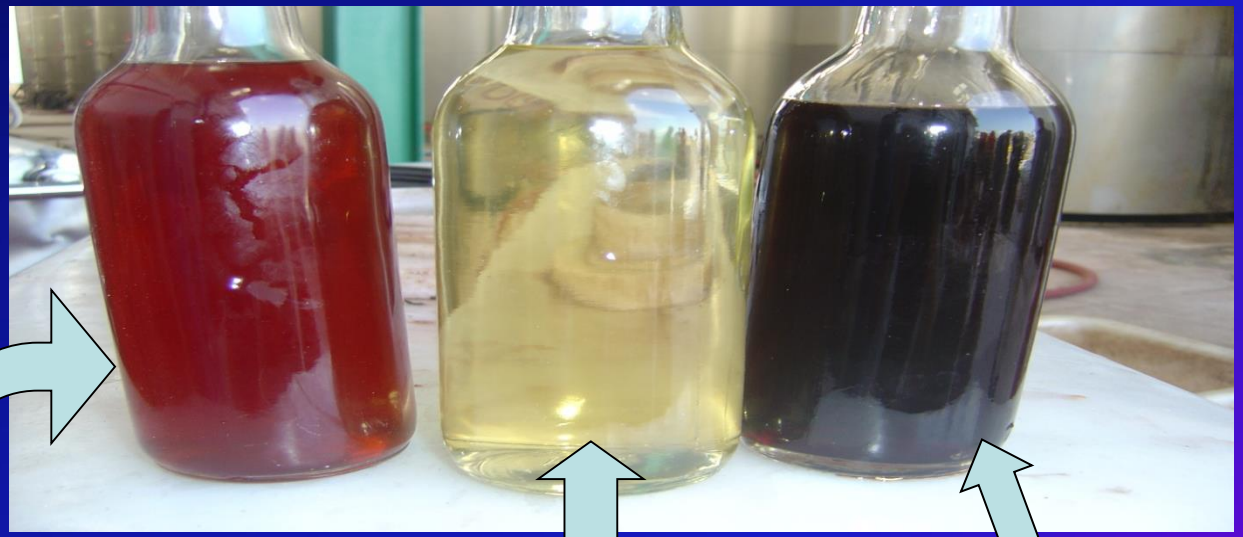
In Greece, every year more than 90.000 tones of grape pomace are being discarded in the environment

DIONYSOS system recovers the high added value polyphenols in three stages:

During the first stage, the grape pomace is air dried and extracted with a mixture of hot water-ethanol for 12 hours.

Then, the extract is diluted with 10 fold water and filtered.

- The second stage concerns the polyphenol adsorption by special resin columns. XAD-4 adsorb exclusively polyphenols, while XAD-7 adsorb tannins and colorants
- When columns were eluted with ethanol, they furnished the corresponding adsorbed natural products (and can be re-used, regeneration)
- The eluent solvent (water-alcohol mixture) is a odorless, off-yellow liquid, free of polyphenols.



Water-alcohol extract

Liquid product of resin columns

Polyphenol extract (alcoholic)

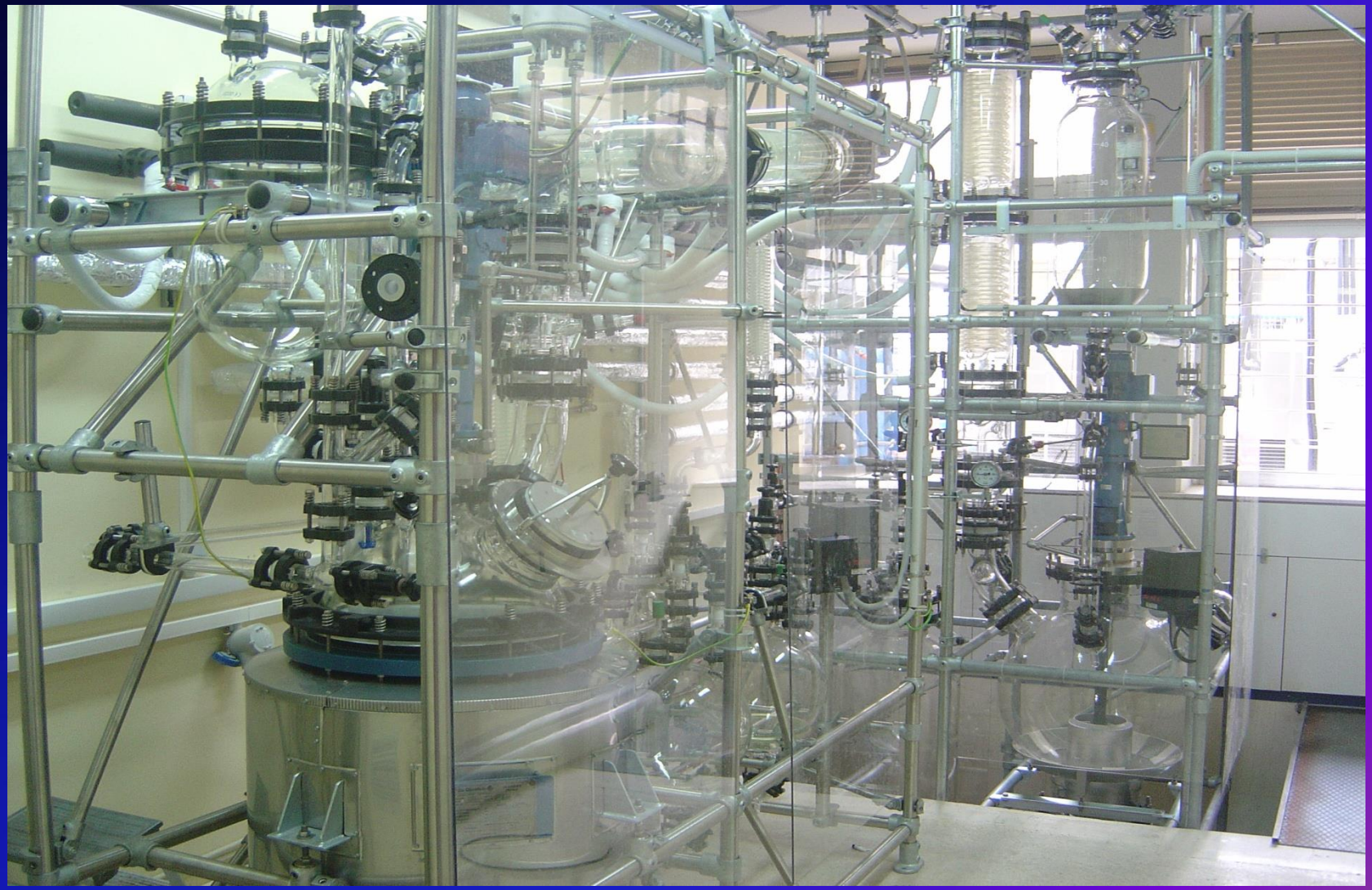
Adsorption Resin Columns

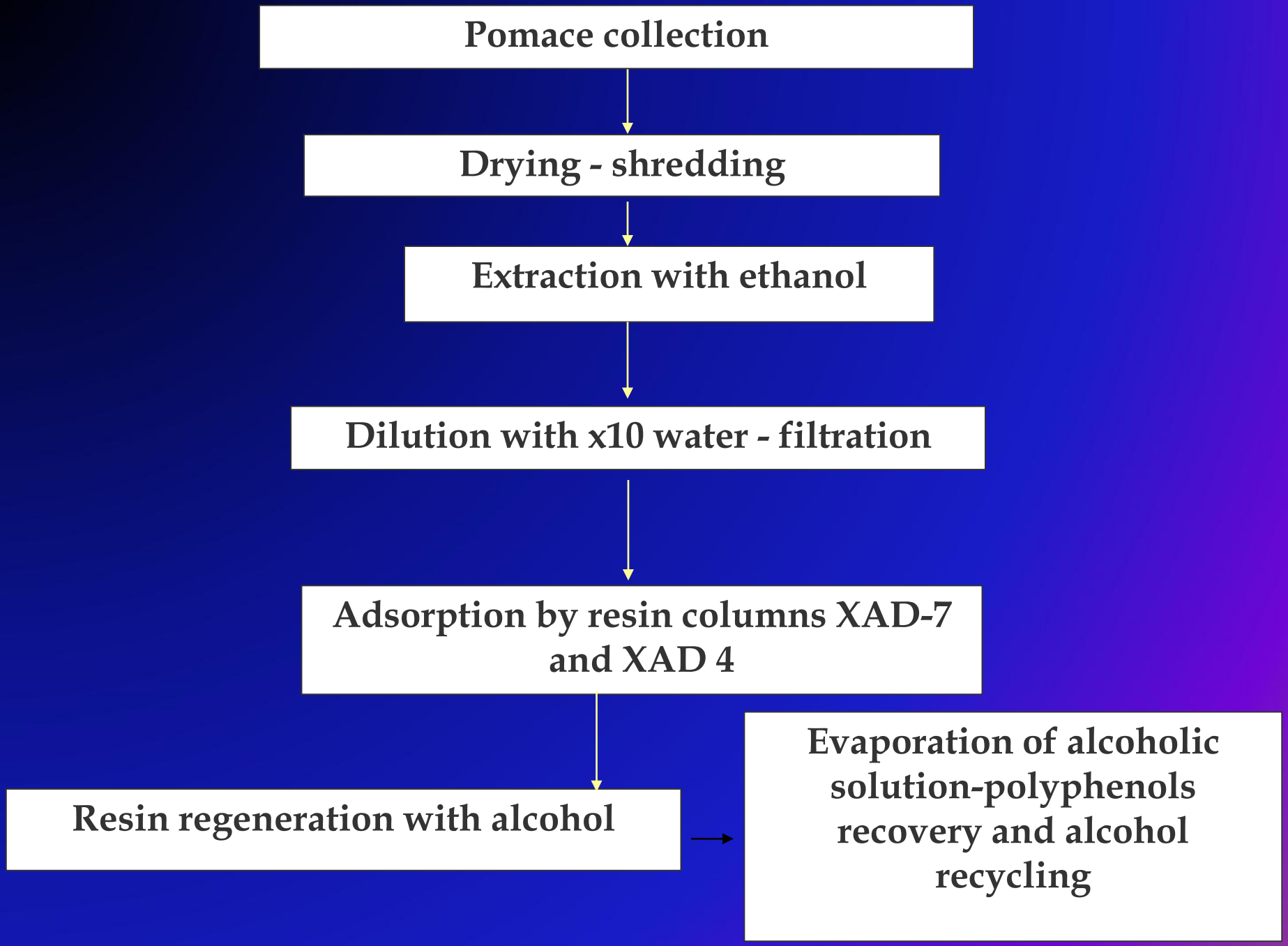
Adsorption resin columns for the separation of polyphenols

- The third stage of waste management process refers to the thermal evaporation (under high vacuum) to recover the polyphenols and recycle the ethanol used.

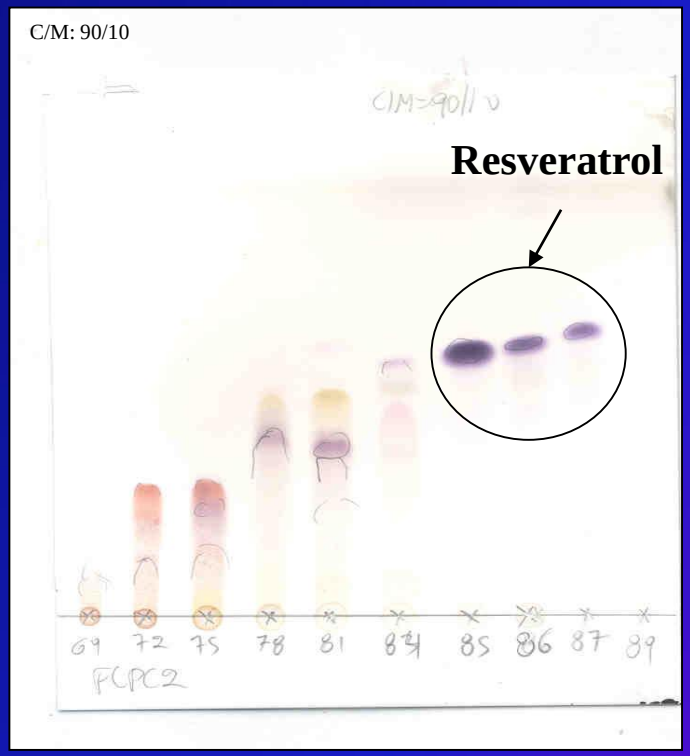
25 liters capacity evaporation system

Larger capacity systems
(63 and 2x200 liters respectively)




Pomace collection
Drying - shredding
Extraction with ethanol
Dilution with x10 water - filtration
Adsorption by resin columns XAD-7 and XAD 4
Resin regeneration with alcohol
Evaporation of alcoholic solution-polyphenols recovery and alcohol recycling

FCPC system for the isolation of active compoundsfrom the polypehnolic extract



With this technique it is feasible to isolate high added value polyphenols as pure compounds, eg. *trans*-resveratrol

1g = 1100 euro

POLYPHENOL EXTRACT

- The polyphenol rich extract obtained from the resin is a slurry, which can not be used for commercial applications
- Thus, the extract is blended with maltodextrine and the mixture is spray dried in order to be

transform to a water soluble red powder, which can easily be incorporated in pharmaceutical, cosmetic, food products or consumed as food supplement

100 Kg POMACE

↓

10 Kg EXTRACT

↓

1 Kg ALCOHOLIC ELUENT OF RESIN (POLYPHENOL RICH EXTRACT)

↓

1 gr RESVERATROL

COMMERCIAL USES OF THE EXTRACTS

Food supplements

Cosmetics

Production of animal food

ANIMAL FEEDING EXPERIMENTS

16 SHEEP

16 GOATS

ASSAY OF BIOACTIVE CONSTITUENTS IN MILK SAMPLES

CLA and TVA comprise the most active constituents of milk lipids.

Conjugated Linoleic Acid (CLA) constitutes the average of 28 different geometrical isomers of linoleic acid. The most active is the *cis-9, trans-11* isomer, which accounts for the 75-95% of total CLA of milk lipids

On the other hand, the *trans*-vaccenic acid (TVA) is the *trans*-11 C18:1 acid and constitutes the precursor of the CLA in animals

Table 1. Average **CLA concentration** *(as % of the total lipid acids)* in milk fat of sheep and goats which were fed with food produced from grape pommace

	Placebo	Experiment
Sheep	**1,64**	**2,64**
Goat	**0,93**	**1,48**

Table 2. Average **TVA concentration** *(as % of the total lipid acids)* in milk fat of sheep and goats which were fed with food produced from grape pommace

	Placebo	**Experiment**
Sheep	**3,19**	**5,11**
Goat	2,71	2,98

CONCLUSIONS

Feeding with grape pommace increased substantially the

1. CLA content in both sheep and goat milks
2. TVA of sheep milks (this parameter was not affected in goat milk).

Production of organic fertilizer by composting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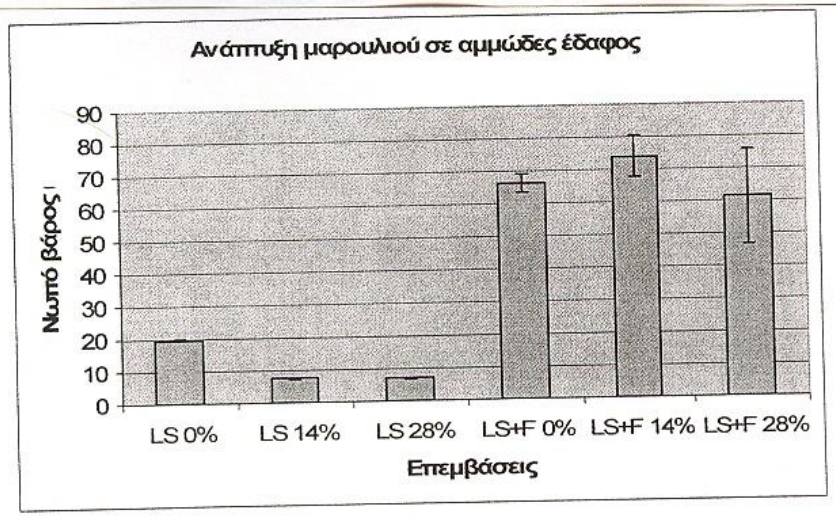
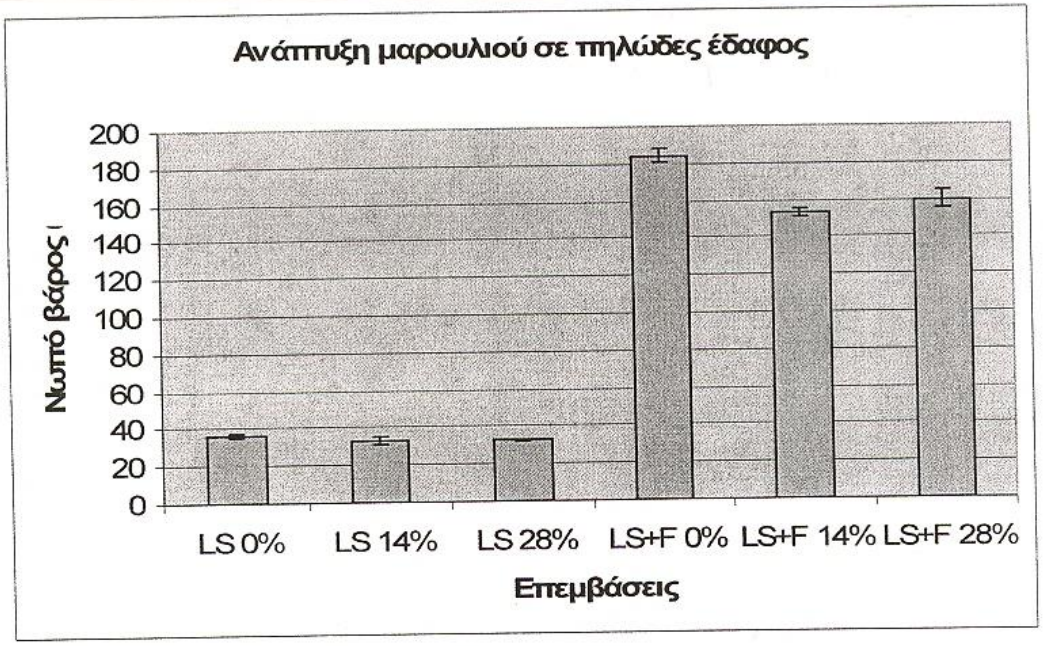
28 12 2004

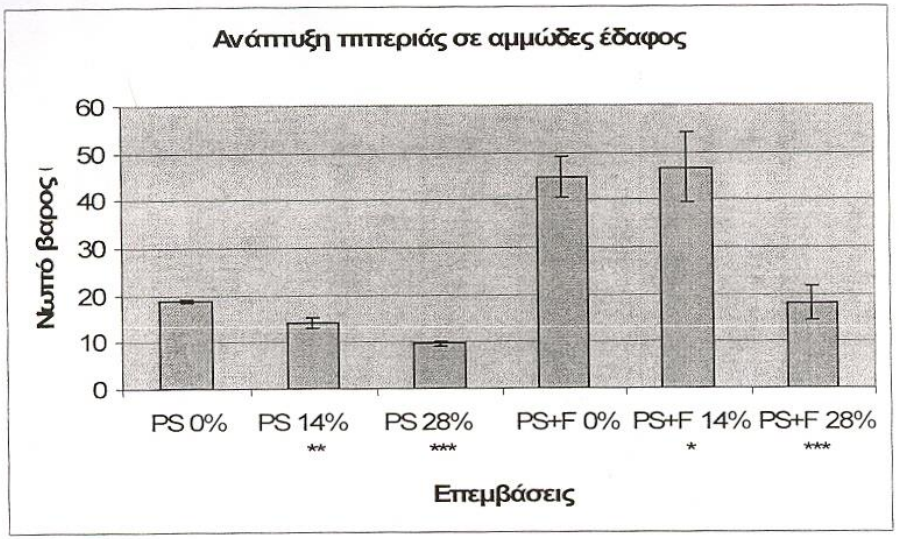

25 4 2005


25 4 2005


KONTOMIHAKOS
85
104


Ανάπτυξη μαρουλιού σε αμμώδες έδαφος
Νωπό βάρος
90
80
70
60
50
40
30
20
10
0
LS 0%
LS 14%
LS 28%
LS+F 0%
LS+F 14%
LS+F 28%
Επεμβάσεις


Ανάπτυξη μαρουλιού σε πηλώδες έδαφος
Νωπό βάρος
200
180
160
140
120
100
80
60
40
20
0
LS 0%
LS 14%
LS 28%
LS+F 0%
LS+F 14%
LS+F 28%
Επεμβάσεις


Ανάπτυξη πιπεριάς σε αμμώδες έδαφος
Νωπό βαρος
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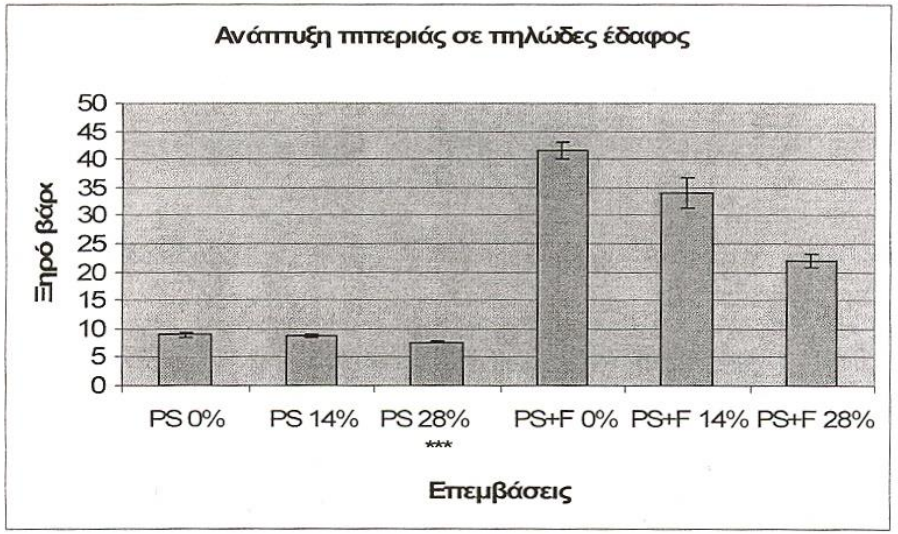
Επεμβάσεις


Ανάπτυξη πιπεριάς σε πηλώδες έδαφος
Ξηρό βάρ
50
45
40
35
30
25
20
15
10
5
0
PS 0%
PS 14%
PS 28%
PS+F 0%
PS+F 14%
PS+F 28%
***
Επεμβάσεις

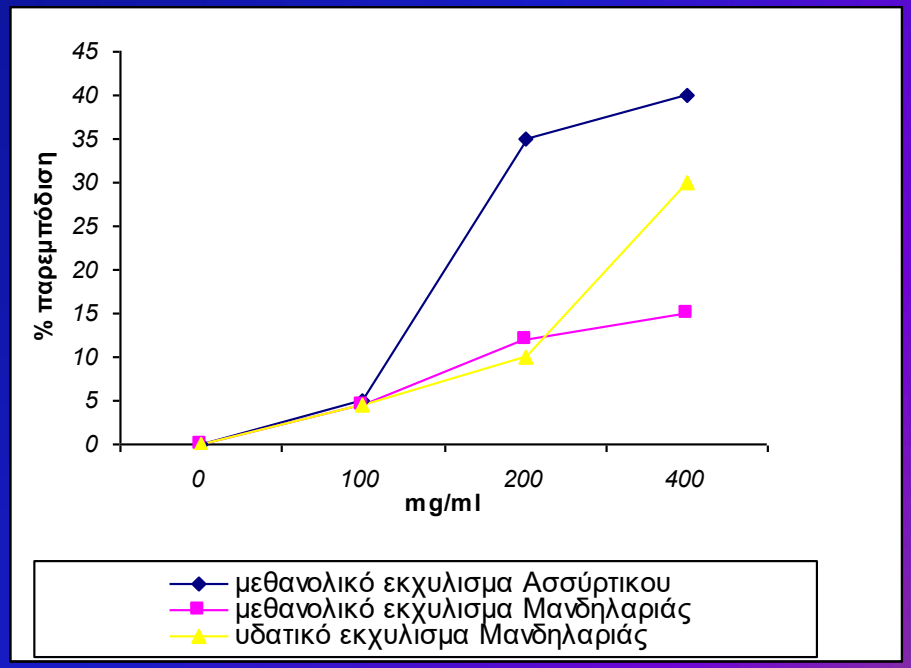

Βιολίπασμα Σαντορίνης
Natural Compost
Εδαφοβελτιωτικό
μεγάλης απόδοσης
12 L

POLYPHENOLIC EXTRACTS BIOACTIVITIES

A. Chemoprotective-anticancer *in vitro* activities of grape pomace extracts obtained from Mandilaria and Assyriko varieties cultivated at Santorini island

Strong inhibition of Topoisomerase I and Mitomycin C:

Strong evidence of the protective role of polyphenol extracts against the DNA chain damage.



EXTRACTS BIOACTIVITIES

B. Chemoprotective-anticancer *in vitro* activities of grape pomace extracts rich in polyphrnol quercetin

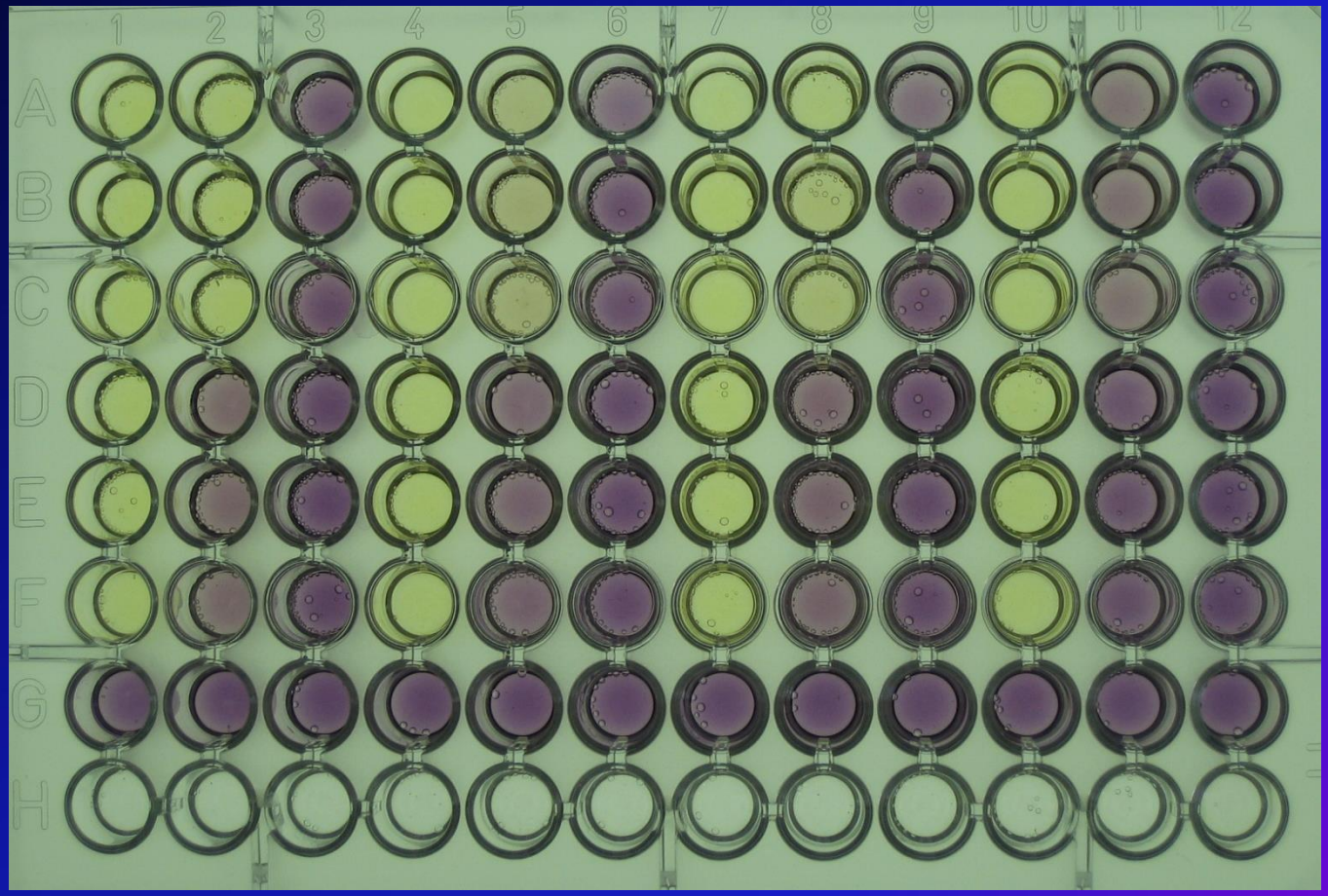
- Inhibition of proliferation of advanced stage cancer cells (DLD-1 cells) is indicative of the quercetin chemoprotective role against various types of cancers

- In a series of experiment qurcetin was assayed to decrease the level of Ras protein of human cells, infected with the H-*ras* gene of the large intestine cancer. Furthermore, a sharp decrease of Rho protein level was also observed.

All aforementioned results constitute strong evidences that quercetin exhibits remarcable chemorpotective role in the development of large intestine cancers.

EXTRACTS BIOACTIVTIES

C. In vitro antioxidant activities

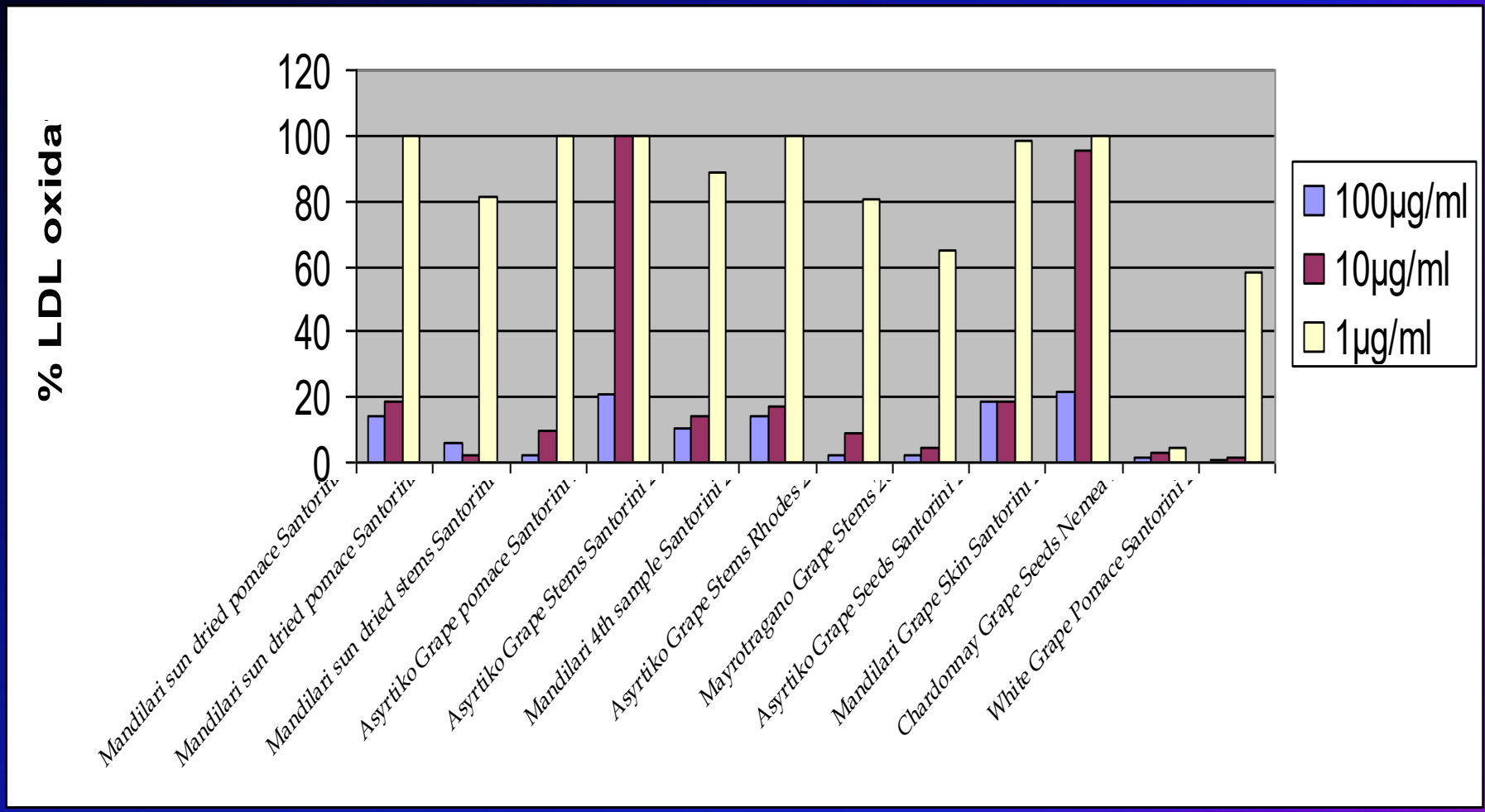


Antioxidant capacity comparisons for reference polyphenols determined by DPPH and Frap assays

DPPH	30 min	FRAP	30min
Compound	IC_{50} (mM)	Compound	reducing ability 1mM FeSO4
Gallic acid	0.012	Quercetin	0.084
Quercetin	0.015	Gallic acid	1.029
(-)Epicatechin	0.013	(-)Epicatechin	1.033
(+)Catechin	0.016	Syringic acid	1.042
Syringic acid	0.029	3.4-dihydroxy Benzoic acid	1.056
Caffeic acid	0.039	(+)Catechin	1.180
Trolox	0.052	Caffeic acid	1.413
3.4-dihydroxy Benzoic acid	0.057	Ferulic acid	1.440
Ferulic acid	0.102	trans-Resveratrol	1.487
trans-Resveratrol	0.130	Trolox	2.035
4-hydroxy Bbenzoic acid	>5mM	p-Coumaric	13.998
p-Coumaric	>5mM	trans-Cinnamic acid	1272.006
trans-Cinnamic acid	>5mM	4-hydroxy Benzoic acid	2220.751

LDL cholesterol oxidation assay

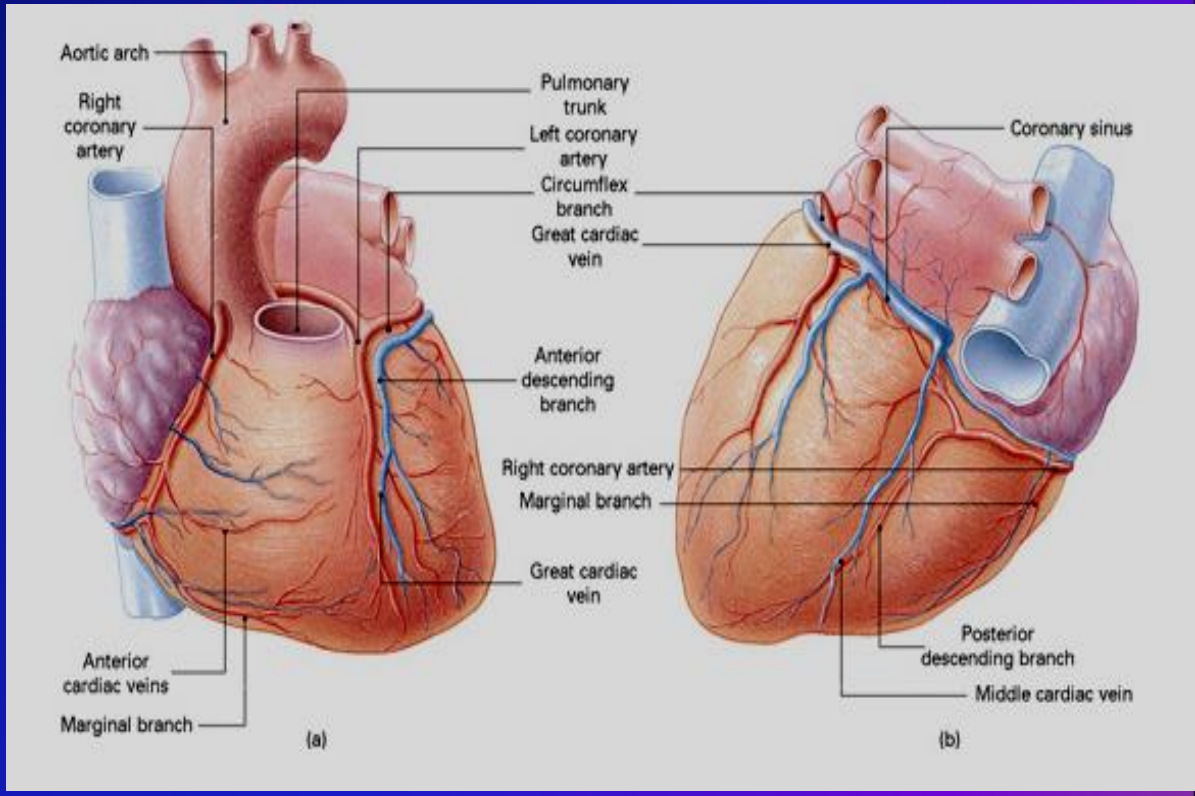
	%LDL cholesterol oxidation		
	100µg/mL	10µg/mL	1µg/mL
Mandilari sun dried pomace Santorini 2003	14.1	18.7	100
Mandilari sun dried pomace Santorini 2004	2.4	6.2	81.3
Mandilari sun dried stems Santorini 2003	2.1	9.8	100
Asyrtiko Grape pomace Santorini 2004	21.1	100	100
Asyrtiko Grape Stems Santorini 2004	10.4	14.1	88.7
Mandilari 4th sample Santorini 2004	14.1	16.8	100
Asyrtiko Grape Stems Rhodes 2003	2.6	8.9	80.5
Mayrotragano Grape Stems 2003	2.1	4.6	65
Asyrtiko Grape Seeds Santorini 2004	19	18.3	98.6
Mandilari Grape Skin Santorini 2004	21.8	95.7	100
Chardonnay Grape Seeds Nemea 2004	1.5	3.1	4.2
White Grape Pomace Santorini 2003	0.9	1.5	58.1


% LDL oxida
120
100
80
60
40
20
0
100μg/ml
10μg/ml
1μg/ml
Mandilari sun dried pomace Santorini
Mandilari sun dried pomace Santorini
Mandilari sun dried stems Santorini
Asyrtiko Grape pomace Santorini
Asyrtiko Grape Stems Santorini
Mandilari 4th sample Santorini
Asyrtiko Grape Stems Rhodes
Mayrotragano Grape Stems
Asyrtiko Grape Seeds Santorini
Mandilari Grape Skin Santorini
Chardonnay Grape Seeds Nemea
White Grape Pomace Santorini

CLINICAL TRIALS

ASSAY OF POLYPHENOL EXTRACT ACTIVITY ON PATIENTS WITH CORONARY HEART DISEASE (CHD)

CHD is considered as one of the major pathological disorders of the Western World. WHO (World Health Organization) predicts that by 2020 CHD will become the major reason of death.



Atherosclerosis

Foam cells

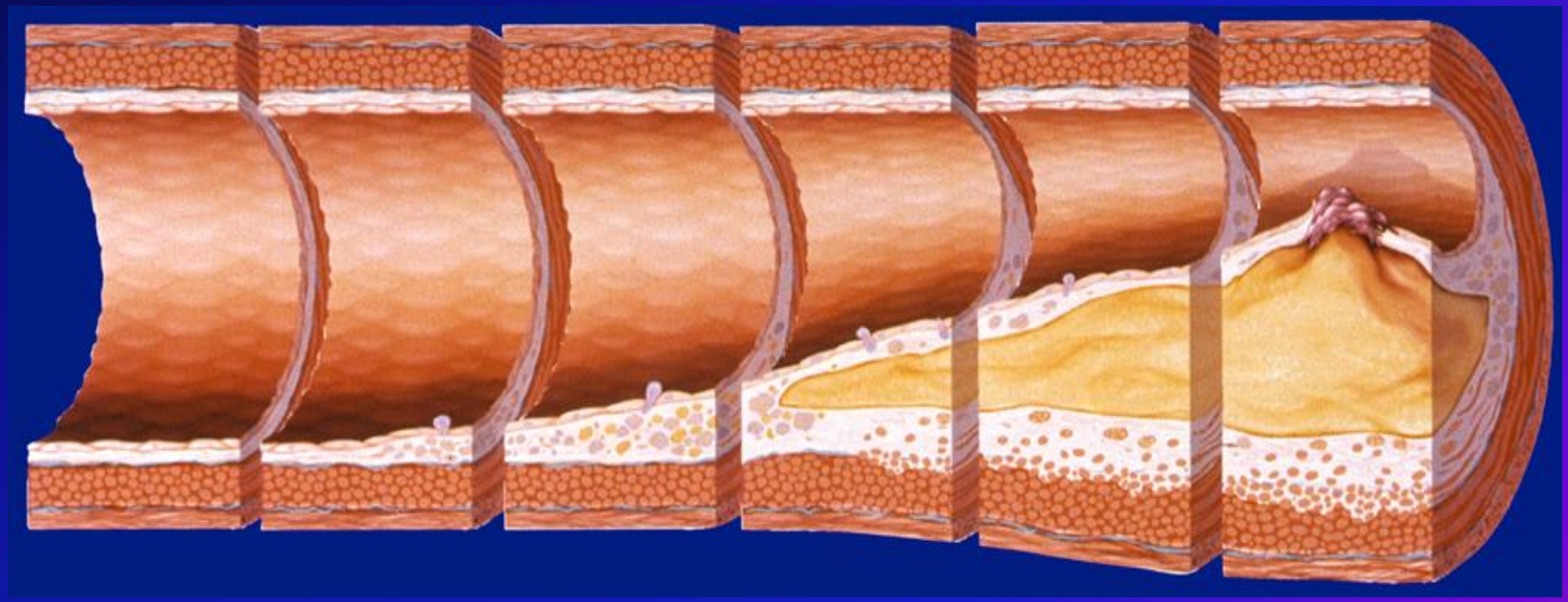
Lipid lineation

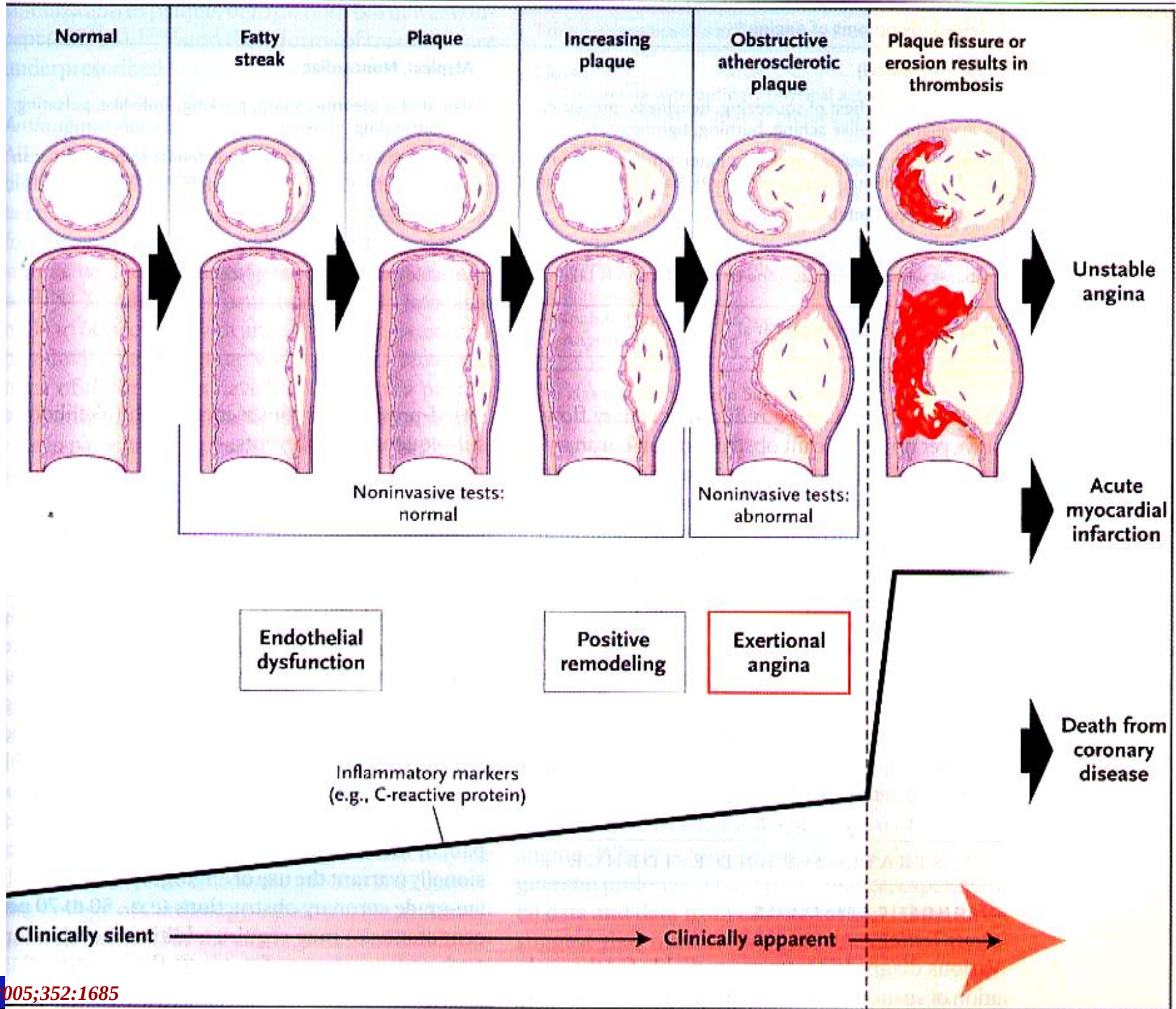
Initial damages - proatehroma

atheroma

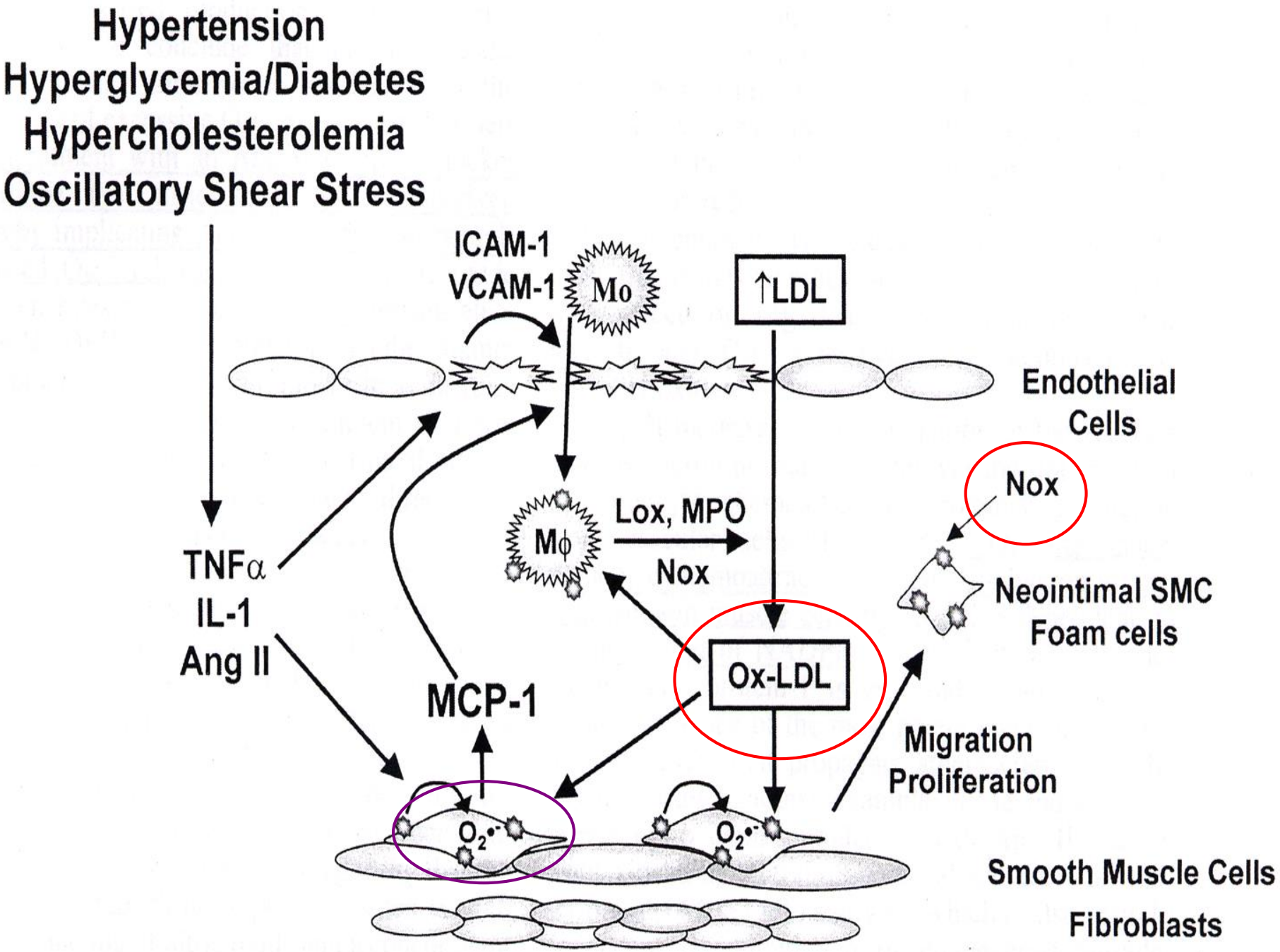
Development of fibrous plaque

Surface corrosion
Bleeding of artery walls
Surface thrombosis

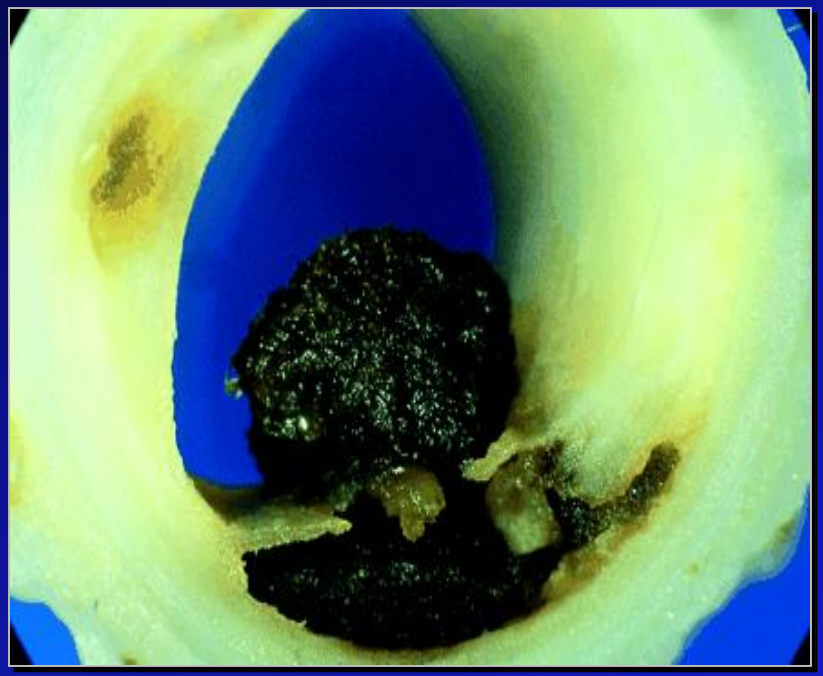


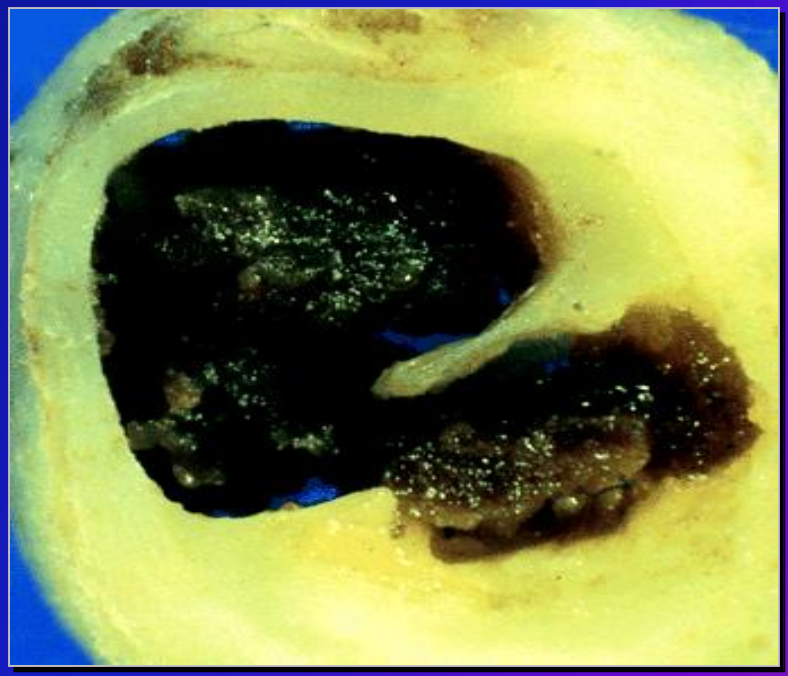


New Engl J Med 2005;352:1685


Hypertension
Hyperglycemia/Diabetes
Hypercholesterolemia
Oscillatory Shear Stress
ICAM-1
VCAM-1
Mo
↑LDL
Endothelial Cells
Nox
Mϕ
Lox, MPO
Nox
TNFα
IL-1
Ang II
MCP-1
Ox-LDL
Neointimal SMC
Foam cells
Migration
Proliferation
$O_2^{\bullet-}$
$O_2^{\bullet-}$
Smooth Muscle Cells
Fibroblasts

Acute Coronary Syndrome (ACS)





Endothelium malfunction
LDL oxidation

Oxidation of LDL is a hallmark for atherosclerosis and coronary heart disease development. One of the earliest steps in the generation of oxidized LDL is the lipid peroxidation of polyunsaturated fatty acids. Lipid peroxidation and its chain reaction in LDL, can be interrupted if LDL lipids are protected from free radicals by antioxidants.

Polyphenol extract

- **0.6 grams of extract is equal to 1 Kg grape berries (based on *trans*-resveratrol)**

Συστατικό	mg/g
Επικατεχίνη	4.32
Κατεχίνη	2.72
Γαλλικό οξύ	2.07
Trans-ρεσβερατρόλη	0.9
Ρουτίνη	0.47
ε-Βινιφερίνη	0.42
p-κουμαρικό οξυ	0.28
φερουλικό οξύ	0.14
Κερκετίνη	0.04

CRITERIA OF PATIENT SELECTION

Males with confirmed CHD by coronary angiography, <70 year

30 patients were divided randomly into 2 groups:

First group (ν=15) consumed 2.4 g of polyphenol extract dissolved in 20 ml of water

Second group (ν=15) consumed 20 ml of water (placebo)

ATTIKO HOSPITAL, University Clinic

The experiment was performed in the morning after a 24 h stop of any medication.

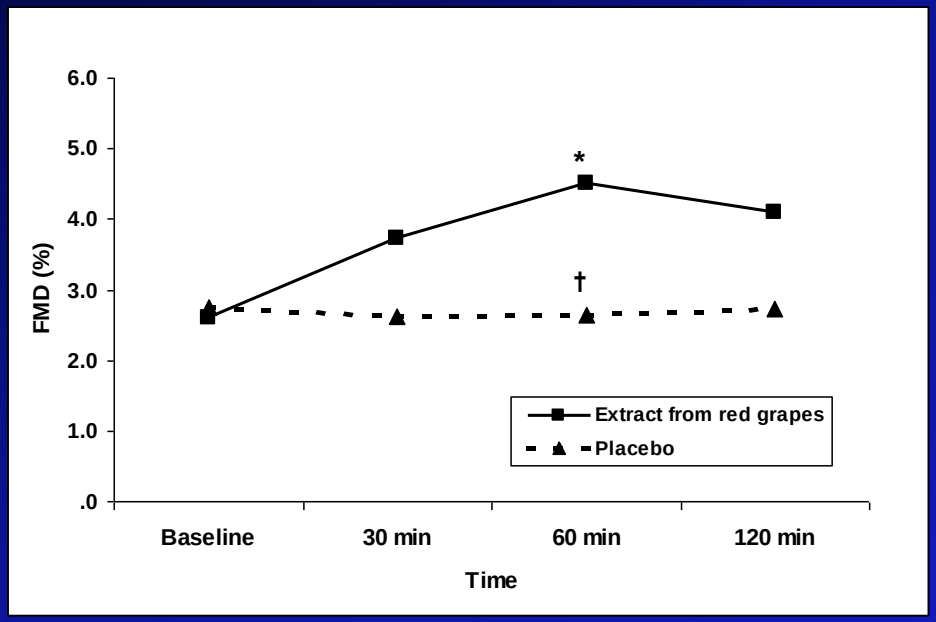
The endothelium function was determined by the measurement of the flow-mediated dilatation (FMD), using high definition ultrasound tomography.

The measurements were performed every 30 minutes, staring before the polyphenol extract consumption and last for 3 additional hours.

CLINICAL TRIALS RESULTS

	0 min	30 min	60 min	120 min
		ingestion of red grape extract		
Diameter size at rest (mm)	4,78 ± 0,5	4,67 ± 0,45	4,7 ± 0,44	4,71 ± 0,48
Flow at rest	152,6 ± 51	135 ± 62	151 ± 68	139 ± 54
Hyperemia (%)	190 ± 96	235 ± 105	225 ± 110	235 ± 141
FMD (%)	2,6 ± 1,5	3,73 ± 2,1	4,52 ± 1,34	4,1 ± 2,34
		ingestion of placebo (n=15)		
Diameter size at rest (mm)	4,5 ± 0,28	4,6 ± 0,28	4,58 ± 0,3	4,5 ± 0,37
Flow at rest	107 ± 67	109 ± 57	114 ± 59	140 ± 78
Hyperemia (%)	257 ± 76	278 ± 107	274 ± 123	248 ± 123
FMD (%)	2,75 ± 1,85	2,65 ± 1,65	2,64 ± 1,85	2,74 ± 1,8

CLINICAL TRIALS



- Increase of FMD value.
- Effect after 30 min and peak on 60 min after the polyphenol consumption.
- beneficial effect on endothelium function on patients with CHD.

Project implications

1. recovery of high added value polyphenols with considerable financial and market interest and demonstration of their uses as:
   - Cosmetics
   - Food supplements
   - Additives in food preparations, eg. dairy products (yogurt, ice creams etc)
2. animal food production
3. organic fertilizer by composting through a controlled process.

All actions are

- **environmentally safe**, since use as solvents only water and ethanol (both are fully recycled)
- adaptable by all wineries, since the compilation of sub-units is simple and do not require large space and/or money investments

Additional Info

http://www.pharm.uoa.gr/dionysos

DIONYSOS project,

LIFE ENV/GR/000223

Agricultural University of Athens

COORDINATOR

University of Athens

GAIA, Goulandris Museum of Natural History

TERRA NOVA Ltd

Union of Cooperative Wineries of Greece