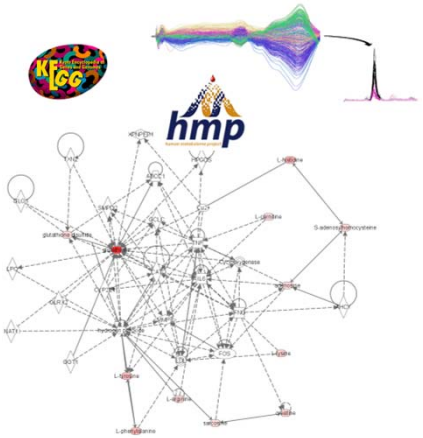
Foodomics: Food Science & Omics Tools in the 21st Century

Alejandro Cifuentes

a.cifuentes@csic.es
Foodomics Laboratory, CIAL, CSIC, Madrid, Spain



Stockholm, May 2015

Thanks!

Alberto Valdés[1], Clara Ibáñez[1], Virginia García-Cañas[1], Carolina Simó[1], Elena Ibáñez[1], Giuseppe Sullini[1,2], Vicente Micol[3], José A. Ferragut[3]

[1] Foodomics Laboratory, CIAL, CSIC, Madrid, Spain
[2] Dept. Scienze del Farmaco e dei Prodotti per la Salute, Univ. Messina, Italy
[3] Institute of Molecular and Cellular Biology, Univ. Miguel Hernandez, Alicante, Spain

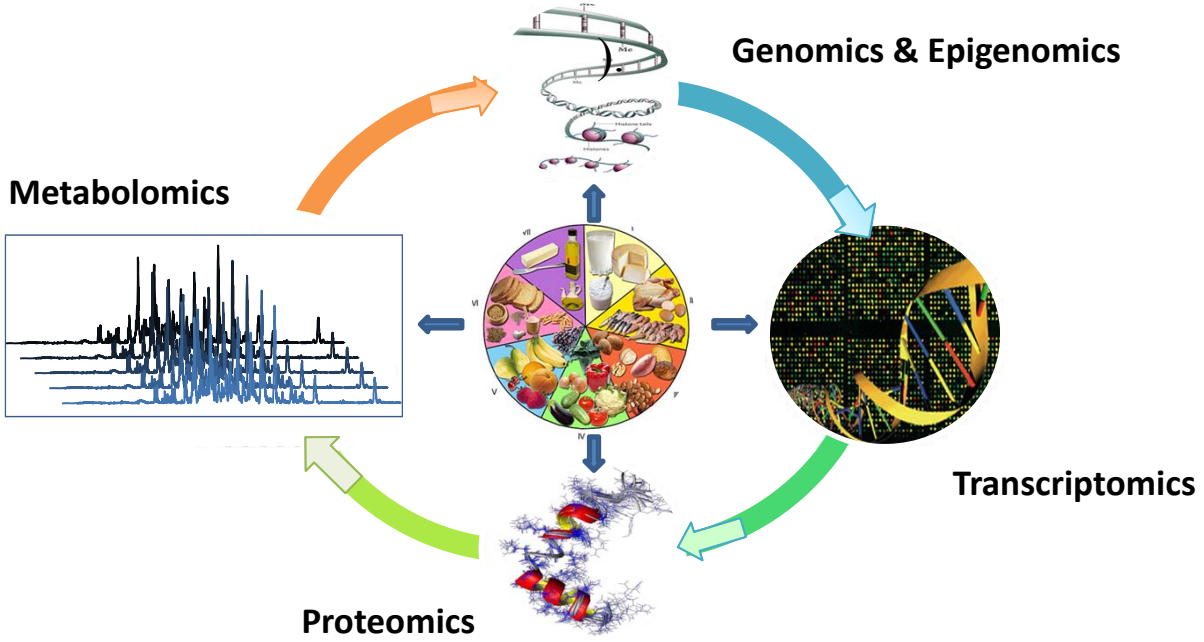
CURRENT & FUTURE CHALLENGES IN FOOD SCIENCE AND NUTRITION

1. To produce new functional foods with scientifically proved claims
2. To understand the effects of gene-food interaction on human health (Nutrigenomics)
3. To explain the different answers from individuals to food (Nutrigenetics)
4. To establish the global role and functions of gut microbiome
5. To reduce through diet the impact of cardiovascular diseases, obesity and cancer (discovering the molecular mechanisms behind).
6. To understand the stress adaptation responses of food-borne pathogens
7. To reduce food allergy and food allergens
8. To understand the molecular basis of biological processes essential for improving agronomic and farm animal production+ sustainability!
9. To develop, produce and monitor new transgenic foods
10. To understand postharvest phenomena through a global approach (genetics linked to environmental responses: biological networks)
11. Bioinformatics (including data processing, clustering, dynamics, or integration of the various 'omics' levels) will have to progress.
12. To get a personalized nutrition.

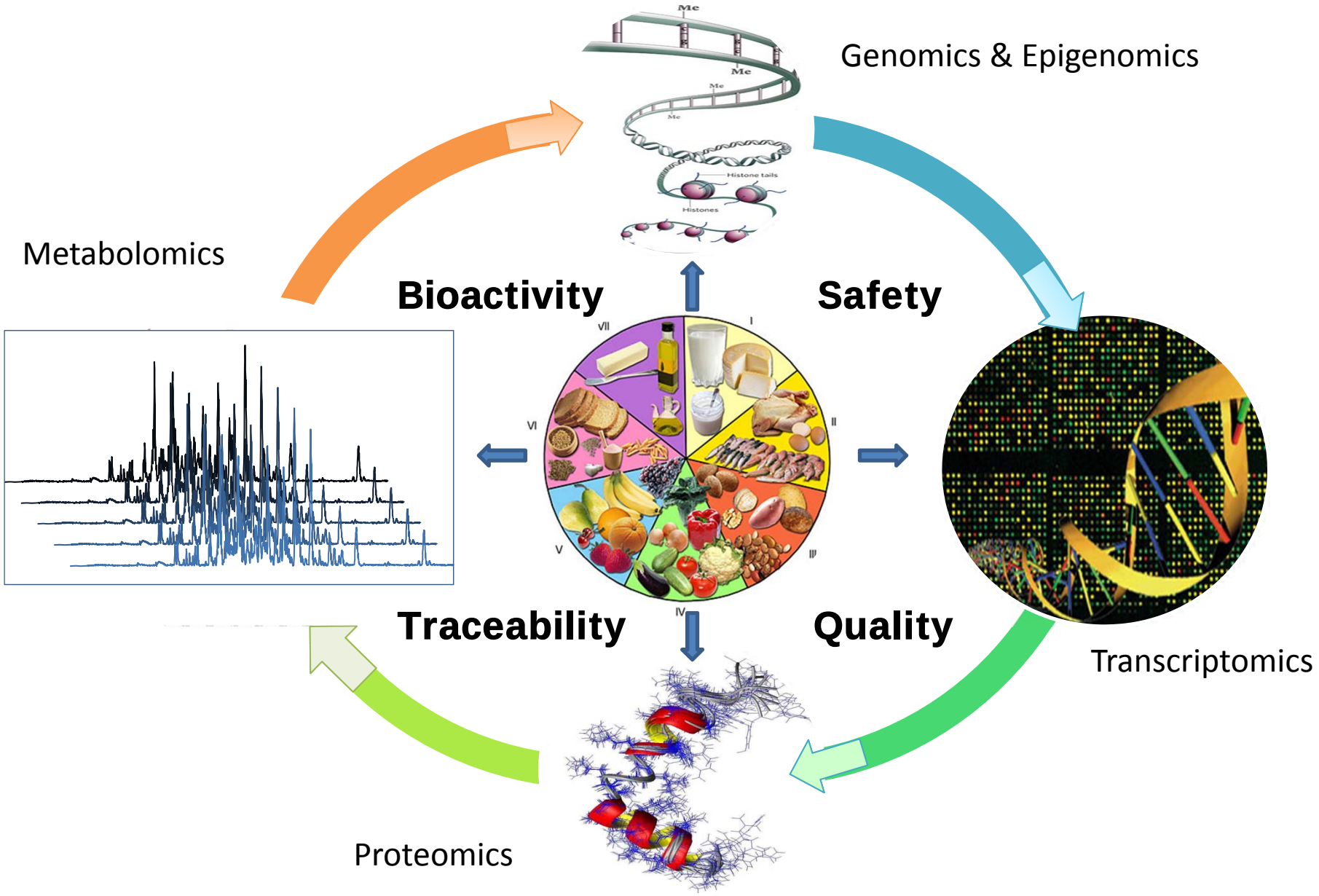
NEW CHALLENGES USUALLY REQUIRE NEW ANSWERS…

Foodomics

Foodomics has been defined by our group as:
A discipline that studies the Food and Nutrition domains through the application and integration of advanced omics technologies to improve consumer's well-being, health, and knowledge
(Cifuentes et al.; *J. Chromatogr. A* 1216 (2009) 7109;
Electrophoresis 31 (2010) 205;
Mass Spec. Rev. 31 (2012) 49–69;
Anal. Chem. 84 (2012) 10150–10159).



Foodomics tools and applications



ELECTROPHORESIS

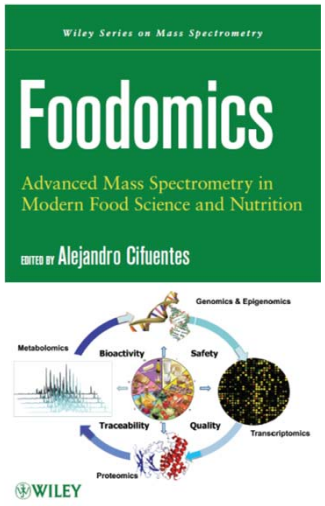
(impact factor: 3.303)

"Foodomics and Advanced Food Analysis"

June 2014. Editor: A. Cifuentes

WILEY-BLACKWELL

March 2013

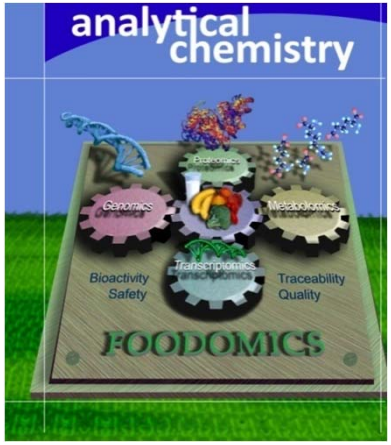


analytical chemistry

(impact factor: 5.856)

"Foodomics" Cover and Feature Article

December 2012



ELSEVIER

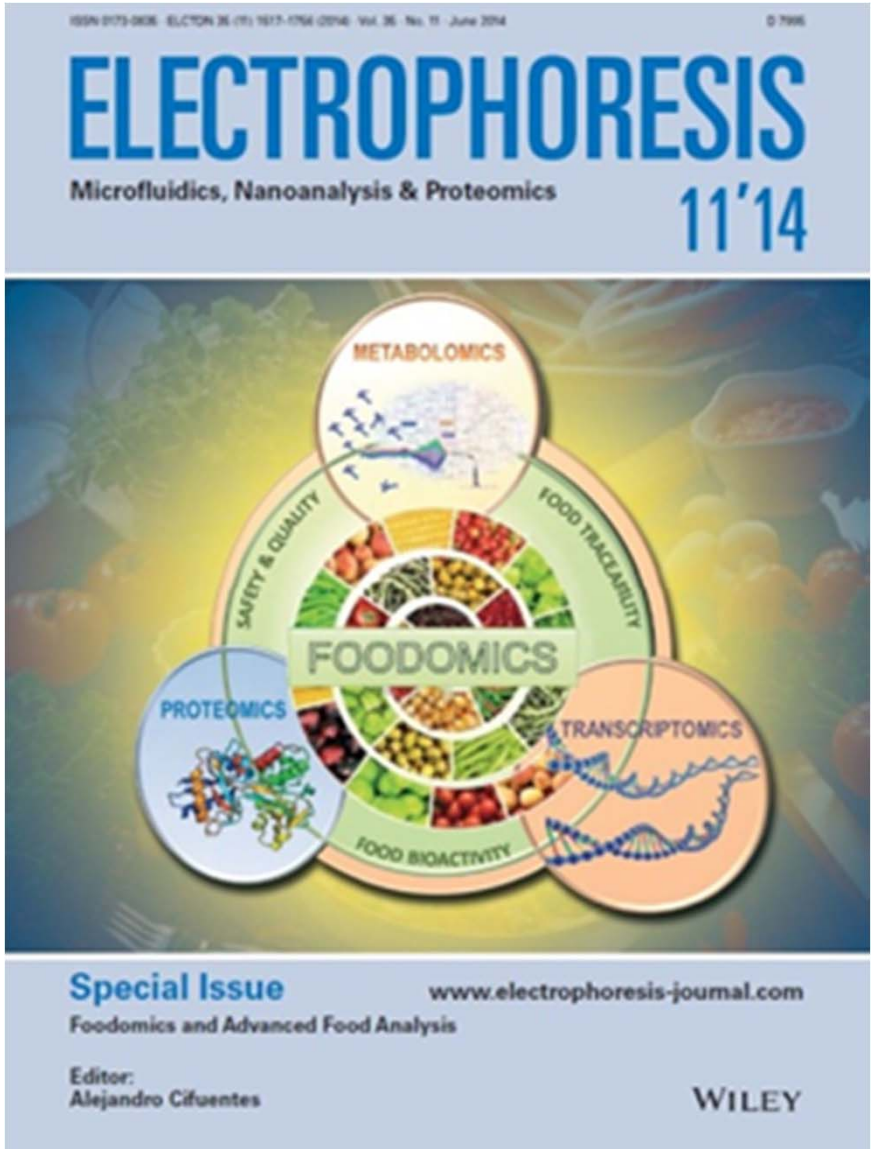
TRENDS IN ANALYTICAL CHEMISTRY

(impact factor: 6.273)

"Modern *Food Analysis and Foodomics*"

December 2013

Editors:
A. Cifuentes
D. Rutledge



(Impact factor: 3.303)

PAPERS ARE WELCOME ON:

Foodomics and
Advanced Food Analysis

(using e.g., omics-approaches, electrodriven methods, liquid-based separation methods)

(deadline, Nov 1st 2015)

a.cifuentes@csic.es

Current Opinion in Food Science

New journal from Elsevier

REVIEW PAPERS ON FOODOMICS & ADVANCED FOOD ANALYSIS ARE WELCOME ANY TIME!

a.cifuentes@csic.es

Foodomics projects in our lab on:

Safety, quality and traceability of Transgenic foods Other foods & ingr

GM corn, GM soya, GM yeasts…

DNA, proteins and metabolites

In collaboration with **GSF** (Munich, Germany)

Bioactivity of food ingredients against:

Alzheimer

Population study

Biological sample: Cebrospinal fluid (CSF)

In collaboration with **Karolinska Institute** (Stockholm, Sweden)

Colon cancer

Human cell lines Animal models

Biological samples: DNA, RNA, proteins and metabolites

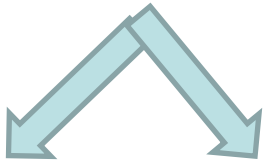
Leukemia

Human cell lines

Biological samples: DNA, RNA, proteins and metabolites

In collaboration with **Univ. Miguel Hernandez**, Elche, Spain **University of Granda**, Granada, Spain

Foodomics projects in our lab on:



Safety, quality and traceability of

Transgenic foods

Other foods & ingr

GM corn, GM soya, GM yeasts…

DNA, proteins and metabolites

In collaboration with **GSF** (Munich, Germany)

Bioactivity of food ingredients against:

Alzheimer

Population study

Biological sample: Cebrospinal fluid (CSF)

In collaboration with **Karolinska Institute** (Stockholm, Sweden)

Colon cancer

Human cell lines
Animal models

Biological samples: DNA, RNA, proteins and metabolites

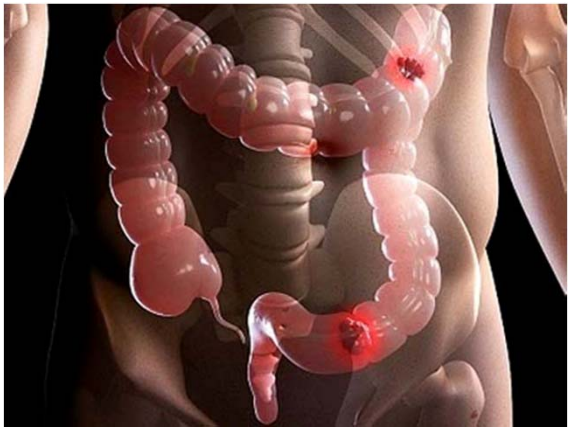
Leukemia

Human cell lines

Biological samples: DNA, RNA, proteins and metabolites

In collaboration with **Univ. Miguel Hernandez**, Elche, Spain
University of Granda, Granada, Spain

Why study colon cancer and diet?



The most diagnosed cancer in Spain: 25000 new cases every year

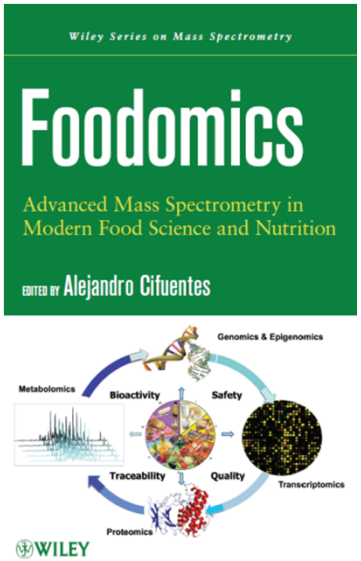
The 2nd cause of death by cancer in Europe and 4th in the world

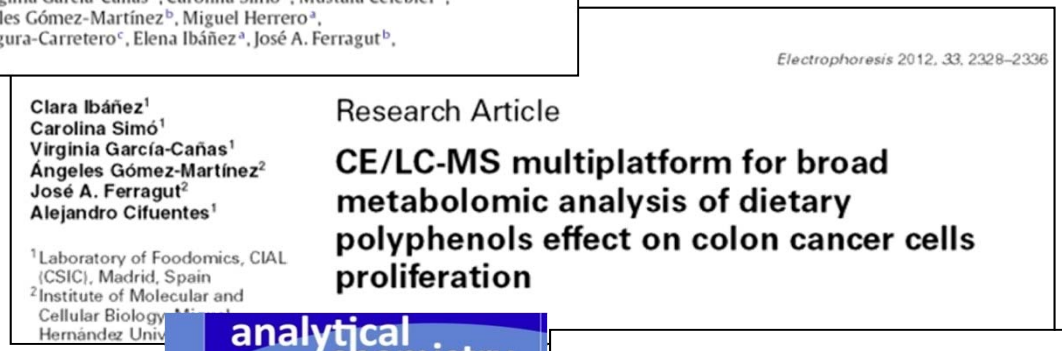
According to several studies, **80% of the cases are related to diet**

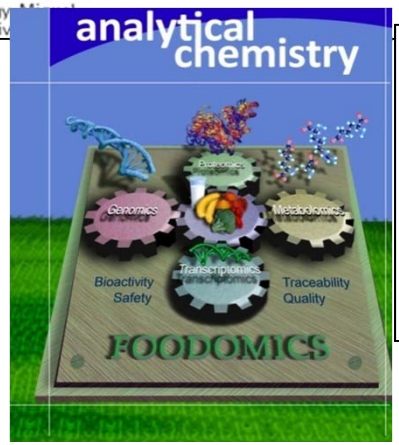
Can we reduce the proliferation speed of colon cancer through diet? This would be a great help since this cancer has a high percentage of recovery if intervention can commence before metastasis

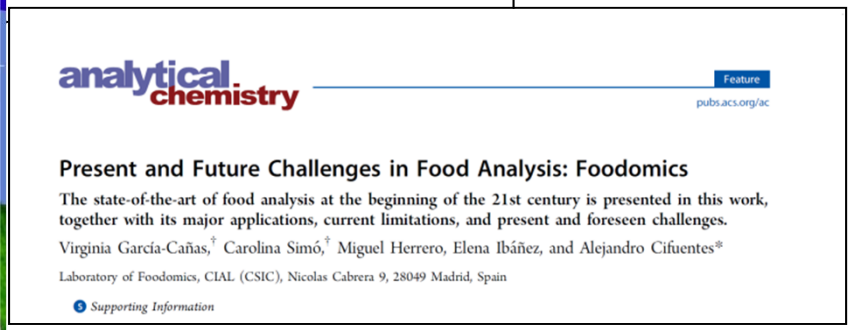
PREVIOUS RESULTS

In previous works, we have demonstrated that rosemary extracts (REs) obtained in our laboratory using SFE exhibited inhibitory effect against proliferation of several cancer cell lines. However, due to the complexity of the REs the observed activity was not correlated to any specific compound present in the extract. **The goals of this work are i) to establish and understand this correlation and ii) to corroborate the RE activity using an *in vivo* model.**







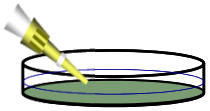


Goal I: To link the antiproliferative activity to specific compounds and to understand how they work at molecular level

Pure compounds

vs.

Natural extract



Treated

HT29 colon cancer cells

Control

In vitro study

RNAs and metabolites obtained from control and treated cells

Foodomics evaluation

Transcriptomics

Metabolomics

Data processing

Statistical analysis

Biomarkers identification and confirmation. Pathway analysis and biological process understanding

Goal II: To corroborate the *in vitro* activity by an *in vivo* model

Rosemary

Green extraction

Selected extract

Control, treated and pre-treated mice

RNAs and metabolites obtained from control and treated individuals

Foodomics evaluation

Transcriptomics

Metabolomics

Data processing

Statistical analysis

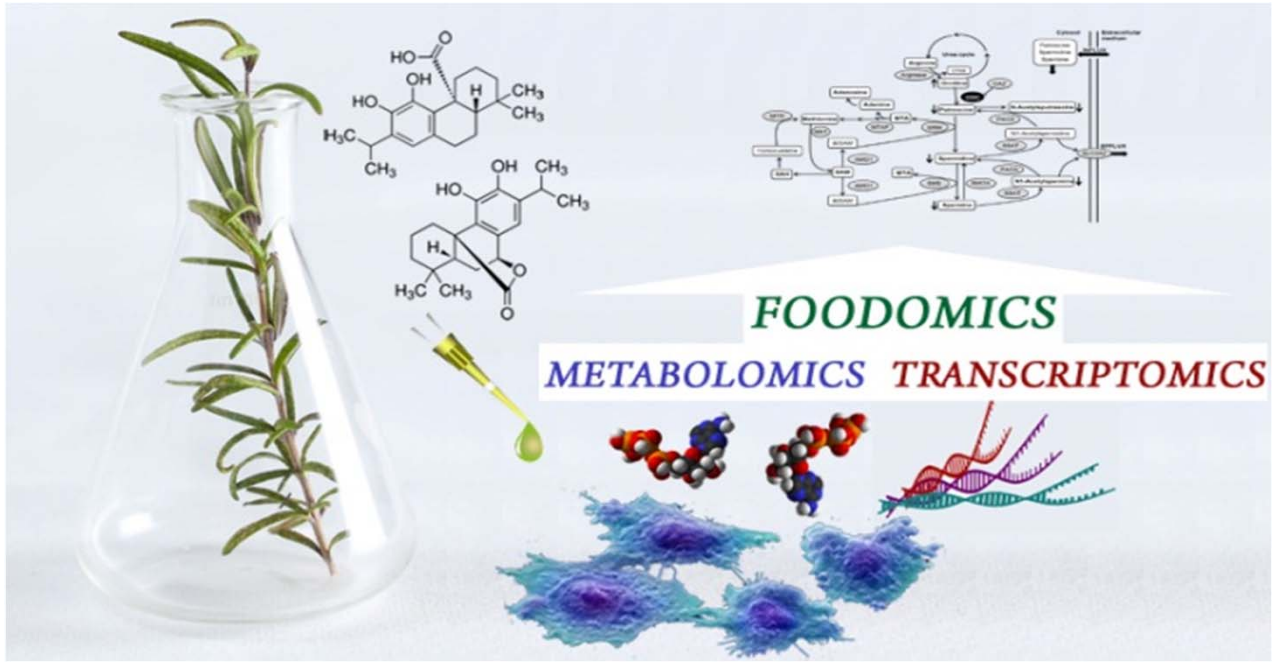
Biomarkers identification and confirmation. Pathway analysis and biological process understanding

PARTS OF THIS WORK

1. To optimize an effective protocol for cell metabolomics with especial emphasis in the sample preparation step and subsequent analysis of the intracellular metabolites from control and treated human HT-29 colon cancer cells.

2. To investigate using a comprehensive Foodomics approach the contribution of carnosic acid (CA) and carnosol (CS), two major compounds present in the active rosemary extract (RE), against proliferation of human HT-29 colon cancer cells.

3. To corroborate the *in vitro* results by testing the antiproliferative activity of RE *in vivo.*



CELL METABOLOMICS OPTIMIZATION AND VALIDATION

(CASE STUDY: METABOLOMICS OF COLON CANCER CELLS HT-29)

Cells pellet

- How many cells do we need?
- Quenching protocol
- Solvent extraction

Pellet (10^7 cells)

300 µL extraction solvent + glass balls

Snap-freeze (Liq N_2, 30 s)

Thawing (Ultrasound bath)

Grinding Ball Mill

3 min, 30/s frec

(x 3)

Centrifugation (60000 x g, 10 min)

200 µL supernatant

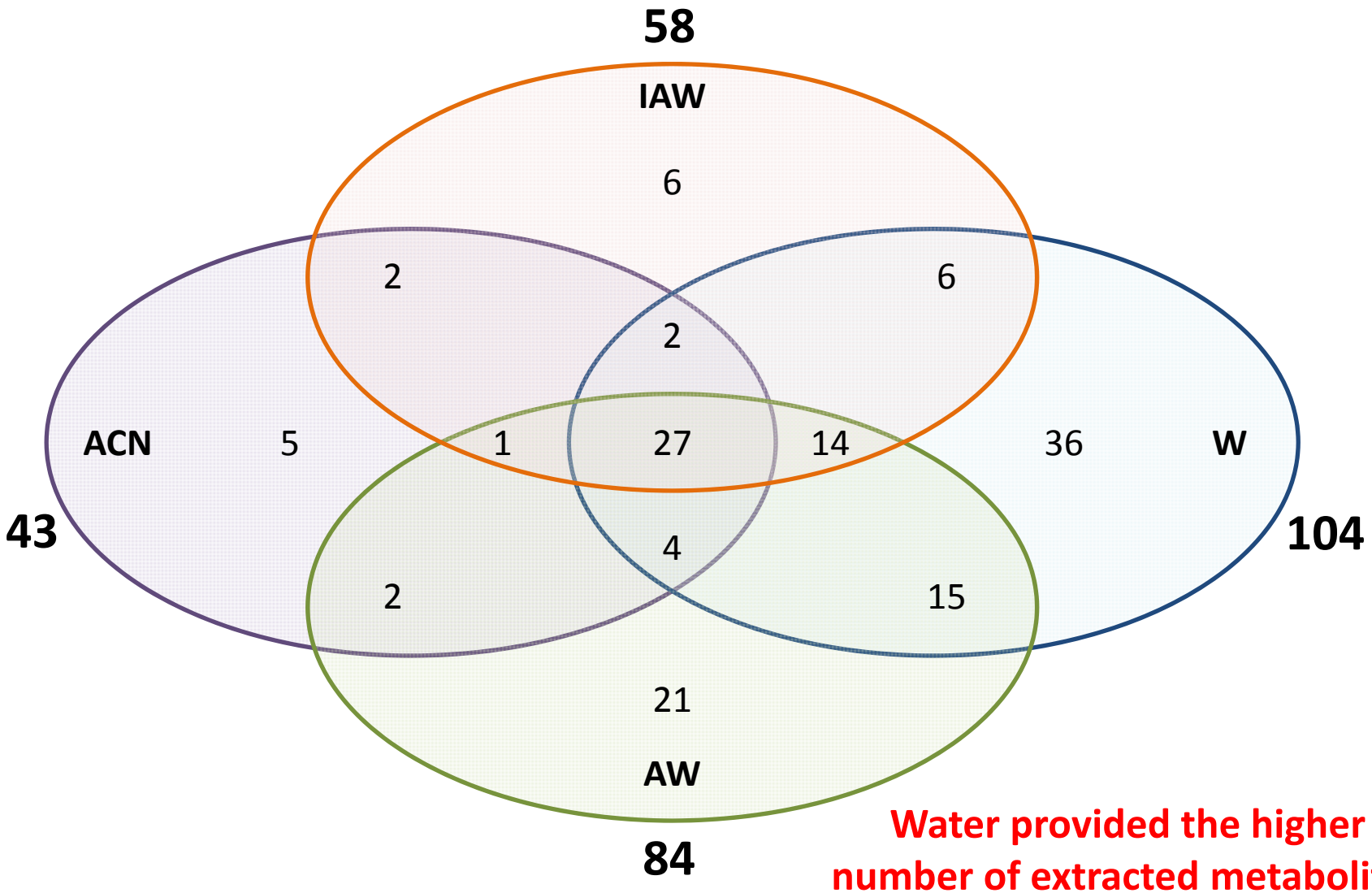
<3kDa fraction

4 Different extraction solvents:

1. ACN
2. IspOH-ACN-Water (3:3:1, v/v/v)
3. Water
4. Water, 5% HCOOH

CE-TOF MS

CELL METABOLOMICS: Solvent extraction selection



Water provided the higher number of extracted metabolites

Venn diagram representation of number of HT-29 intracellular metabolites extracted with acidized water (AW), water (W), acetonitrile (ACN) and isopropanol-acetonitrile-water (IAW) following a non-targeted metabolomic approach.

CELL METABOLOMICS: Solvent extraction selection

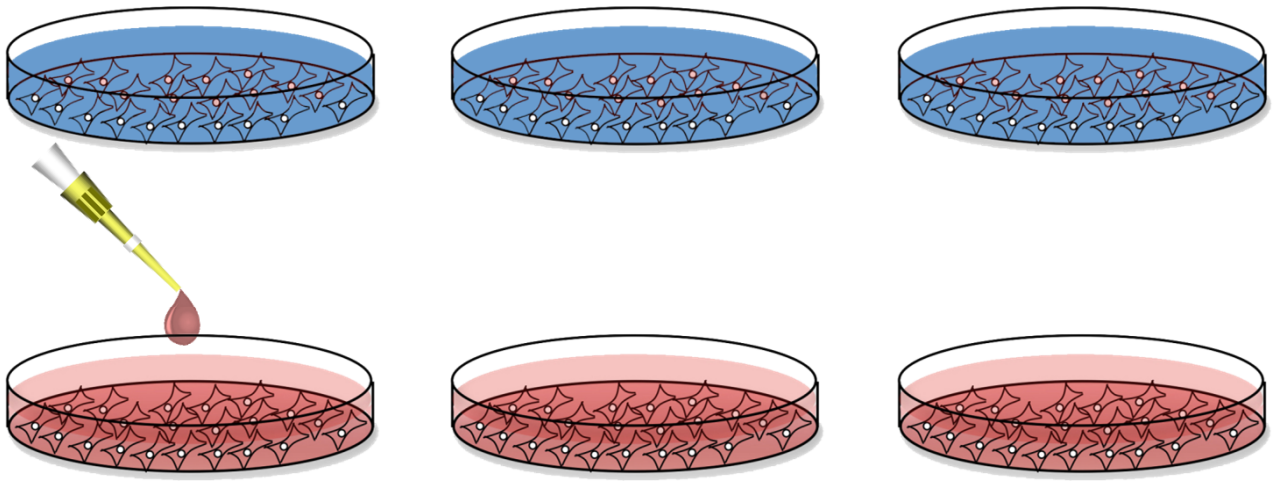
Repeatability of metabolite extraction (five extractions each condition) using Water (W), acidified water (AW), acetonitrile (ACN) and isopropanol-acetonitrile-water (IAW)

		Metabolite database				% RSD peak area (n=5)			
Compound	Molecular formula	METLIN	KEGG	HMDB	m/z $[M+H]^+$	W	AW	ACN	IAW
Spermine	$C_{10}H_{26}N_4$	255	C00750	HMDB01256	203.2230	6	6	ND	54
Spermidine	$C_7H_{19}N_3$	254	C00315	HMDB01257	146.1652	9	6	29	41
Putrescine	$C_4H_{12}N_2$	3226	C00134	HMDB01414	89.1073	ND*	ND	ND	ND
Acetylspermine	$C_{12}H_{28}N_4O$	3369	C02567	HMDB01186	245.2336	ND	ND	88	ND
Ornithine	$C_5H_{12}N_2O_2$	27	C01602	HMDB00214	133.0972	ND	ND	ND	ND
Arginine	$C_6H_{14}N_4O_2$	13	C00062	HMDB00517	175.119	6	14	27	10
S-Adenosylmethionine	$C_{15}H_{23}N_6O_5S$	3289	C00019	HMDB01185	399.1445	6	6	32	9
Adenine	$C_5H_5N_5$	85	C00147	HMDB00034	136.0618	9	10	23	23
Acetylputrescine	$C_6H_{14}N_2O$	3252	C02714	HMDB02064	131.1179	ND	ND	ND	ND
S-Adenosyl-L-homocysteine	$C_{14}H_{20}N_6O_5S$	296	C00021	HMDB00939	223.0747	9	11	51	19
Methionine	$C_5H_{11}NO_2S$	26	C00073	HMDB00696	150.0583	8	12	15	9
Adenosine	$C_{10}H_{13}N_5O_4$	86	C00212	HMDB00050	268.104	7	13	52	29
5'-Deoxy-5'-(methylthio)adenosine	$C11H_{15}N_5O_3S$	3425	C00170	HMDB01173	298.0968	9	ND	ND	15
***ND: Not detected**									

Water provided the higher reproducibility between extractions (n=5)

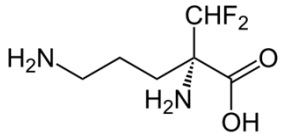
CELL METABOLOMICS VALIDATION

METABOLIC PROFILING OF THE EFFECT OF DFMO ON COLON CANCER CELLS (HT-29)



Untreated

5 mM DFMO



α-difluoromethylornithine

10^7 CELLS

METABOLITE EXTRACTION

METABOLOMICS

ORNITHINE → STOP → PUTRESCINE

ENZYMATIC INHIBITION
ornithine decarboxylase

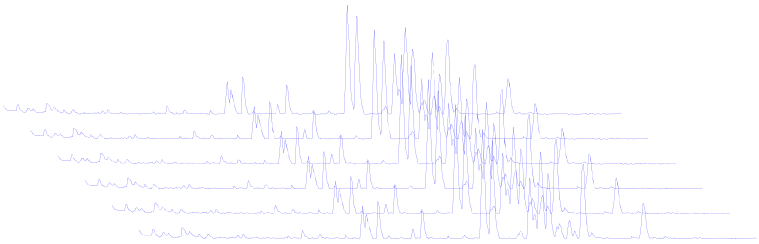
↓ Polyamine levels

polyamine metabolism unbalance

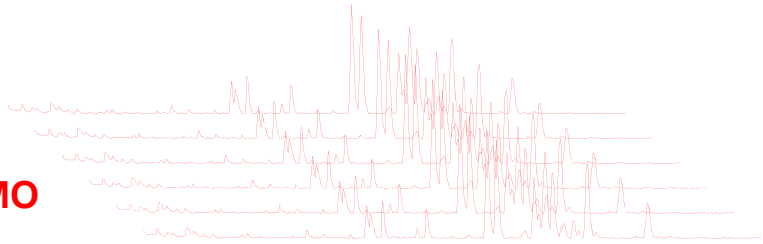
CELL METABOLOMICS VALIDATION

Analysis for global metabolic profiling of polyamine-related metabolites

Untreated



DFMO



Data processing: **MZmine** 2.7.2.

Open source software

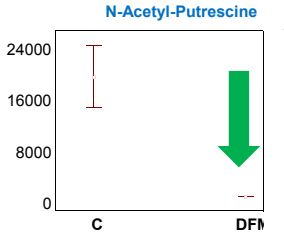
66 compounds related to polyamine pathway detected in the cell culture samples

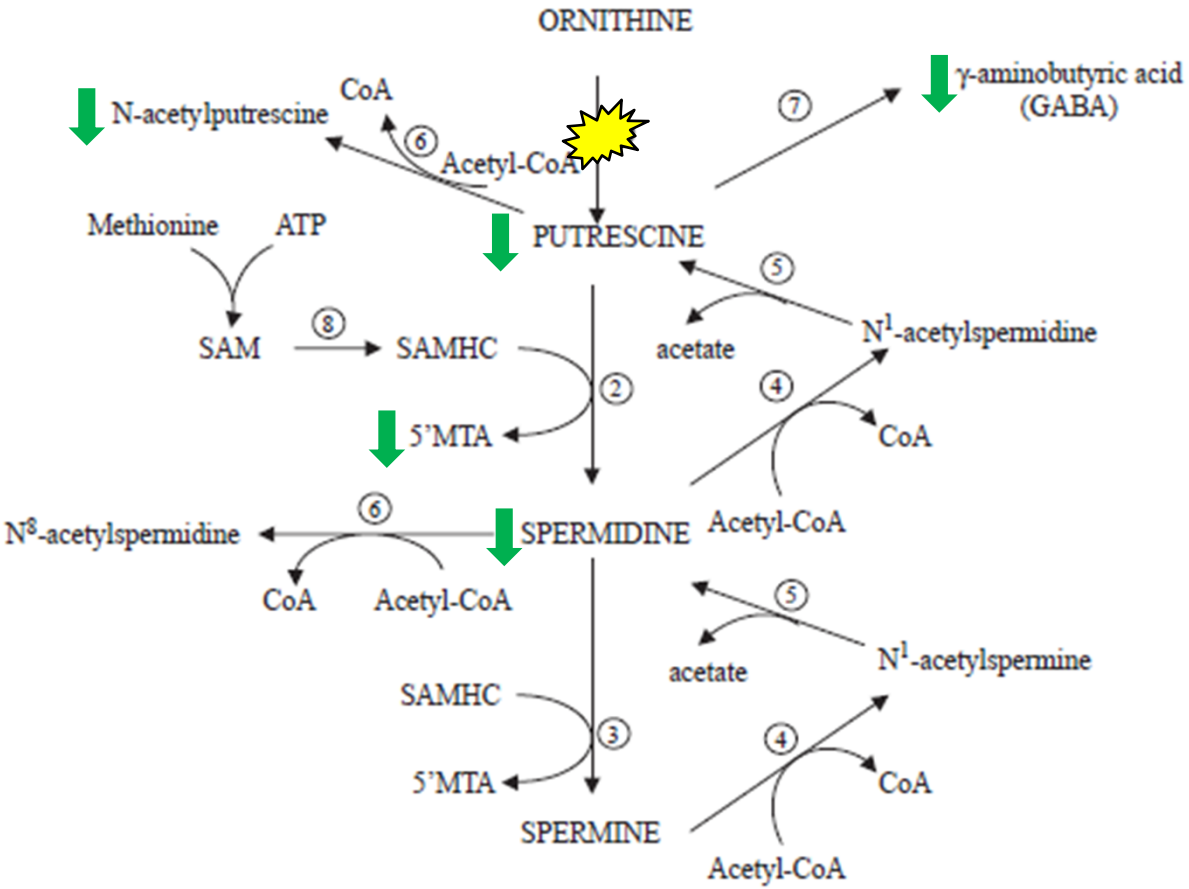
Statistical analysis
STATISTICA

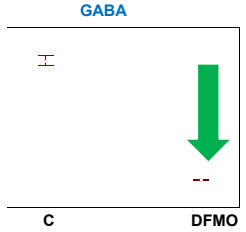
8 compounds related to polyamine pathway were statistically different ($p<0.05$)

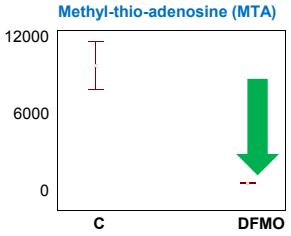
CELL METABOLOMICS VALIDATION

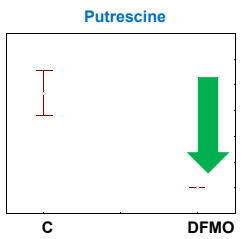
DFMO effect on polyamine-related compounds (marked with a green arrow) are confirmed demonstrating the usefulness of the whole cell metabolomics approach.

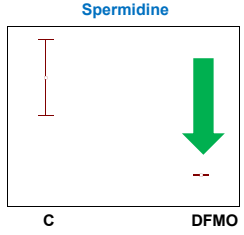












MORE INFORMATION ON THIS WORK CAN BE FOUND IN:

Journal of Pharmaceutical and Biomedical Analysis 110 (2015) 83–92

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Metabolomics of adherent mammalian cells by capillary electrophoresis-mass spectrometry: HT-29 cells as case study

Clara Ibáñez[a], Carolina Simó[a,*], Alberto Valdés[a], Luca Campone[b], Anna Lisa Piccinelli[b], Virginia García-Cañas[a], Alejandro Cifuentes[a]

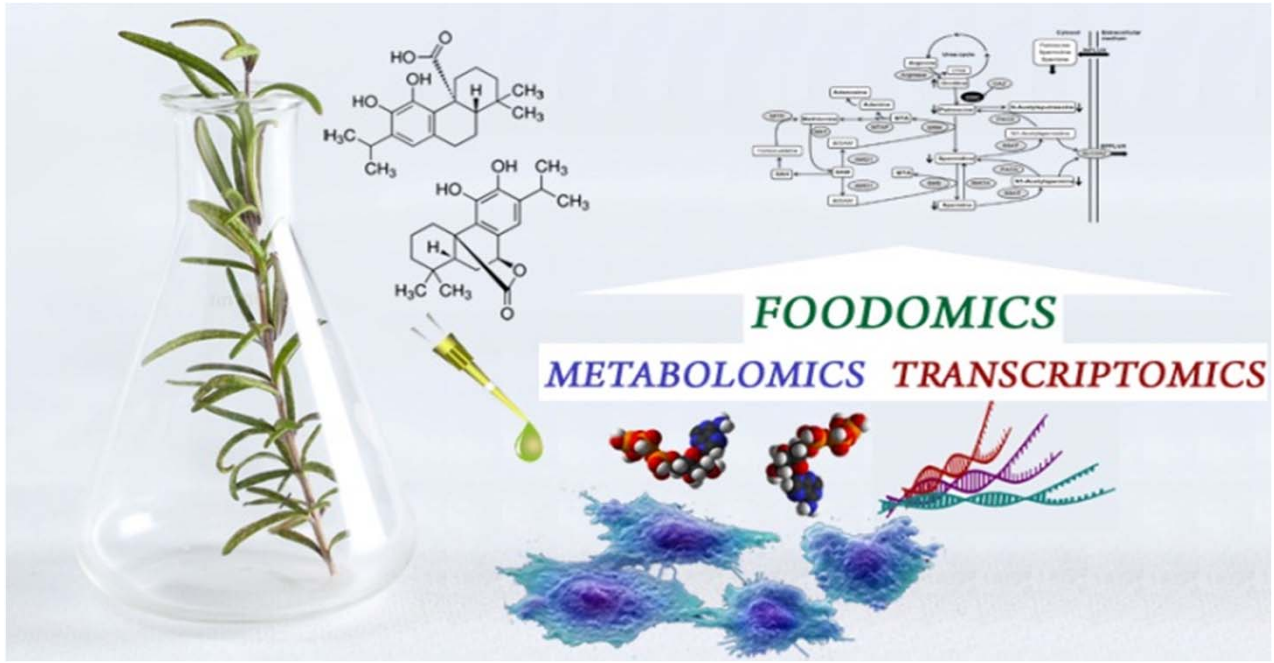
[a] *Laboratory of Foodomics, Institute of Food Science Research (CIAL), CSIC, Nicolas Cabrera 9, Cantoblanco Campus, 28049 Madrid, Spain*
[b] *Dipartimento di Farmacia, University of Salerno, Via Giovanni Paolo II 132, 84084, Fisciano (SA), Italy*

PARTS OF THIS WORK

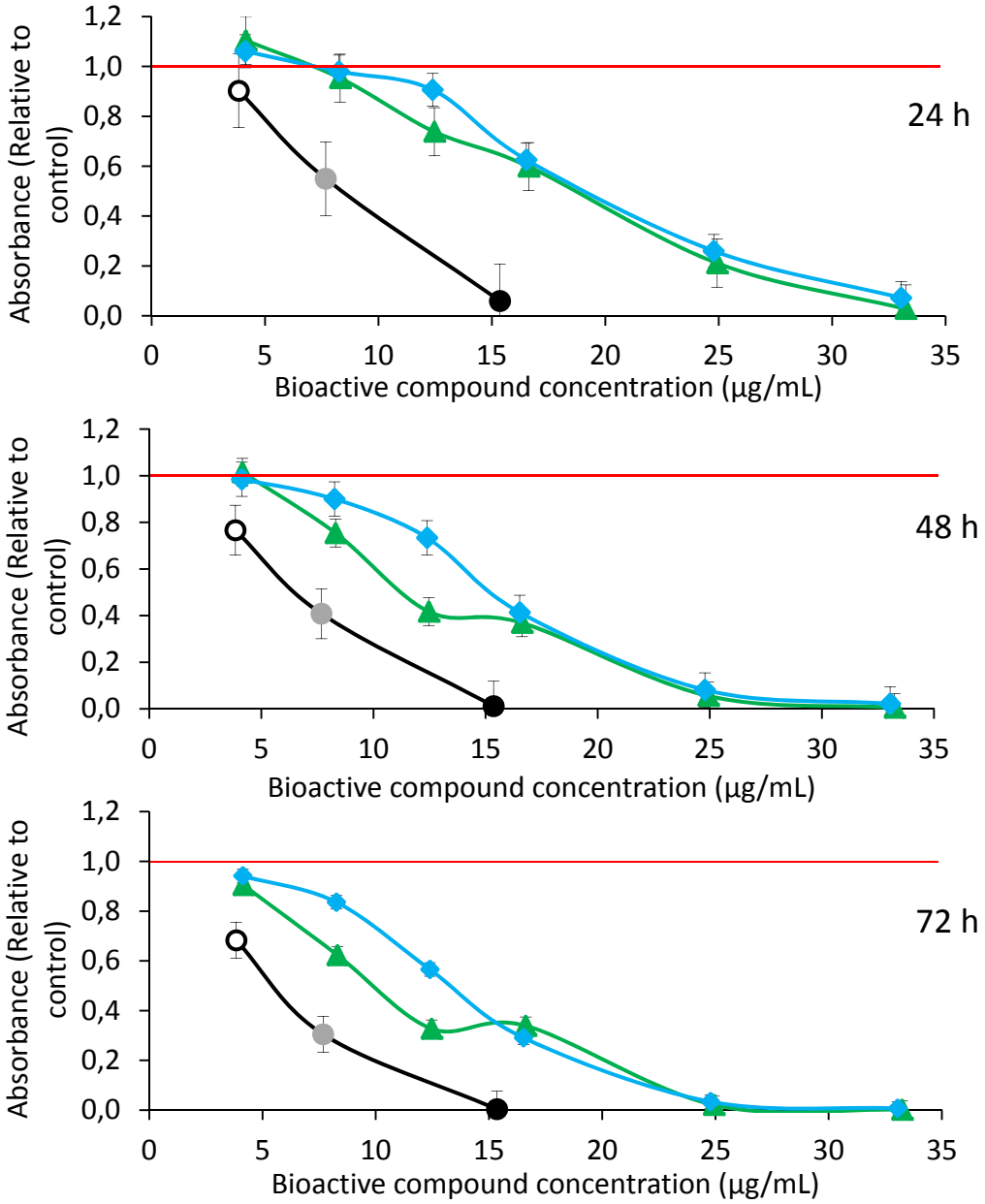
1. To optimize an effective protocol for cell metabolomics with especial emphasis in the sample preparation step and subsequent analysis of the intracellular metabolites from control and treated human HT-29 colon cancer cells

2. To investigate using a comprehensive Foodomics approach the contribution of carnosic acid (CA) and carnosol (CS), two major compounds present in the active rosemary extract (RE), against proliferation of human HT-29 colon cancer cells.

3. To corroborate the *in vitro* results by testing the antiproliferative activity of RE *in vivo.*



CELL PROLIFERATION INHIBITION (HT-29)
MTT ASSAY



◊ Carnosol (CS)
Δ Carnosic acid (CA)
○ Rosemary extract (RE)
○ 15 µg/mL
● 30 µg/mL
● 60 µg/mL

1-Dose-dependent reduction of cell proliferation observed in all cases.

2-CA and CS antiproliferative activity lower than rosemary extract.

3-CA activity better than CS.

Hypothesis:
May the inhibitory activity of RE on cell proliferation be due to the combined effect of both compounds?.
To check this: a combination of both compounds, CA and CS, was investigated in terms of synergistic, additive or antagonistic effects on inhibiting cell proliferation in HT-29 cell culture.

COMBINATION OF CA + CS
(synergistic, additive or antagonistic effect on cell proliferation inhibition)

Proportion of CA:CS in rosemary extract

7:1

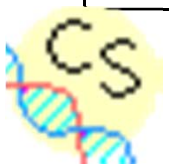
Combination (mixture) assayed at different concentration levels

→ Carnosol µg/mL

Carnosic Acid µg/mL

Carnosol µg/mL	61.4	30.7	15.4	7.7
8.9	61.4+8.9 (240µg RE/ml)			
4.5		30.7+4.5 (120µg RE/ml)		
2.2			15.4+2.2 (60µg RE/ml)	
1.1				7.7+1.1 (30µg RE/ml)

Data treatment → COMPUSYN



CI > 1 → Antagonistic
CI = 1 → Additive
CI < 1 → Synergistic

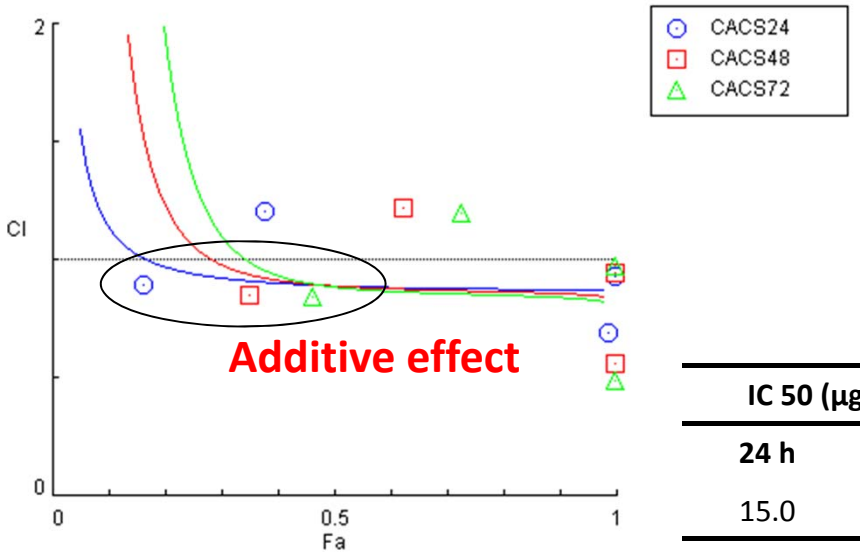
Chou TC, Pharmacol. Rev. 58:621–681, 2006.

COMBINATION OF CA + CS
(synergistic, additive or antagonistic effect on cell proliferation inhibition)

Proportion of CA:CS in rosemary extract

7:1

Combination (mixture) assayed at differnt concentration levels



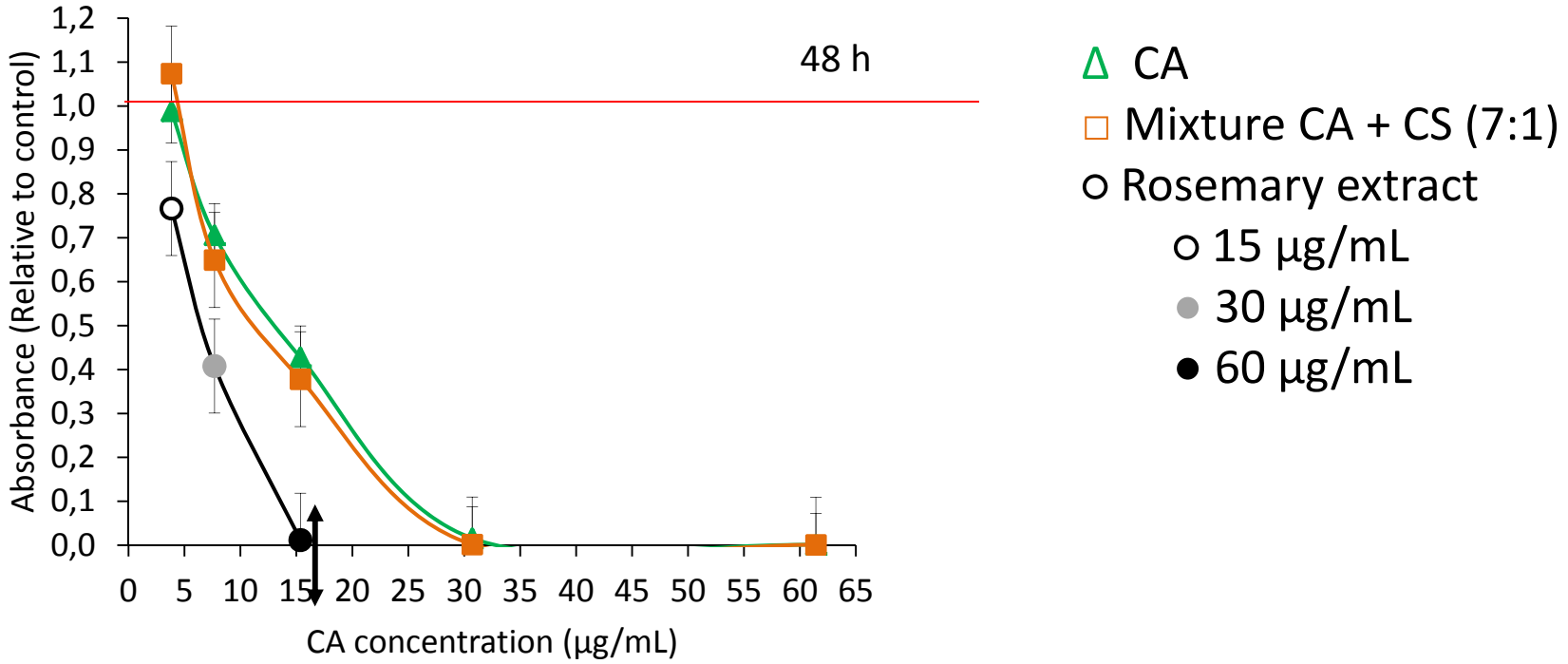
IC 50 (µg CA/mL + µg CS/mL)		
24 h	**48 h**	**72 h**
15.0	11.2	9.8

Combination index values for actual experimental points

µg RE/mL	µg CA/mL + µg CS/mL	Total Dose µg/mL	24 h		48 h		72 h	
			Fa	CI value	Fa	CI value	Fa	CI value
	61.4+8.9	70.3	0.997	0.9	0.999	0.9	0.999	1.0
	30.7+4.5	35.2	0.986	0.7	0.998	0.6	0.999	0.5
60	**15.4+2.2**	17.6	0.378	1.2	0.622	1.2	0.727	1.2
30	**7.7+1.1**	8.8	0.163	**0.9**	0.351	**0.9**	0.463	**0.8**
				r: 0.969		r: 0.946		r: 0.926

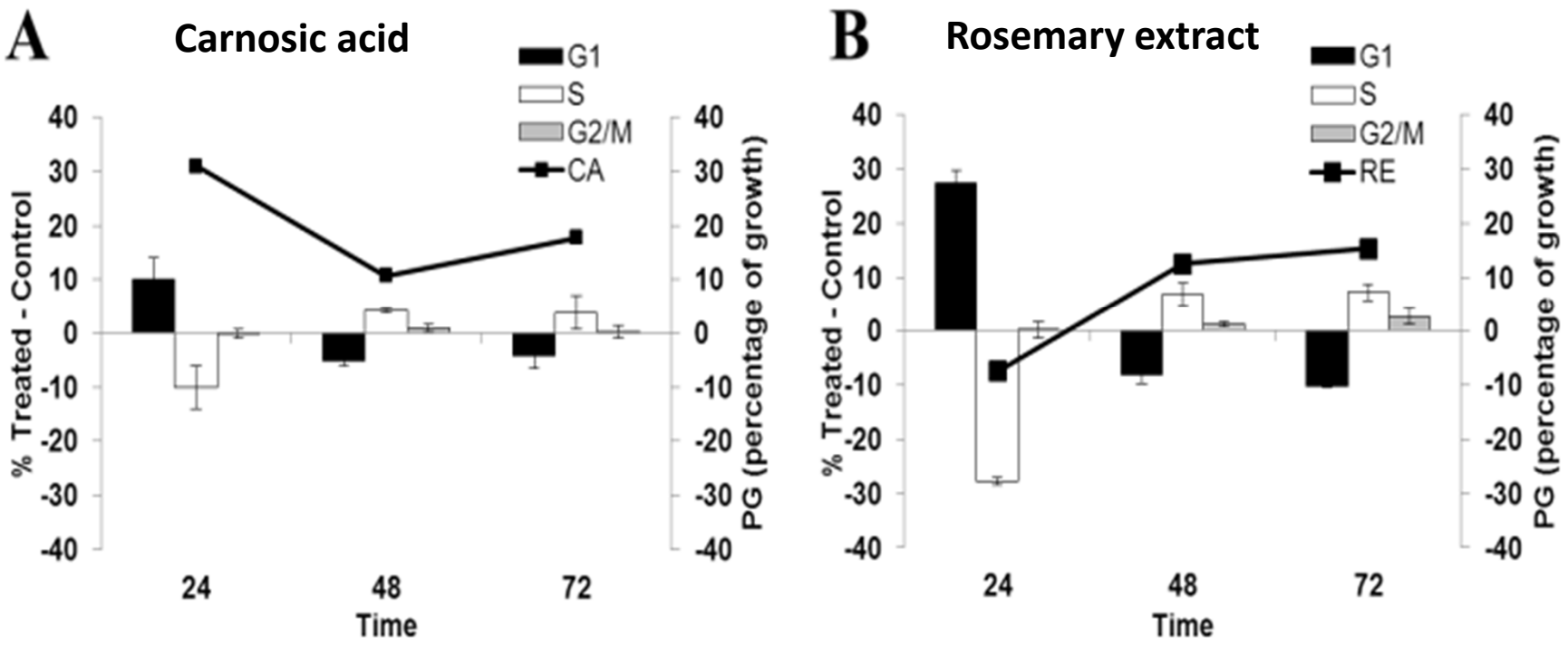
Additive effect

COMPARISON OF ANTIPROLIFERATIVE ACTIVITY OF CA vs. CA + CS COMBINATION vs. ROSEMARY EXTRACT



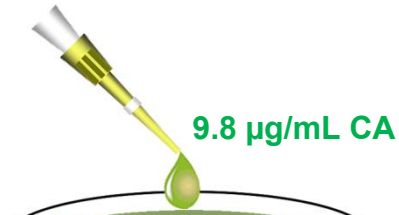
Every RE concentration possess stronger antiproliferative activity than the CA + CS mixture. This suggests other constituents of the extract might be also contributing to the activity of RE on HT-29 cells, however, **most of the observed antiproliferative activity of the extract is exerted by CA and CS**. Furthermore, CA has the higher content in RE and slightly superior antiproliferative effect., therefore, the cellular and **molecular mechanisms underlying the antiproliferative activity of CA against HT-29 cells were investigated by Foodomics**.

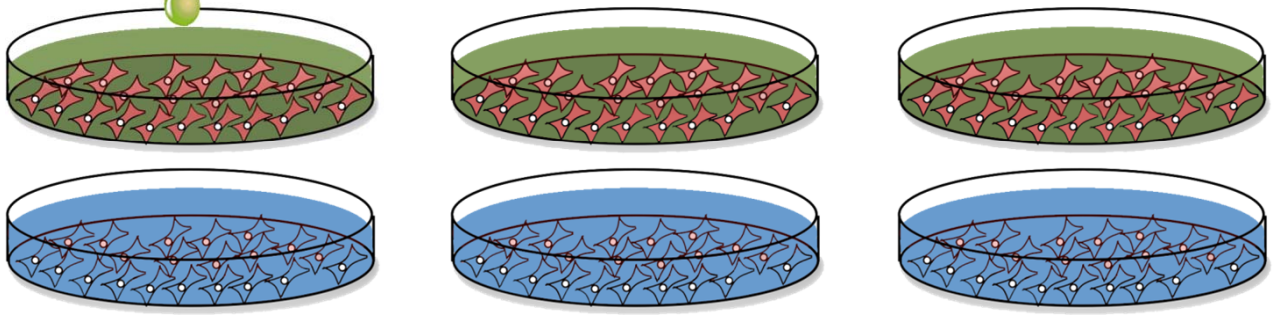
ANTIPROLIFERATIVE ACTIVITY OF CA: CELL CYCLE ANALYSIS WITH PROPIDIUM IODIDE AND FLOW CYTOMETRY



Cell cycle distribution patterns (bars) determined by flow cytometry and calculated percentage of growth (line) from MTT assay data of HT-29 cells exposed to 12.5 µg/mL of CA and 30 µg/mL of RE for up to 72 h of incubation. **CA and RE show similar effect on HT29-cell cycle, G1-S arrest after 24h, indicating a cytostatic effect**. Although both treatments exert similar alterations on the cell cycle phases, the percentage of growth did not correlated equally in both treatments. Thus, the antiproliferative effect of CA was increasing over 48 h while with RE reached the maximum at 24 h and recovered afterwards

FOODOMICS EVALUATION OF THE EFFECT OF CARNOSIC ACID





CARNOSIC ACID

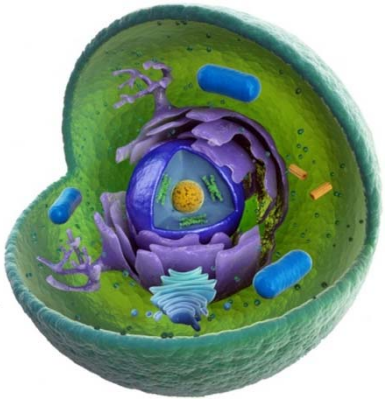
Untreated

Incubation for 48 h

CELLS WERE DETACHED AND COUNTED

10^7 CELLS

METABOLITE EXTRACTION



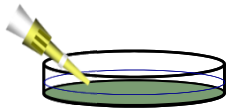
10^6 CELLS

RNA EXTRACTION

METABOLOMICS

TRANSCRIPTOMICS

CELL METABOLOMICS: EFFECT OF CARNOSIC ACID



Treated

Control

Human HT29 colon cancer cell line:
treated vs. control

ANALYSIS OF INTRACELLULAR METABOLITES

CE-TOF-MS

RP-UPLC-QTOF-MS

High Resolution Separation Techniques Coupled to mass spectrometry

Bare silica capillary: 87 cm,
50 µm ID BGE: 3 M HCOOH
Voltage: +27 kV
Sample injection: 80 s (0.5 psi)

Positive ion mode
Seath liquid: IspOH-H_2O (1:1, v/v)
Seath liquid flow: 0.24 mL/h
Nebulizar gas pressure: 0.4 bar
Drying gas flow: 4L/min
Drying gas temperature: 200º C
Mass scan: 50-500 m/z

ZORBAX C18, Rapid Resolution HT
(2.1 50 mm, 1.8 µm)
Gradient: 0-6 min: 2-20% B; 6-10 min:
20-100% B; 10-12 min: 100% B
A: water with 5 mM ammonium
formate at pH 5.8
B: ACN 0.1% formic acid

Positive ion mode
Nebulizar gas pressure: 40 psi
Drying gas flow: 10L/min
Drying gas temperature: 300º C
Mass scan: 50-1000 m/z

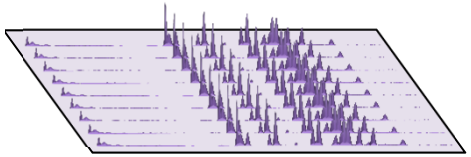
CELL METABOLOMICS: EFFECT OF CARNOSIC ACID

DATA TREATMENT PIPELINE

Data processing

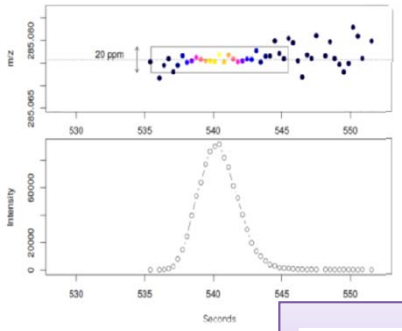
(Peak picking, alignment, grouping, filtering, etc.)

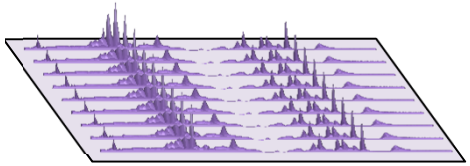
Statistical analysis



CE-TOF MS dataset

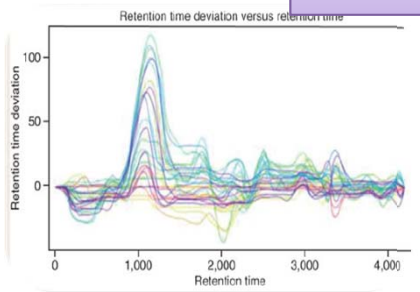
MZmine 2



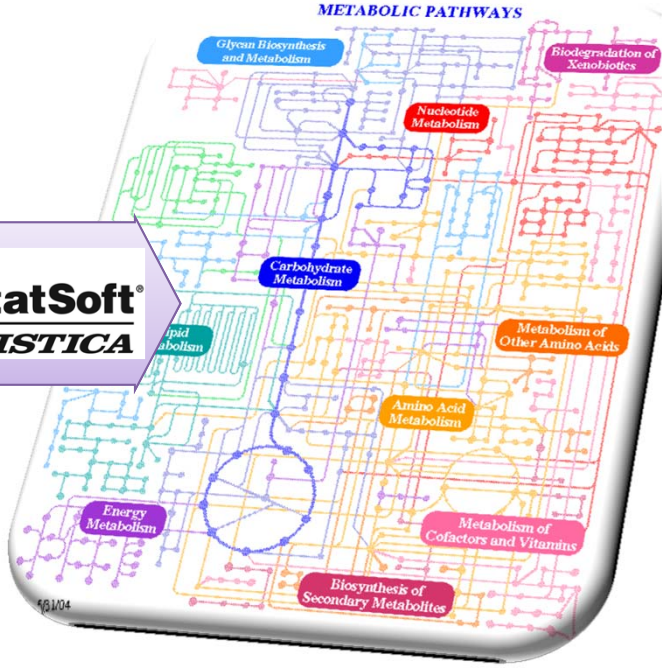


UPLC-TOF MS dataset

XCMS
mzMatch



StatSoft
STATISTICA



Biological interpretation

CELL METABOLOMICS: EFFECT OF CARNOSIC ACID

Identification of metabolites differentially expressed in HT-29 cells after carnosic acid treatment, determined by CE-TOF-MS and UHPLC-TOF-MS.

Migration/ Retention time	m/z	Ion	Formula	Error (ppm)	Tentative identification	Standard coinjection	Database identifier	Expression
4.21[a]	131.118	M+H	C6H14N2O	-2.9	N-acetyl-putrescine	YES	HMDB02064	DOWN
6.07[a]	104.070	M+H	C4H9NO2	-6.7	γ-aminobutyric acid	YES	HMDB00650	DOWN
7.73[a]	298.096	M+H	C11H15N5O3S	-3.8	Methyl-thio-adenosine	YES	HMDB01173	DOWN
8.96[a]	179.049	M+H	C5H10N2O3S	4.5	Cysteinyl-glycine	NO	HMDB28775	UP
8.32[a]	307.086	M+2H	C20H32N6O12S2	8.5	Oxidized glutathione	YES	HMDB03337	DOWN
8.96[a]	308.092	M+H	C10H17N3O6S	1.8	Reduced glutathione	YES	HMDB00125	UP
7.28[a]	244.094	M+H	C9H13N3O5	5.5	Cytidine	YES	HMDB00089	DOWN
2.38[b]	456.246	M+NH4	C14H22O3	5.0	Oxo-tetradecadienoic acid	NO	LMFA01060185	DOWN
2.64[b]	306.059	M+K	C10H13N5O4	6.5	Adenosine	YES	HMDB00050	DOWN
2.70[b]	393.194	M+NH4	C15H25N3O8	4.2	Tripeptide (I/L, D, E)	NO	MID20124	UP
2.71[b]	161.092	M+H	C6H12N2O3	-3.7	Alanyl-Alanine	NO	HMDB28680	DOWN
3.21[b]	471.171	M+H	C23H26N4O5S1	-1.3	Tripeptide (C, Y, W)	NO	MID22112	DOWN
3.28[b]	112.041	M+H	C5H5NO2	3.7	Pyrrole-carboxylic acid	NO	HMDB04230	DOWN
3.40[b]	453.168	M+Na	C21H26N4O6	2.5	Tripeptide (W, E, P)	NO	MID 19517	DOWN
3.42[b]	244.092	M+H	C9H13N3O5	3.3	Cytidine	YES	HMDB00089	DOWN
3.59[b]	455.176	M+H	C23H26N4O4S1	1.4	Tripeptide (F, W, C)	NO	MID18550	DOWN
3.64[b]	327.324	M+H	C21H42O2	-6.1	Dodecyl-nonanoate	NO	LMFA07010453	UP
5.79[b]	274.201	M+H	C14H27NO4	-0.8	Heptanoyl-carnitine	NO	HMDB13238	DOWN
6.48[b]	127.123	M+H-H2O	C7H16N2O	4.8	N-Acetyl-cadaverine	YES	HMDB02284	DOWN
6.81[b]	308.092	M+H	C10H17N3O6S	-0.5	Reduced glutathione	YES	HMDB00125	UP
7.00[b]	162.115	M+H	C7H15NO3	-1.9	Carnitine	YES	HMDB00062	UP

[a] identified metabolites from CE-ESI-TOF MS analysis. [b] identified metabolites from UHPLC-ESI-TOF MS analysis.

CELL METABOLOMICS: EFFECT OF CARNOSIC ACID

STATISTICAL ANALYSIS

CE-TOF MS

7 metabolites significantly altered

- **N-Acetyl-Putrescine**
- **GABA**
- **Methyl-thio-adenosine**
- **Cysteinyl-glycine**
- **GSSG (oxidized glutathione)**
- **GSH (reduced glutathione)**
- **Cytidine**

UPLC-TOF MS

14 metabolites significantly altered

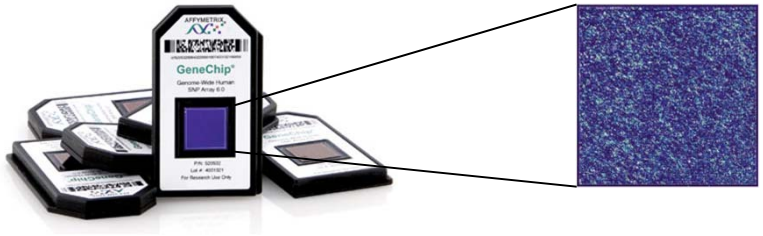
- **N-Acetyl-Cadaverine**
- **Carnitine**
- **Heptanoyl-Carnitine**
- **Adenosine**
- **Pyrrole-Carboxylic acid**
- **GSH**
- **Cytidine**
- **Alanyl-alanine**
- **Tripeptide (I/L, D, E)**
- **Tripeptide (C, Y, W)**
- **Tripeptide (F, W, C)**
- **Tripeptide (W, E, P)**
- **Dodecyl-nonanoate**
- **Oxo-Tetradecanoic acid**

Only 2 out of the 21 metabolites were detected by both CE-TOF MS and UHPLC-TOF MS approaches, demonstrating the complementarity of these two metabolomic methodologies

TRANSCRIPTOMIC EFFECT OF CARNOSIC ACID

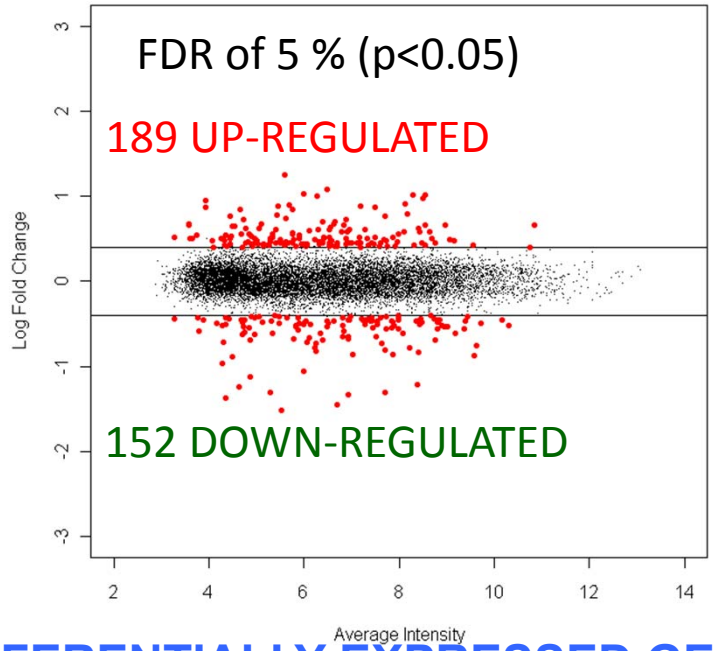
GENE EXPRESSION MICROARRAY ANALYSIS

QUALITY CONTROL → **DATA PRE-PROCESSING** → **DATA PROCESING** → STATISTICAL ANALYSIS



Affymetrix Human Gene 1.0 ST microarray

Core Analysis function included in IPA was applied to analyze the lists of differentially expressed genes (DEGs) identified in microarray analysis. Cut-offs were set at 0.4 as M-value cutoff that corresponds to expression ratios (fold change) ≥ 1.3 for up-regulated and ≤ 0.8 for down-regulated genes. Thus, 213 genes from microarray data were eligible for biological function and pathway analysis



341 DIFFERENTIALLY EXPRESSED GENES I CARNOSIC ACID

(1.2% of the genes covered by the whole-transcriptome microarray)

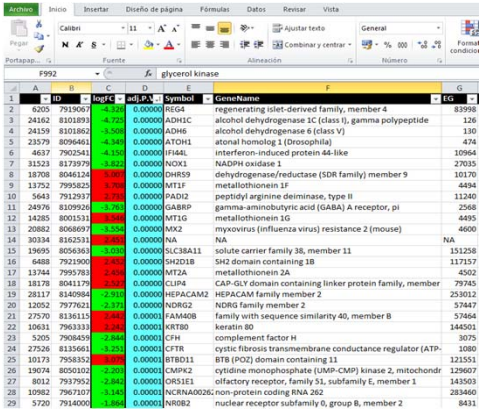
TRANSCRIPTOMIC EFFECT OF CARNOSIC ACID

BIOINFORMATICS STRATEGIES

BIOLOGICAL FUNCTIONS POTENTIALLY MODIFIED BY CARNOSIC ACID

Based on the list of identifiers (DEG), IPA performs **functional enrichment analysis** in order to identify the biological processes and functions over-represented in a given list of genes.

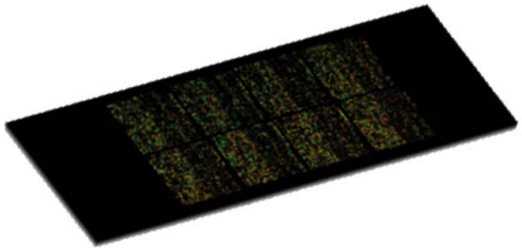
LIST OF DIFFERENTIALLY EXPRESSED GENES (DEGs)



	ID	logFC	adj.P.Val	Symbol	GeneName	EG
2	6205	7919067	-4.326	0.00000	REG4	regenerating islet-derived family, member 4	83998
3	24162	8101893	-4.725	0.00000	ADH1C	alcohol dehydrogenase 1C (class I), gamma polypeptide	126
4	24159	8101862	-3.508	0.00000	ADH6	alcohol dehydrogenase 6 (class V)	130
5	23579	8096461	-4.349	0.00000	ATOH1	atonal homolog 1 (Drosophila)	474
6	4637	7902541	-4.150	0.00000	IFI44L	interferon-induced protein 44-like	10964
7	31523	8173979	-3.822	0.00000	NOX1	NADPH oxidase 1	27035
8	18708	8046124	-5.087	0.00000	DHRS9	dehydrogenase/reductase (SDR family) member 9	10170
9	13752	7995825	-3.756	0.00000	MT1F	metallothionein 1F	4494
10	5643	7912937	-2.750	0.00000	PADI2	peptidyl arginine deiminase, type II	11240
11	24976	8109926	-3.760	0.00000	GABRP	gamma-aminobutyric acid (GABA) A receptor, pi	2568
12	14285	8001531	-3.546	0.00000	MT1G	metallothionein 1G	4495
13	20882	8068697	-3.554	0.00000	MX2	myxovirus (influenza virus) resistance 2 (mouse)	4600
14	30334	8162531	-2.430	0.00000	NA	NA	NA
15	19695	8056363	-3.030	0.00000	SLC38A11	solute carrier family 38, member 11	151258
16	6488	7921900	2.452	0.00000	SH2D1B	SH2 domain containing 1B	117157
17	13744	7995783	2.458	0.00000	MT2A	metallothionein 2A	4502
18	18178	8041179	2.532	0.00000	CLIP4	CAP-GLY domain containing linker protein family, member	79745
19	28117	8140984	2.910	0.00000	HEPACAM2	HEPACAM family member 2	253012
20	12052	7977621	2.371	0.00000	NDRG2	NDRG family member 2	57447
21	27570	8136115	2.462	0.00001	FAM40B	family with sequence similarity 40, member B	57464
22	10631	7963333	2.297	0.00001	KRT80	keratin 80	144501
23	5205	7908459	2.844	0.00001	CFH	complement factor H	3075
24	27526	8135661	3.251	0.00001	CFTR	cystic fibrosis transmembrane conductance regulator (ATP-	1080
25	10173	7958352	3.875	0.00001	BTBD11	BTB (POZ) domain containing 11	121551
26	19074	8050102	2.269	0.00001	CMPK2	cytidine monophosphate (UMP-CMP) kinase 2, mitochondr	129607
27	8012	7937952	2.842	0.00001	OR51E1	olfactory receptor, family 51, subfamily E, member 1	143503
28	10982	7967107	3.140	0.00001	NCRNA00262	non-protein coding RNA 262	283460
29	5720	7914000	1.866	0.00001	NR0B2	nuclear receptor subfamily 0, group B, member 2	8431

INGENUITY PATHWAY ANALYSIS

WHOLE MICROARRAY DATA



GSEA
Gene Set Enrichment Analysis

GENE SET ENRICHMENT ANALYSIS

Over-represented biological processes in the transcriptomic profile

TRANSCRIPTOMIC EFFECT OF CARNOSIC ACID

IPA analysis of the molecular and cellular functions over-represented in the list of differentially expressed genes in HT-29 cells after CA treatment.

Annotation	p-val[a]	Activation z-score	Genes[b]
Transport of molecules	5.10E-04	-2.379 **Inhibition**	↓CA2,↓CFTR,↓CLDN2,↓EDN1,↓GPC3,↓NDRG2,↓NR4A1,↓SLC14A1,↓SLC16A7,↓SLC5A1,↓SLC7A2
Metabolism of terpenoid	1.07E-04	-2.016 **Inhibition**	↓ADH1C,↓EDN1,↓NR4A1, ↓PRLR
Metabolism of ROS	3.81E-03	+2.015 **Activation**	↓NR4A1,↑IL18,↓HEPH, ↓SPRY2,↓SESN1,↑MAOB ,↓mir-21, ↑IL8, ↑NCF1

[a] significance value calculated with the right-tailed Fisher's exact test; [b] the arrows indicate an increase (↑) or decrease (↓) in the transcript levels.

TRANSCRIPTOMIC EFFECT OF CARNOSIC ACID

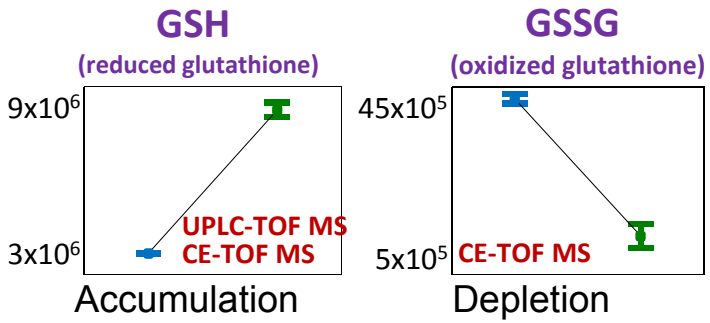
RT-qPCR confirmation of gene expression ratios determined by microarray analysis in response to carnosic acid treatment of HT-29 colon cancer cells.

Gene symbol	**Microarray**		**RT-qPCR**	
	FC[a]	**p-value**[b]	**FC**[a]	**p-value**[c]
ALDH3A1	1.50	<0.001	2.15	0.024
NOX1	0.54	<0.001	0.52	0.011
GPX3	1.74	<0.001	1.74	0.034
ADH1C	0.40	<0.001	0.18	0.034
MAOB	1.34	0.013	1.54	0.031

[a] For comparison purposes, M-values (log_2-fold change) obtained from microarray analysis were converted to Fold Change (FC) values.
[b] Adjusted p-value (FDR). [c] Statistical significance calculated by REST.

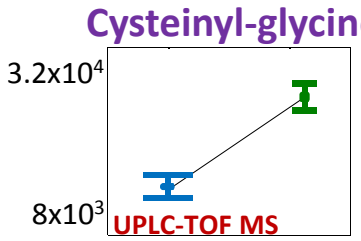
CELL METABOLOMICS: EFFECT OF CARNOSIC ACID

BIOLOGICAL MEANING: OTHER FINDINGS

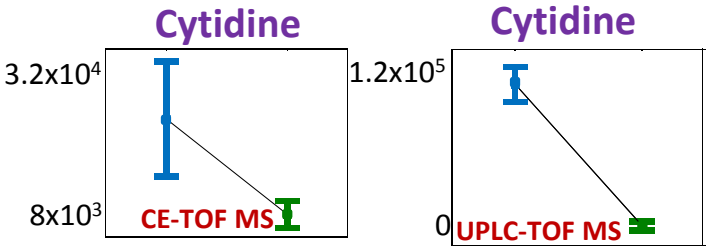


CARNOSIC ACID INDUCED ACCUMULATION OF REDUCED GLUTAHIONE AND DECREASED OXIDIZED GLUTATHIONE

CONTROL REDOX STATUS INCLUDING REACTIVE OXYGEN SPECIES (ROS)



Cysteinylglycine is a naturally occurring dipeptide. It is a breakdown product of glutathione (GSH) and an intermediate in the gamma-glutamyl cycle. **Its increase is related to the GSH accumulation induced by CA.**

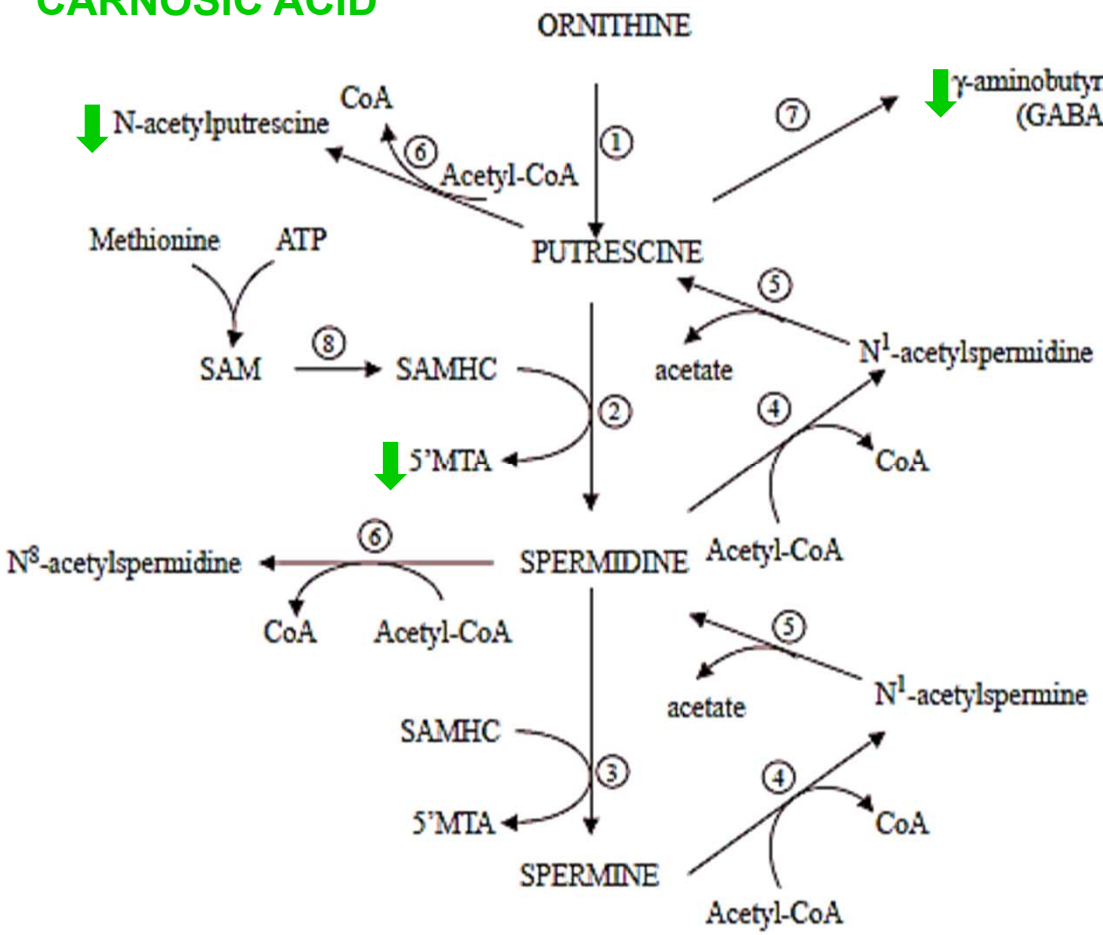


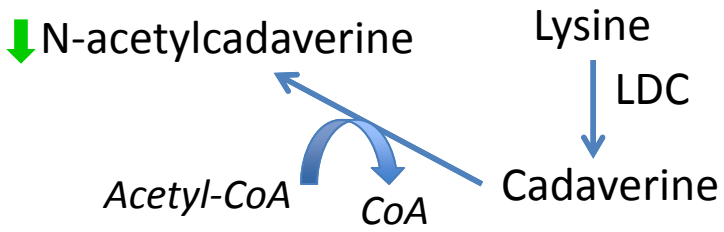
A significant intracellular depletion of the nucleoside cytidine by CA treatment was corroborated by both analytical platforms. **Further investigations are required** to elucidate the role and the underlying mechanisms of the effect of cytidine depletion in HT-29 cell proliferation.

CELL METABOLOMICS: EFFECT OF CARNOSIC ACID

BIOLOGICAL MEANING: POLYAMINES PATHWAY

TREATMENT WITH CARNOSIC ACID

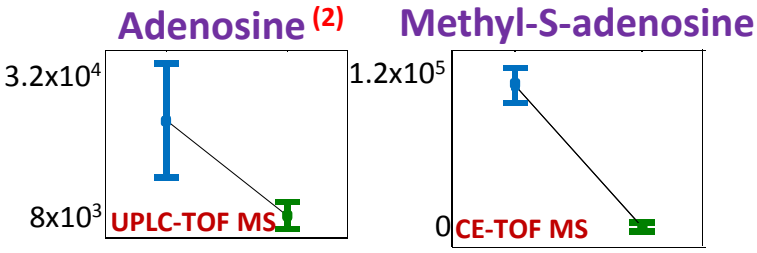




Decreased levels of N-acetylputrescine, N-acetylcadaverine, 5'MTA and γ-aminobutyric acid have already been connected to a reduction of cell proliferation and viability of different cancer cells.

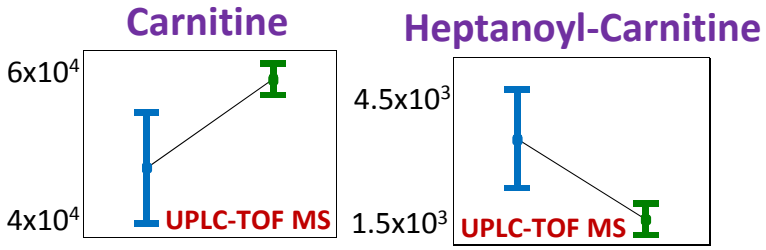
CELL METABOLOMICS: EFFECT OF CARNOSIC ACID

BIOLOGICAL MEANING: OTHER FINDINGS



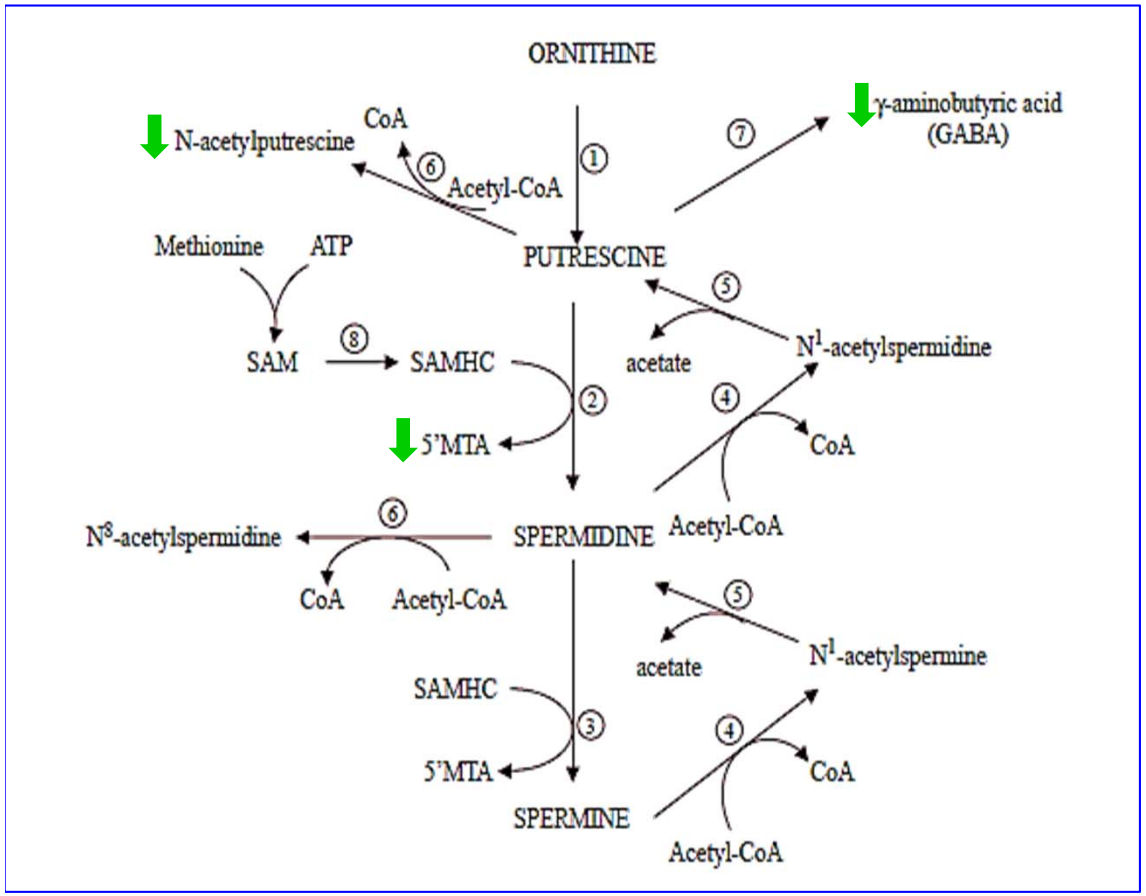
LINKED TO NUCLEIC ACID METABOLISM

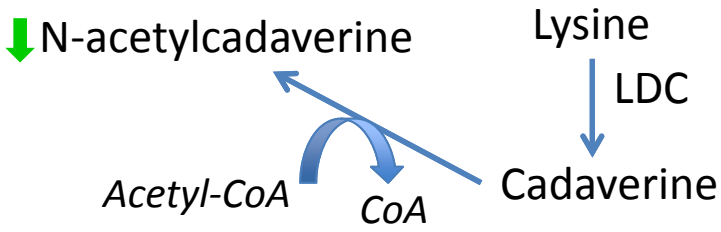
Metabolomics data also indicated a decrease in methylthioadenosine and adenosine levels. Chemically-induced inhibition of polyamine metabolism in HT-29 cells has been associated with unexpected changes in these methionine cycle intermediates and a decrease in the nucleoside thymidine pool, causing a **cytostatic effect**.

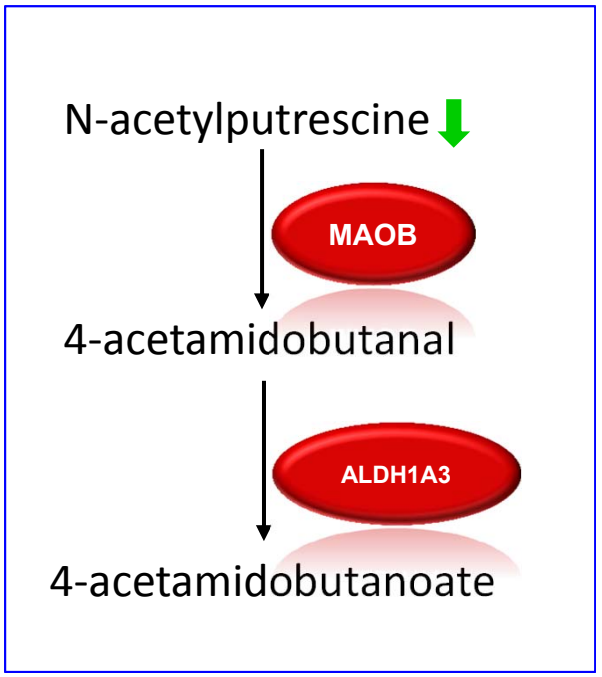


PROMOTES FATTY ACID OXIDATION AND DECREASES PROLIFERATION IN HT-29

Foodomics effect of carnosic acid: Integration of Transcriptomics and Metabolomics results



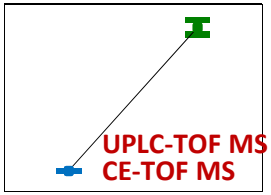




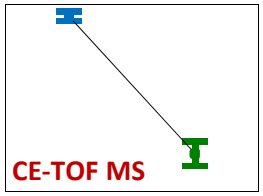
Decreased levels of N-acetylputrescine, N-acetylcadaverine, 5'MTA and γ-aminobutyric acid reduce cell proliferation and viability of HT29 colon cancer cells. E.g., N-acetylputrescine depletion could be explained by the up-regulation of MAOB gene product, involved in the enzymatic transformation of N-acetylputrescine to 4-acetamidobutanal, that can be further transformed to 4-acetamidobutanoate by ALDH1A3 gene product (also induced by the CA treatment).

Foodomics effect of carnosic acid: Integration of Transcriptomics and Metabolomics results

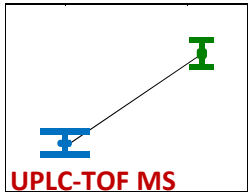
GSH

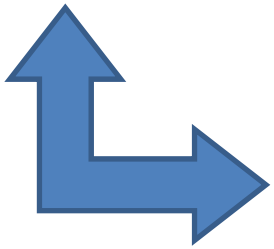


GSSG

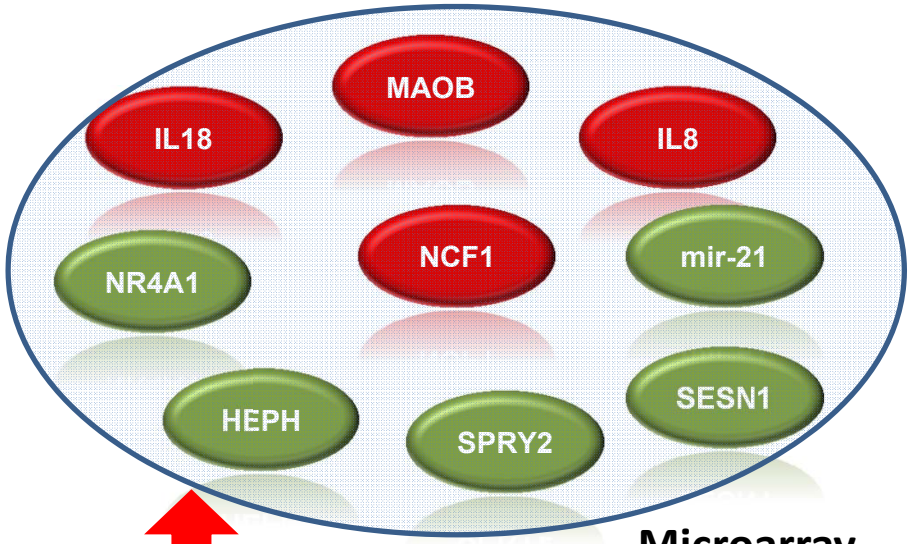


Cys-Gly



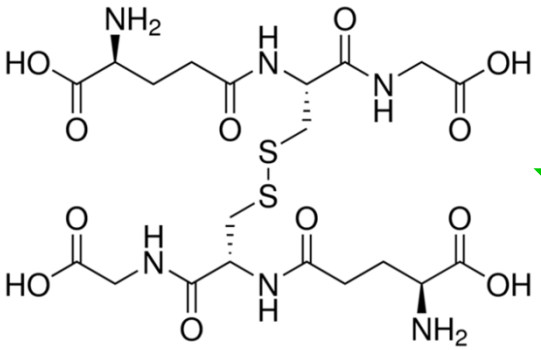


Chemopreventive activity of CA against HT29 colon cancer is corroborated by the **activation of the metabolism of Reactive Oxygen Species (ROS)** and the increase of the levels of GSH, Cys-Gly and the reduction of GSSG



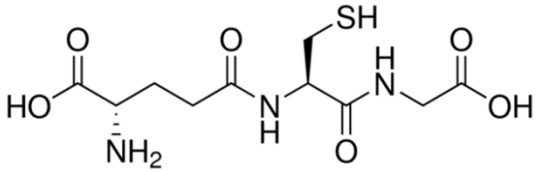
Microarray

Foodomics effect of carnosic acid: Integration of Transcriptomics and Metabolomics results



GSSG
(oxidized glutathione)

GSH
(reduced glutathione)



GPXs

CONTROL
GSH/GSSG = 0.7

H_2O
Alcohols

H_2O_2
Lipid peroxides

CARNOSIC ACID TREATMENT
GSH/GSSG = 6.4

Enhanced detoxifying cellular capability

Combined with transcriptomic data, these results indicate higher GSH availability, necessary for the activity of GPXs encoded enzymes (i.e., glutathione peroxidase), up-regulated by CA treatment, suggesting an enhanced detoxifying cellular capability.
The balance between amine-oxidizing enzymes and antioxidant enzymes is a crucial point for cancer growth inhibition or progression.

CONCLUSIONS

1. Although carnosic acid (CA) and carnosol (CS) exhibit additive antiproliferative effect when they are combined in solution at a molar ratio of 6.9:1, the results reveal that CA contributes more significantly than CS to the activity against colon cancer cells proliferation.
2. The Foodomics study reveals that CA induces transcriptional activation of genes that encode detoxifying enzymes and altered the expression of genes linked to transport and biosynthesis of terpenoids in the colon cancer cells.
3. Functional analysis highlighted the activation of the ROS metabolism and alteration of several genes involved in pathways describing oxidative degradation of relevant endogenous metabolites, providing new evidences about the transcriptional change induced by CA in HT-29 cells.
4. Metabolomics analysis showed that the treatment with CA affected the intracellular levels of glutathione. Elevated levels of GSH provided additional evidences to transcriptomic results regarding chemopreventive response of cells to CA treatment. Moreover, the Foodomics approach was useful to establish the links between decreased levels of polyamines and their degradation pathway at the gene level.

MORE INFORMATION ON THIS WORK CAN BE FOUND IN:

analytical chemistry

Article

A comprehensive Foodomics study on the mechanisms operating at various molecular levels in cancer cells in response to individual rosemary polyphenols

Alberto Valdés, Virginia Garcia-Cañas, Carolina Simó, Clara Ibañez, Vicente Micol, Jose Antonio Ferragut, and Alejandro Cifuentes

Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/ac502401j • Publication Date (Web): 04 Sep 2014

ELSEVIER

JOURNAL OF FUNCTIONAL FOODS

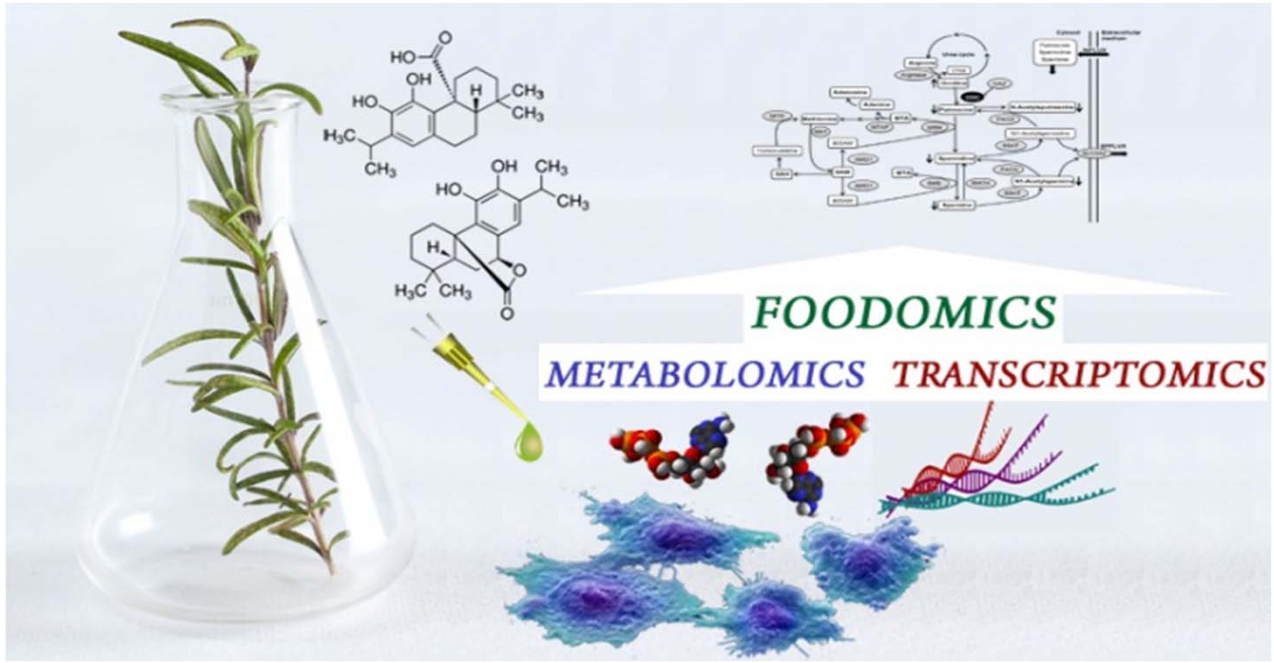
-A. Valdes, G. Sullini, E. Ibañez, A. Cifuentes, V. Garcia-Cañas "Rosemary polyphenols induce unfolded protein response and changes in cholesterol metabolism in colon cancer cells“ **15 (2015) 429-439**

PARTS OF THIS WORK

1. To optimize an effective protocol for cell metabolomics with especial emphasis in the sample preparation step and subsequent analysis of the intracellular metabolites from control and treated human HT-29 colon cancer cells

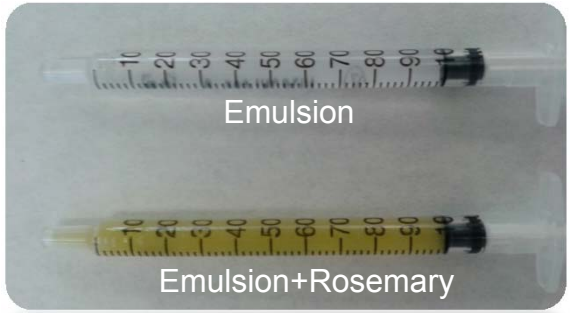
2. To investigate using a comprehensive Foodomics approach the contribution of carnosic acid (CA) and carnosol (CS), two major compounds present in the active rosemary extract (RE), against proliferation of human HT-29 colon cancer cells.

3. To corroborate the *in vitro* results by testing the antiproliferative activity of RE *in vivo*.



In vivo confirmation:

Inhibition of human colon cancer xenografts (HT-29) in Foxn1 (nude) mice model by oral treatment with rosemary extract



IN VIVO EXPERIMENTS: DESIGN

TREATMENT → **ORAL administration (200 mg/kg)** → 12 mice

PRE-TREATMENT → **ORAL administration (200 mg/kg)** → 10 mice

CONTROL → **ORAL administration (Emulsion or vehicle: 40% PEG400, 10% Tween 80 and 50% PBS)** → 10 mice

Work in progress:

Rosemary

Green extraction

Selected extract

Control, treated and pre-treated mice

Tumor and other samples from different tissues.

RNAs and metabolites obtained from control, treated and pretreated individuals

Foodomics evaluation

Transcriptomics

Metabolomics

Data processing

Statistical analysis

Biomarkers identification and confirmation. Pathway analysis and biological process understanding

Thank you!