Project no. : COOP-CT-2003-508649

Acronym : PARADOX

Project title : French Paradox
Red wine extract food additives

Instrument : Special Research Project for SME`s
Cooperative Research (CRAFT)

FINAL REPORT

Period covered : from 15. 02. 2004 to 14. 02 2006 Date of Preparation : 30.03.2006

Start date of project : 15.02.2004 Duration : 24 months

Project coordinator name : Dr. Barbara Gimeno

Project coordinator organisation name : GAT Formulation GmbH

Revision : Draft

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PARADOX PROJECT

EU CONTRACT COOP-CT-2003-508649

From the lab to the shelf

A successful cooperative EU project in between University, Clinical Institutions and Industry for the streamlined development (from basic science to consumer) of a new and patented Food Ingredient Product.

The **agricultural waste** of the wine making process –grape pomace- is the **raw material for the production of a new Functional Food Ingredient** enriched in **antioxidants**, **beneficial for preventing diverse illnesses** as atherosclerosis, cancer, cardiovascular disease, brain ictus, retinal degeneration, inflammatory processes (rheuma) and many other pathological conditions related to oxidative stress and endothelial misfunctioning.

A **"green" process of extraction** (no use of apolar solvents) of an antioxidant fraction of grape pomaces has been developed industrially, based on the treatment of the grape pomace with acetone or ethyl acetate and final precipitation of the antioxidants by supercritical CO2 fluid extraction.

The extract has shown inhibiting **action** over the enzyme ET-1, particulary involved in **deterioration of blood vessels**, and to **reduce the oxidative stress** to in vivo cells.

The **identification of the main antioxidant compounds** revealed predominantly catechin, gallic acid, gallocatechin, epigallocatechin and epicatechin monomers and oligomers, resveratrol, viniferin E, piceid, a particulary interesting compound with a tetrameric epicatechin structure, not available as an analytical standard and a challenge for synthesis chemistry.

Current grape antioxidants used in functional foods lose their beneficial activity due to oxidation during industrial processing and shelf life. **The technology employed provides for the first time a solution** to this major problem: we have patented a **novel microencapsulation process for the antioxidant extract**, that was developed to protect the extract from light, oxygen, temperature and pressure stress, both in the processing of the Functional Food Ingredient, and in the making of the Functional Food Product(Nutraceutical) where the extract is to be added. As a result Food Products rich in antioxidants showing an **extend shelf life** are obtained.

A pilot trial on chemical stability and efficacy of our microencapsulated extract added to an orange juice showed not only that the **newly developed microencapsulated product has an extraordinary stability** with respect to current antioxidant preparations, but also that the **bioavailability is not aversively affected** by the microencapsulation.

Two clinical trials on the microencapsulated grape pomace extract **showed** that volunteers that ingested our PARADOX-juice for 14 days developed an increased level of plasma circulating antioxidant. This is a conclusive proof (as far as the illness above mentioned are in the vast majority conclusively proven to be due to oxidation stress) that the PARADOX product we have developed is a commercial Functional Food Ingredient that **benefits the antioxidant status of the consumers**, and therefore helps to preserve their health.

The product developed has been subjected to **marketing studies** and was found competitive as a functional food ingredient for liquid and dry food products. T**he tangible result of the PARADOX project is a commercial functional food ingredient, made out of natural waste, whose efficacy is backed up by clinical studies.**

This project has benefited from the successful collaboration of teams from the lab to the customer with the goal to increase the technological level of some European Food Ingredients.

PARADOX: development of the project

FP6-2002-SME-1
Co-Operative Research
Proposal Number 508649
Acronym PARADOX

WP #1 grape pomace providing

Chapoutier / Heinrich / Tilla / Vinceller
⇒ Delivery of the grape pomace (100 kg each)

Step No. 1

extraction

WP #2 extraction

CINS:
⇒ with ethanolic solutions
GAT:
⇒ SLE with organic solvents
NATEX:
⇒ SFE with CO_2

Step No. 2
characterization

WP #3 analytical methods

GAT:
⇒ qualitative / quantitative analyzes:
- chromatographic experiments
- determination of the total phenolic content (Folin-Ciocalteu assay)
- determination of the overall antioxidative capacity (ORAC assay)

Step No. 3

- optimization of the extraction
- purification of the extract

WP #2 & WP #3 analysis / extraction

GAT:
⇒ extractions:
SLE
⇒ purifications:
L/L - TLC - SPE - precipitations
⇒ characterization:
HPLC - Folin-Ciocalteu - ORAC

Step No. 4
Development of an extraction / purification protocol.

WP #2 & WP #3 analysis / extraction

CINS:
⇒ Increase of the extraction yield
NATEX:
⇒ Purification of the polyphenols by forced precipitation with CO_2

Step No. 5

analyzes

WP #3 analytical methods

GAT:
⇒ characterization:
HPLC - Folin-Ciocalteu - ORAC
CHIROBLOCK:
⇒ capillary zone electrophoresis-CZA

Step No. 6
Final optimization and production

WP #2 & WP #3 analysis/extractions

CINS: alcoholic extraction
GAT: purification by L/L partition + quality control
NATEX: purification

Step No. 7

Development of an innovative formulation

WP #4 formulation work

GAT:
⇒ Production of microencapsulated polyphenols as food additives

SLE = Solid Liquid phase Extraction
LL= Liquid Liquid Extraction
TLC = Thin Layer Chromatography
SPE = Solid Phase Extraction
SFE = Supercritical Fluid Extraction
HPLC = High Pressure Liquid Chromatography
CZE = Capillary Zone Electrophoresis

PARADOX: development of the project/cont.

FP6-2002-SME-1
Co-Operative Research
Proposal Number 508649
Acronym PARADOX

WP #5 efficacy testing

Castanas / Corder / Ceriello
⇒ testing of crude pomace extracts
⇒ testing of microencapsulated pomace extracts

Step No. 8

Testing on cell lines

WP #5 efficacy testing in vitro

Castanas: breast, prostate, liver cancer cells (H2O2 V, ROS, ACM)
Corder: Endothelial cells
Ceriello: Hyperglycaemic cells

Step No. 9
Extract Selection

WP #1 & WP #2 grape Pomace providing & preparation of extracts

CINS:
⇒ extraction
NATEX:
⇒ Purification of the polyphenols by forced precipitation with CO_2

Step No. 10

Preparation for in vivo study

WP #3 & WP #4 analysis/formulation

GAT:
⇒ analytical characterization of crude extracts
⇒ Microencapsulation

Step No. 11
Standardization of the microencapsulated product

WP #5 efficacy testing in vivo

Castanas:
⇒ Determination of plasma redox status on healthy volunteers

Step No. 12
Functional orange juice

WP #6 process development

GAT:
⇒ upscaling and documentation of the microencapsulation process for the extract

Step No. 13
Preparation of publications

WP #7 training material

GAT: preparation of communication material: presentations, trainings, brochures, product catalogue

Step No. 14

Dissemination to distribution partners

WP #8 Dissemination

Campi Jove, KUK:
⇒ industrial product development using PARADOX Functional Food Ingredients

H2O2 V = H2O2 Viability
ROS= Reactive Oxygen Species
ACM = Actin Cytoskeleton Modification

Description of work

Outline

This final report covers the work performed from February 2004 to February 2006 for the PARADOX-project (European contract No. COOP-CT-2003-508649).

Introduction

The goal of the PARADOX-project is to obtain and formulate extracts from red wine by-products. The extraction was focused on a restricted set of antioxidants remaining in the grape pomace after pressing. These compounds, the polyphenols, were chosen due to their beneficial effect upon the human health, for which the term “French Paradox” was coined (Prof. Serge Renaud et al.).
In the first part of the study, analytical techniques were developed to characterize qualitatively and quantitatively the content of the grape pomace extracts. This development was made possible by the use of selected polyphenols standards. The analyzes obtained were used to increase the yield of the extraction techniques
In the second part of the study, innovative formulations were developed to formulate the polyphenols extracts as microcapsules for food additives.

The PARADOX-project was divided into eight work packages (WP). Four of these packages were performed during the first year of the project (milestones 1 & 2). The following sections describe the achievements of the project work divided into several steps dependent on time.

WP #1: Raw material providing: red wine residues

Grape pomace raw material was obtained from our wine-producing project partners, representing mayor and minor red wine producing countries in Europe:

- Bodegas Roda S.A. (Spain)
- M. Chapoutier (France)
- Weingut J. Heinrich (Austria)
- Tilia wine cellar (Slovenia)
- Vincellar MPS Kft. (Hungary)

Grape pomace was sampled 6 days after maceration (at 20°C) for the red wine vinification. The grape pomace was packaged in small portions (~1 kg), deep frozen to prevent secondary microbiological contamination and sent to GAT. The French project partner (M.Chapoutier) had no facilities to freeze the pomace. It was candied to prevent further microbiological contamination.

Step No.1: Extraction (WP #2)

Solid-liquid extractions of grape pomace were carried out to obtain extracts with high polyphenol content. Antioxidants either possess hydrophilic or hydrophobic properties. Extraction techniques covering a wide range of polarities are required.

Different technologies for antioxidant extraction were employed either at GAT or in collaboration with industrial partners:

- Extractions with sub and supercritical CO_2 with/without polar modifiers.
  NATEX Prozesstechnologie GesmbH (Austria).

- Alcoholic extraction assisted or not with pressure and acids.
  CINS, Center for isolation of natural substances, d.o.o. (Slovenia).

- Extractions with non alcoholic solvents of different polarities (Water, trichloromethane, ethyl acetate, hexane, tetrahydrofurane, tert-butylmethylether). Alcoholic extractions (ethanol, methanol).
  GAT Formulation (Austria)

All the above extraction were designed according to a multifactorial experimental matrix aiming to cover the maximum polarity range affordable by each technique.
The analytical techniques used to determine the contents of phenolic compounds and the overall antioxidant capacity of the extracts were developed in the work package 3.

Extraction with CO_2

Restrictions have been made concerning the use of solvents in the food industry. Attempts were made to replace these solvents by supercritical CO_2. This solvent has several advantages: low cost, low toxicity, easy elimination. To optimize the polyphenol extraction a multifactor experimental matrix was designed (critical / sub critical / presence of an polar modifier) to optimize the extraction (Fig. 1).

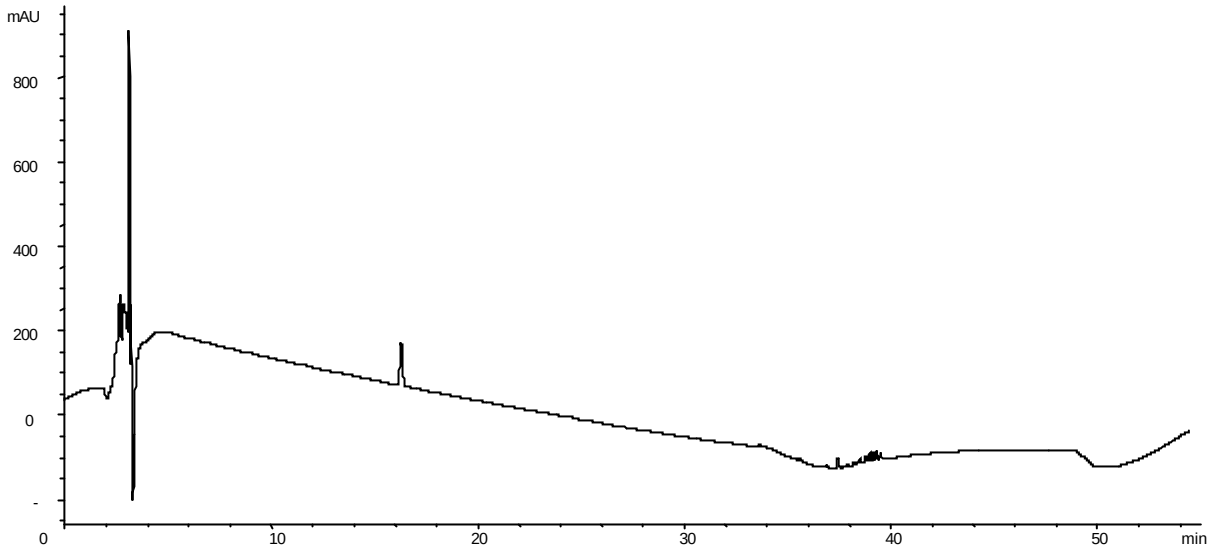


Fig. 1: Austrian grape pomace extracted with CO_2. No polyphenols are visible at their expected retention time.

Fig. 1 shows a chromatogram of the CO_2-extraction. Even with the addition of a polar modifier, it was not possible to extract the polyphenols in a single step from the grape pomace. The intrinsic polarity of CO_2 is not appropriate to extract large amounts of polyphenols.

Extraction: Work performed at GAT

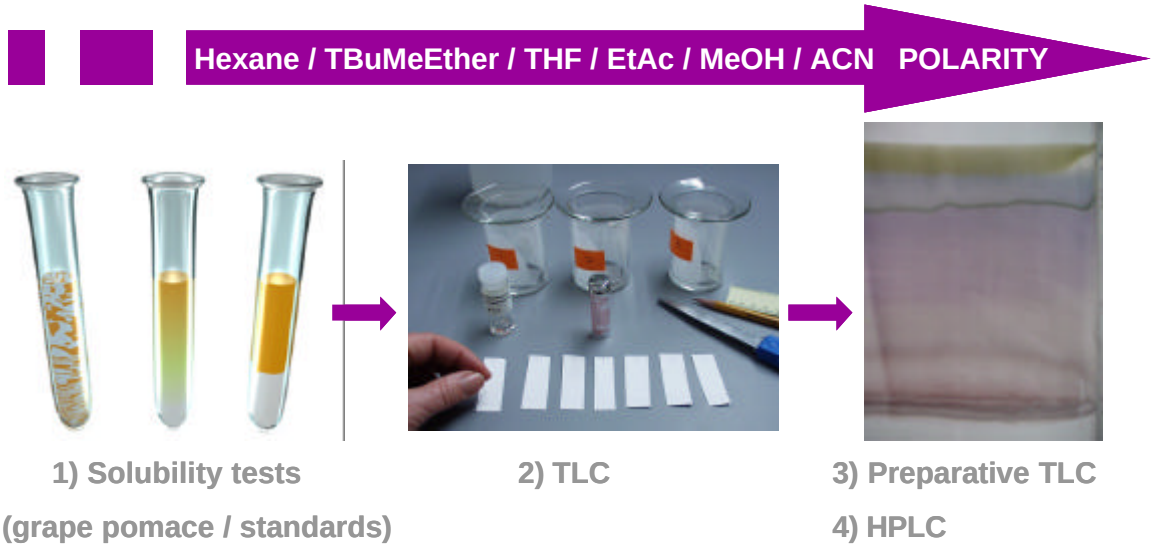
In all extracts obtained by solid/ liquid extraction the content of the polyphenols was low. To overcome possible intrinsic drawbacks of the polyphenol study (i.e. irreversible extraction on silica based material for chromatography), the extracts were hydrolyzed under acidic condition and HPLC columns bearing large pores were employed. As no improvement could be seen with these new conditions, it was proven that the polyphenols were concomitantly

extracted with non-phenolic substances. The extraction step was therefore thoroughly studied by using:

- different extracting phases showing large polarity differences
- a defatting procedure with n-hexane
- thin-layer chromatography (TLC) - analytical and preparative scale
- solid phase extraction

Fig. 2 shows the TLC experiments carried out with the extracts obtained from GAT / CINS / NATEX.

Fig. 2: Attempts made to extract and purify polyphenols



None of the above procedures led to an extract with high polyphenol content. However, the extracts made with ethyl acetate gave better yields than any other extracting phase. This solvent was thereafter used for all the extractions (Fig. 3).

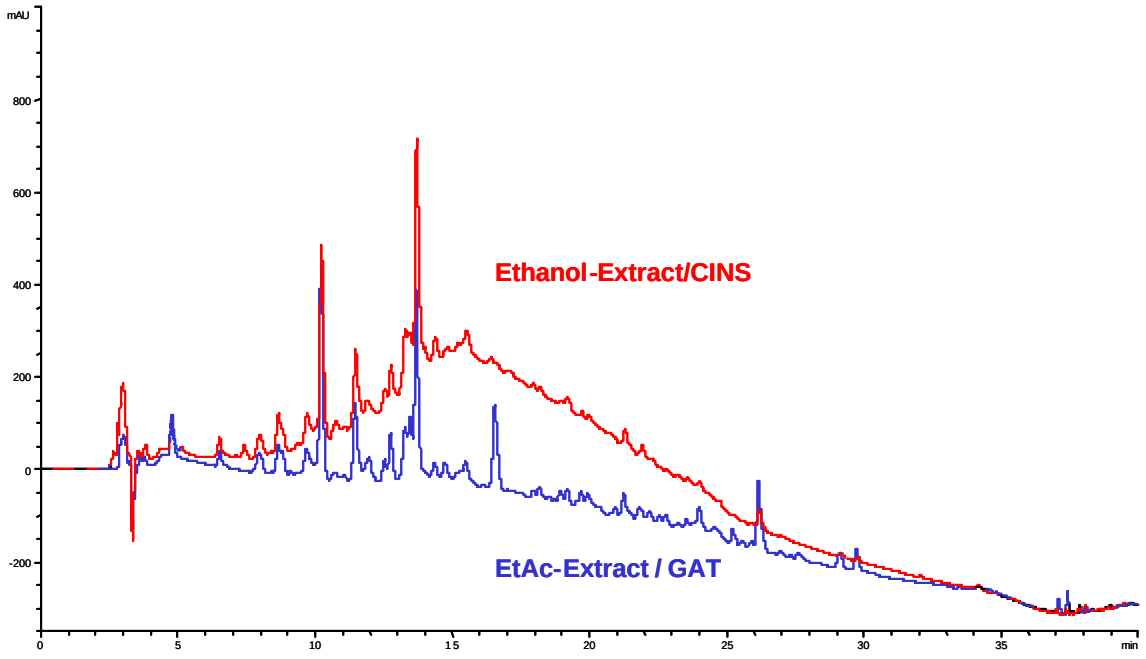


Fig. 3: Selectivity of the solvent for the polyphenol extract. Ethyl acetate is more selective than the alcoholic phase for extraction of the selected analytes (identification not shown); concentration at 15 mg.ml^{-1}

Step No.2: Characterization (WP #3)

Reference compounds

Nine phenolic target molecules with high antioxidant capacity were selected for the analysis and characterization of grape pomace extracts:

- Gallic acid
- trans- and cis-Resveratrol
- Chlorogenic acid
- Catechin and Epigallocatechin gallate
- Epicatechin and Epicatechin gallate
- Proanthocyanidine B_2

Methods

The analytical means for analyzing grape pomace were:

- Chromatography (HPLC) to identify and quantify the main compounds in the extracts
- Folin-Ciocalteau colorimetric assay to estimate concentration of total phenolic compounds
- ORAC fluorimetric assay is used to measure the overall antioxidant capacity

All analytical means were also applied to commercially available grape pomace extracts (BREKO OPC40, BREKO P90) for comparative reasons.

In HPLC, the identification of the analytes was performed by comparing the retention times of the analytes versus the ones obtained for standards (Fig. 4), and by matching their respective UV-absorption spectra. Commercial standards were in principle provided by Sigma-Aldrich (Vienna, Austria).

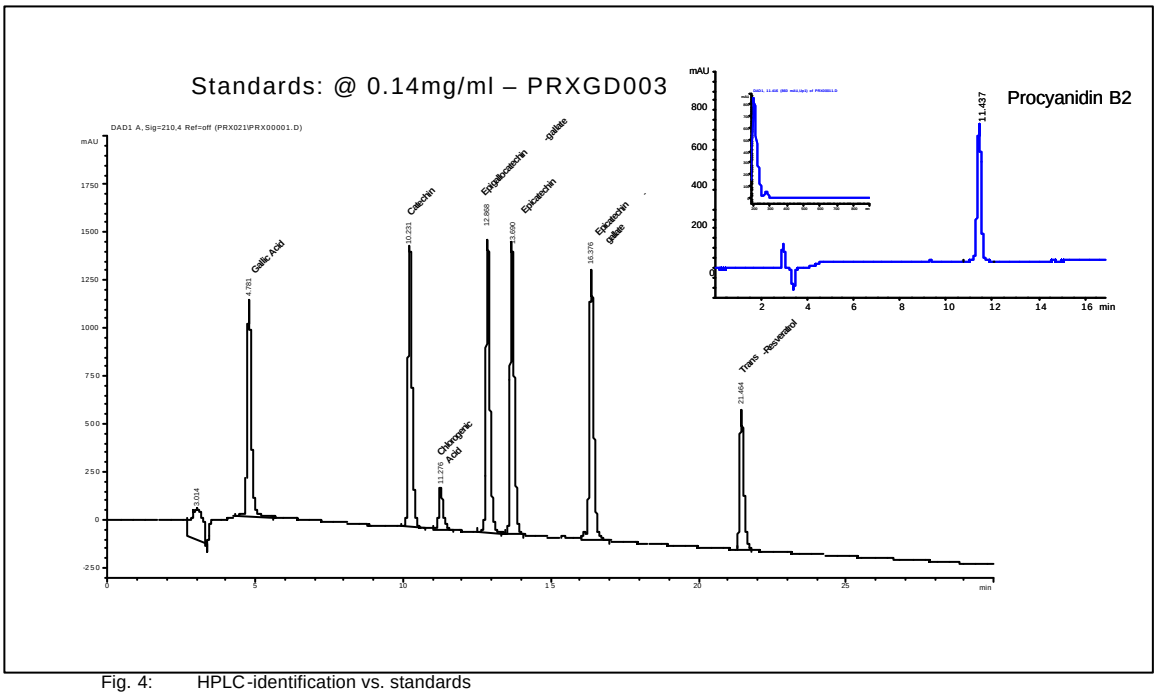


Fig. 4: HPLC-identification vs. standards

The determination of the total polyphenol index is based on the Folin-Ciocalteu reaction in basic medium and subsequent spectrophotometric detection at 750 nm. By applying this colorimetric assay, all the phenolic compounds form colored complexes together with the Folin-Ciocalteu reagent. The color intensity is equivalent to the phenolic compounds and is measured by spectrophotometry.

The overall antioxidative capacity is addressed using the oxygen radical absorption capacity assay (ORAC). This fluorimetric test is based upon the fluorescence decay of a probe when submitted to a radical attack (Fig.4a). The decay is monitored both in the presence and in the absence of antioxidants. The two resulting decay curves are integrated and their area difference gives a value linearly correlated to the antioxidant capacity (Fig.4b).

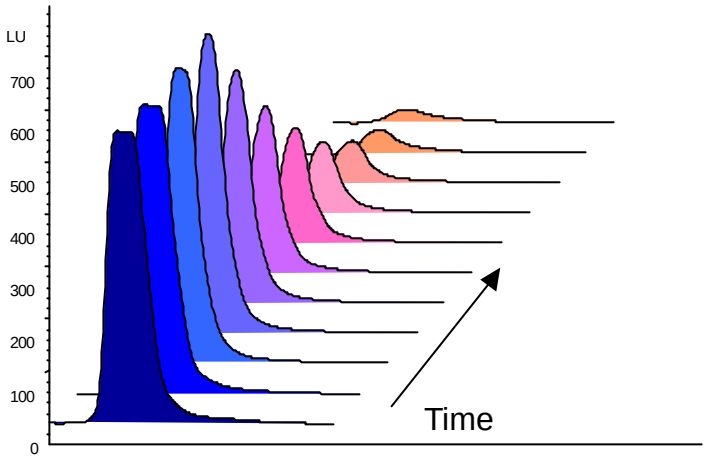


Fig. 4a: Fluorescence decay

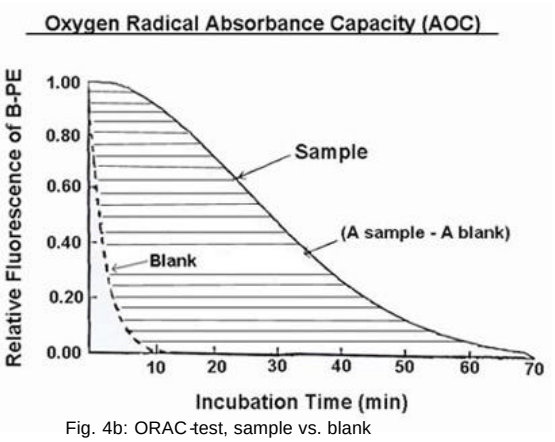


Fig. 4b: ORAC-test, sample vs. blank

The area under the fluorescence decay curve (AUC) is integrated and the net AUC, which is an index of the antioxidant capacity, is calculated by subtracting the AUC of the blank from that of the antioxidant.

Characterization of extracts

The extracts, which were delivered by CINS and NATEX, revealed low ratios of signal/concentration in HPLC experiments. The contents of catechin and epicatechin in CINS extracts were compared with two commercially available grape pomace extracts (BREKO OPC40, BREKO P90). Extracts from CINS (Fig. 5) showed low contents of reference compounds, only the phenolics catechin and epicatechin were present in relevant concentrations (Tab. 1).

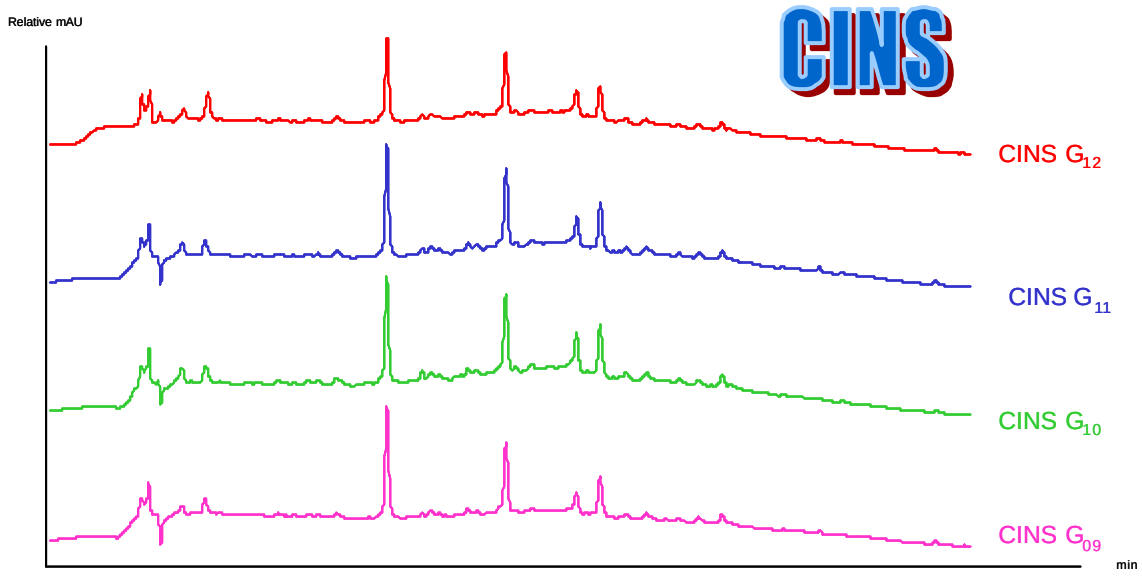


Fig. 5: Chromatograms of ethanolic extracts from CINS at 5 mg.ml^{-1}

Tab.1: Quantification of catechin- and epicatechin by external calibration;

Extracts	Catechin [wt-% extract]	Epicatechin [wt-% extract]
CINS G9	0.45	0.33
CINS G10	0.57	0.40
CINS G11	0.55	0.41
CINS G12	0.57	0.35
BREKO OPC40	5.5	2.62
BREKO P90	3.81	1.88

In extracts from NATEX only traces of reference compounds were detectable.

According to results of chromatography, the Folin-Ciocalteu- and the ORAC-assay were only performed with the CINS extracts. Tab. 2 shows the total phenolic content expressed as gallic acid equivalent [wt-%]. Total phenolic content in CINS extracts was relatively low compared with BREKO OPC40.
The ORAC-assay revealed the same tendency in terms of low antioxidant capacity compared with BREKO OPC40 (Fig. 6). Therefore, the antioxidant capacity depends on the total phenolic content in the grape pomace extracts.

Tab.2: Folin-Ciocalteu-assay

Extracts	[wt-%] gallic acid equivalent
CINS G9	12.44
CINS G10	14.80
CINS G11	15.32
CINS G12	12.57
BREKO OPC40	77.32

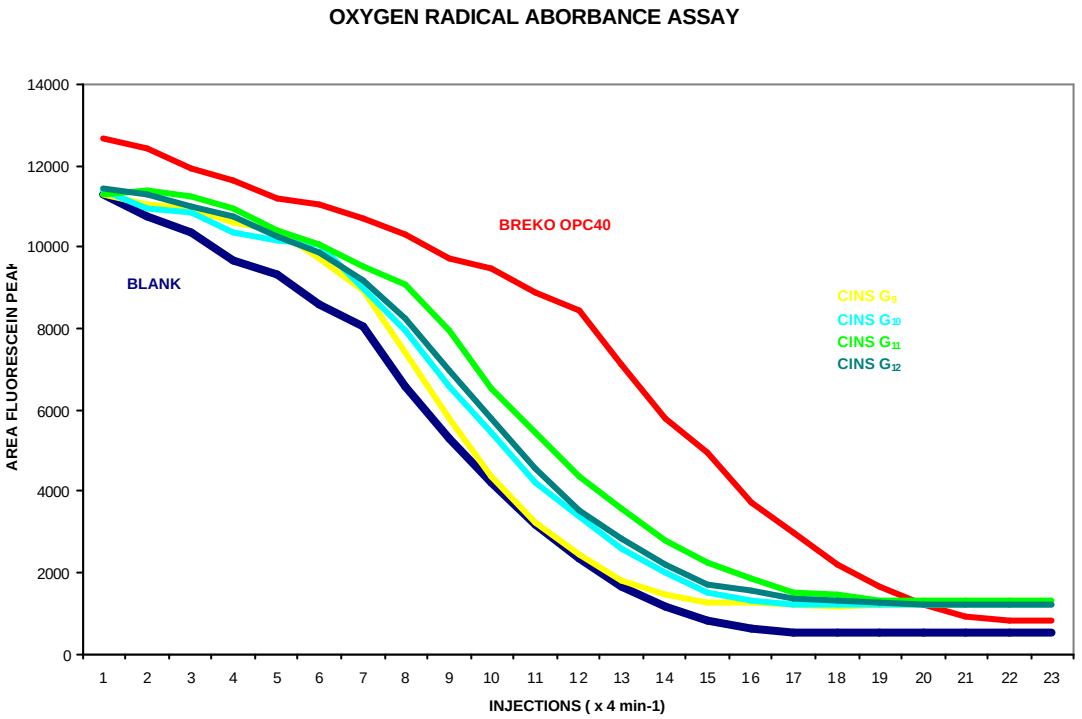


Fig. 6: ORAC-assay

Due to the low initial results, the extract preparation as well as the analytical conditions were furthermore optimized by

- Changing the HPLC-column in order to increase the response of the phenolic compounds

- Applying additional extraction techniques (see next step)

Step No.3: Optimization of the extraction/ purification of the extract (WP #2 & 3)

The nature of the grape pomace was scrutinized in order to increase the yield of polyphenols. Different parameters were studied:

- origin of the grape pomace
- distribution of the polyphenols in the pomace (skins or seeds)
- influence of the crushing
- influence of the fermentation time

Origin of the grape pomace

Grape pomace from different European countries were extracted with ethyl acetate and submitted to the optimized analytical methods developed at GAT (Fig. 7).

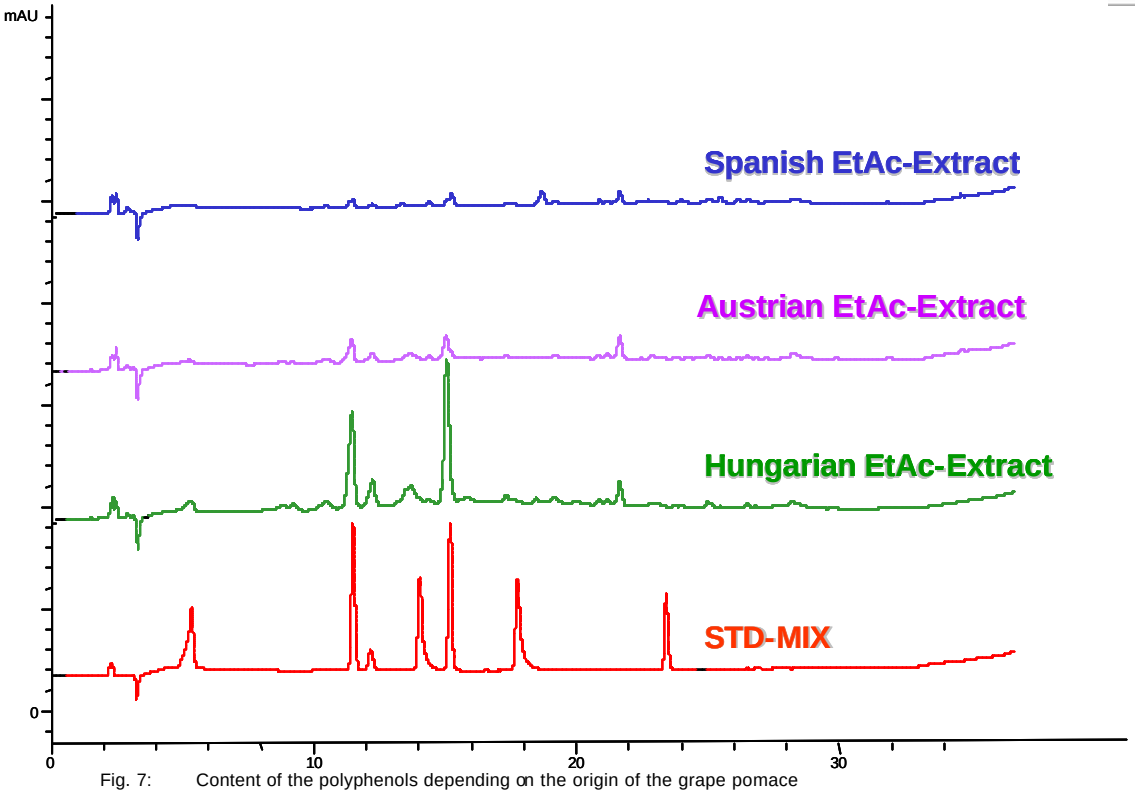


Fig. 7: Content of the polyphenols depending on the origin of the grape pomace

Distribution of polyphenols in the pomace

The grape pomace from Spain was sorted into skins and seeds, extracted with ethyl acetate and analyzed by HPLC. Results showed, that the polyphenols were mainly present in the seeds (Fig. 8).

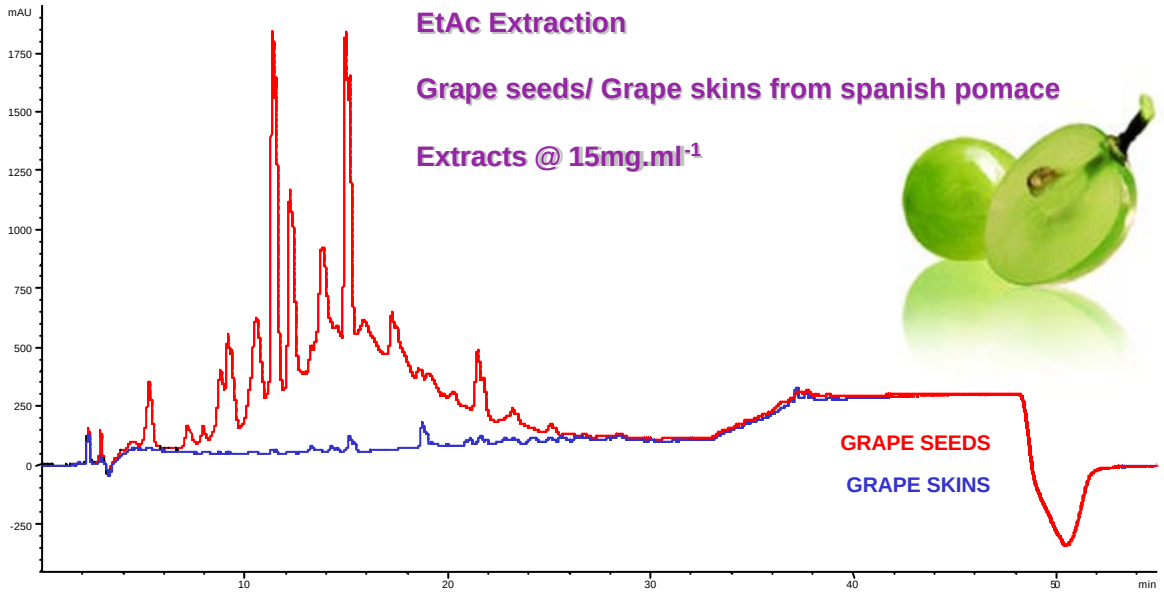


Fig. 8: Chromatograms of grape skins and seeds

The low amount of seeds in the Spanish as well as in the Austrian pomace explained the poor content of polyphenols compared to the Hungarian pomace (see Fig. 7), which consisted primarily of seeds. It is therefore recommended to extract specifically grape pomace rich in seeds to obtain a high polyphenol content.

Influence of the crushing

Crushed and uncrushed grape pomace was submitted to an extraction with ethyl acetate. To further increase the extraction yield of polyphenols, a grinding step of the grape pomace was implemented. Fig. 9 shows the influence of the crushing upon the extraction yield. The crushed material was obtained by passing quickly three times the frozen pomace in an electric grinder. These approach limits the decay of the antioxidants by preventing an excessive heating.

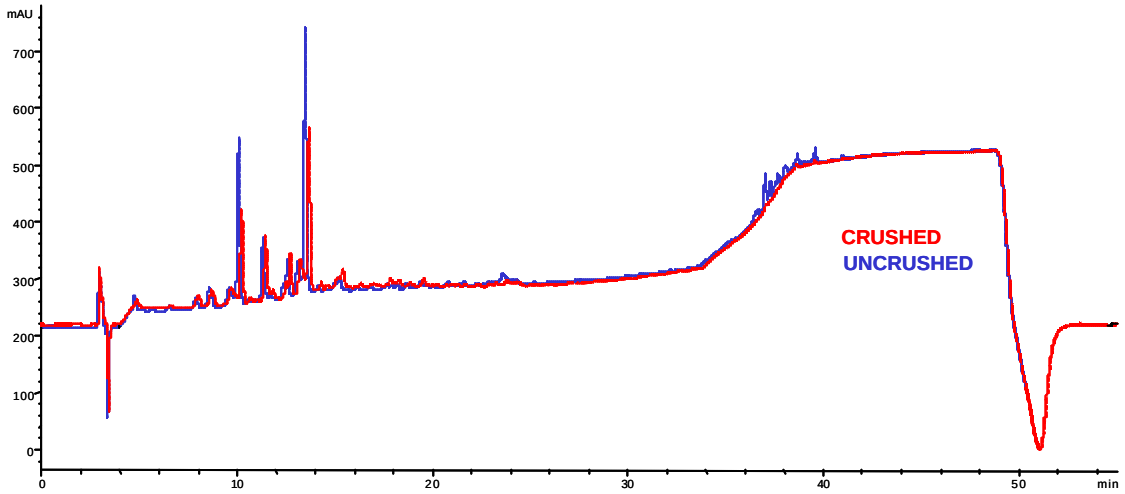


Fig. 9: Extract from the Hungarian grape pomace

Fig. 9 indicates that the crushing does not increase the extraction yield as expected. The higher specific surface of the crushed pomace does not lead to a higher mass transfer. It is therefore recommended to extract the uncrushed grape pomace. This feature is advantageous since the crushing step would have been time and energy consuming.

Influence of the fermentation time

The principal sources of polyphenols in the grapes are the skins and the seeds. The fermentation process for the antioxidants appears to be mostly a mass transfer from the skins to the wine. The polyphenolic content of the seeds seems to be less sensitive to the fermentation process, probably due to the shielding effect of the skins.

The yield of the polyphenols from the grape pomace strongly depends on the extent of the fermentation process (Fig. 10a and 10b represent the extracts obtained from the grape pomace and the wine, respectively, at different extraction times).

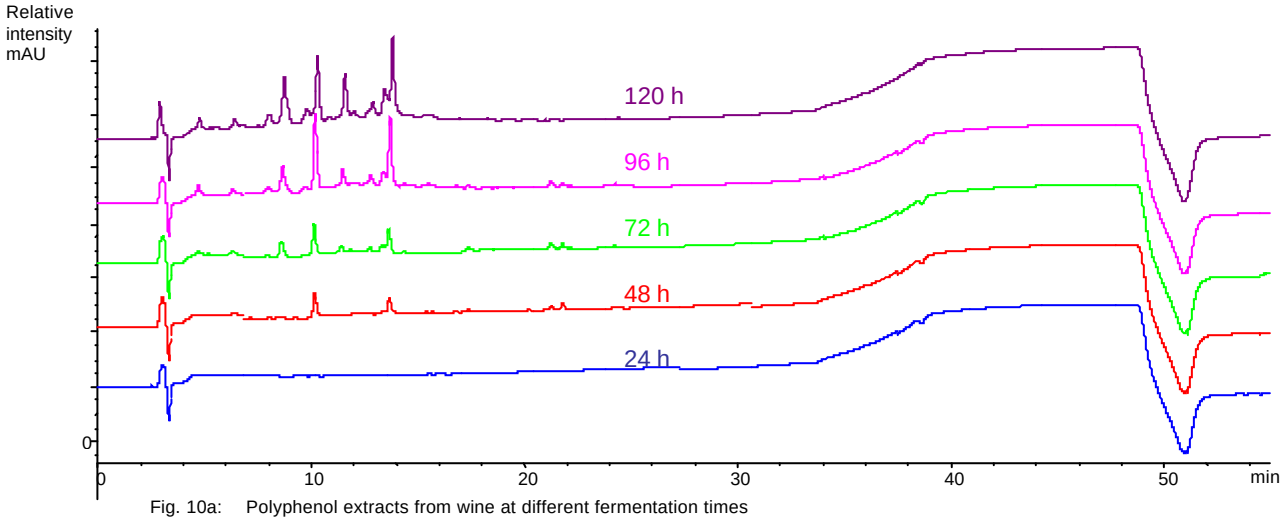


Fig. 10a: Polyphenol extracts from wine at different fermentation times

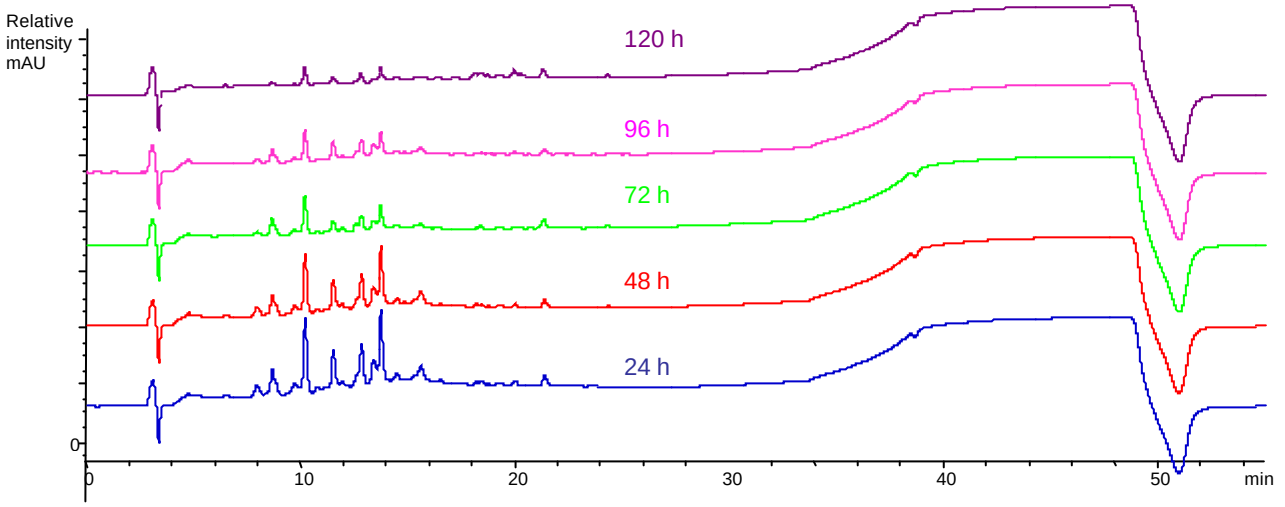


Fig. 10b: Polyphenol extracts from grape pomace at different fermentation times

Fig. 10a and 10b evidence the mass transfer mechanism of the polyphenols. During the fermentation process the polyphenols, originally contained both in the skins and in the seeds, are transferred to the wine. This mechanism is highly complex due to the formation of alcohol which enhances the solubility of such substances.
From the two pictures above it is recommended to use, as far as possible, grape pomaces collected after a short fermentation process.

Optimization of extract preparation - purification

None of the different approaches to increase the extraction yield was conclusive. The best extracts obtained with ethyl acetate still showed a low content of polyphenols (see Fig. 3). The striking feature of Fig 3 is the very low ratio signal/ concentration obtained even by considering a low absorbance of the sample. This characteristic clearly indicates that the polyphenols are concomitantly extracted with a non-polyphenolic material.

By applying all described extraction techniques to the grape pomace extracts, only low contents of polyphenols were detected compared with commercial products. Since no improvement of the extraction could be made with a single step-extraction, a purification step had to be considered during extraction. A new and innovative two steps-extraction process transferable to a large scale was developed aiming to obtain large amounts of polyphenols

out of grape pomace extract. This process is described in Fig. 11, where the optimized as well as the initial extraction processes are depicted.

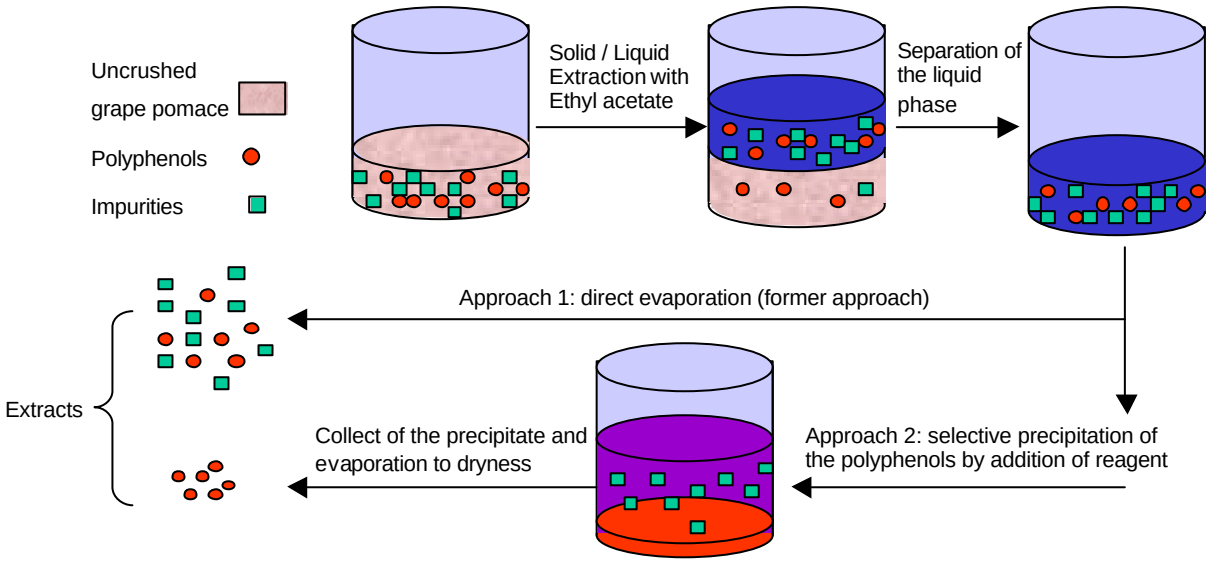


Fig. 11: Extraction scheme of polyphenols; old and new approach

The optimized approach to extract polyphenols out of grape pomace on a laboratory scale was made as follows: grape pomace was soaked for 24 hours with two mass equivalents of ethyl acetate. The organic phase was collected by filtrating the organic phase, then a one-volume equivalent of n-hexane was added. The solution was briefly stirred and the precipitated polyphenols left to settle down for one hour. The red precipitate was collected and dried in the desiccator under reduced atmosphere at room temperature.
The optimized extraction method with forced precipitation was highly effective to concentrate the polyphenols (Fig. 12, at 15 $mg.ml^{-1}$). By comparing the chromatograms of both extraction methods (Fig. 12 & 13, both at 15 $mg.ml^{-1}$), a much higher ratio of signal/concentration was shown.

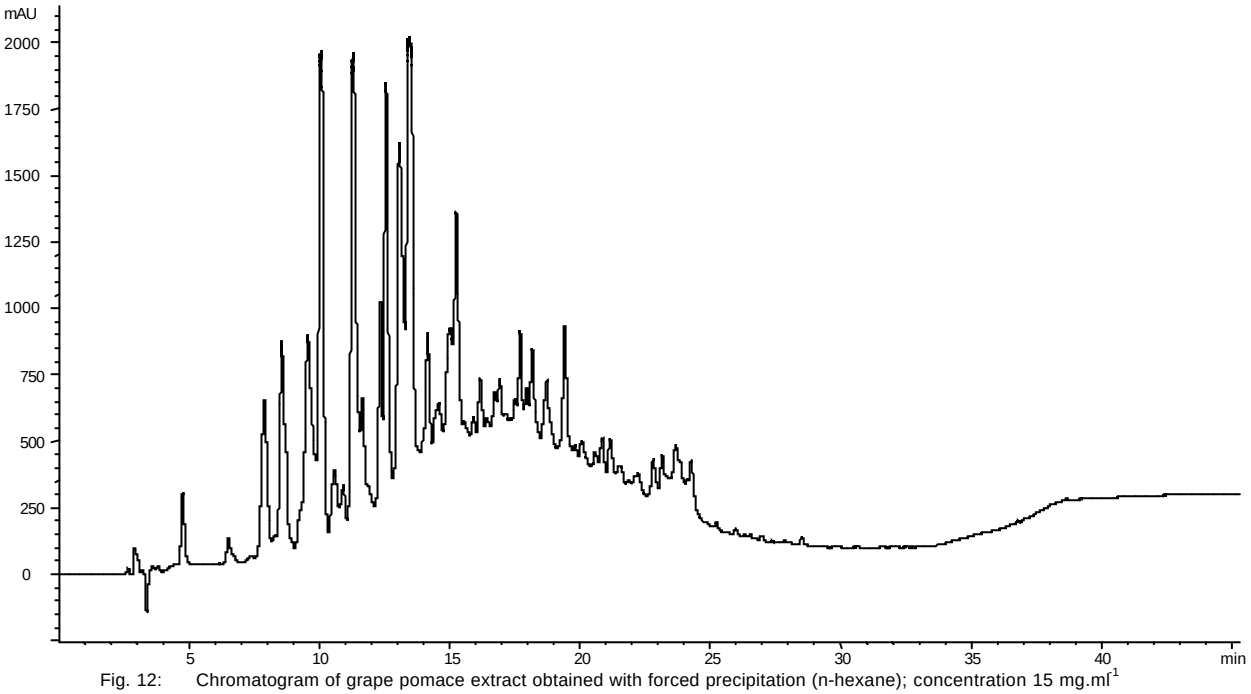


Fig. 12: Chromatogram of grape pomace extract obtained with forced precipitation (n-hexane); concentration 15 mg.ml^{-1}

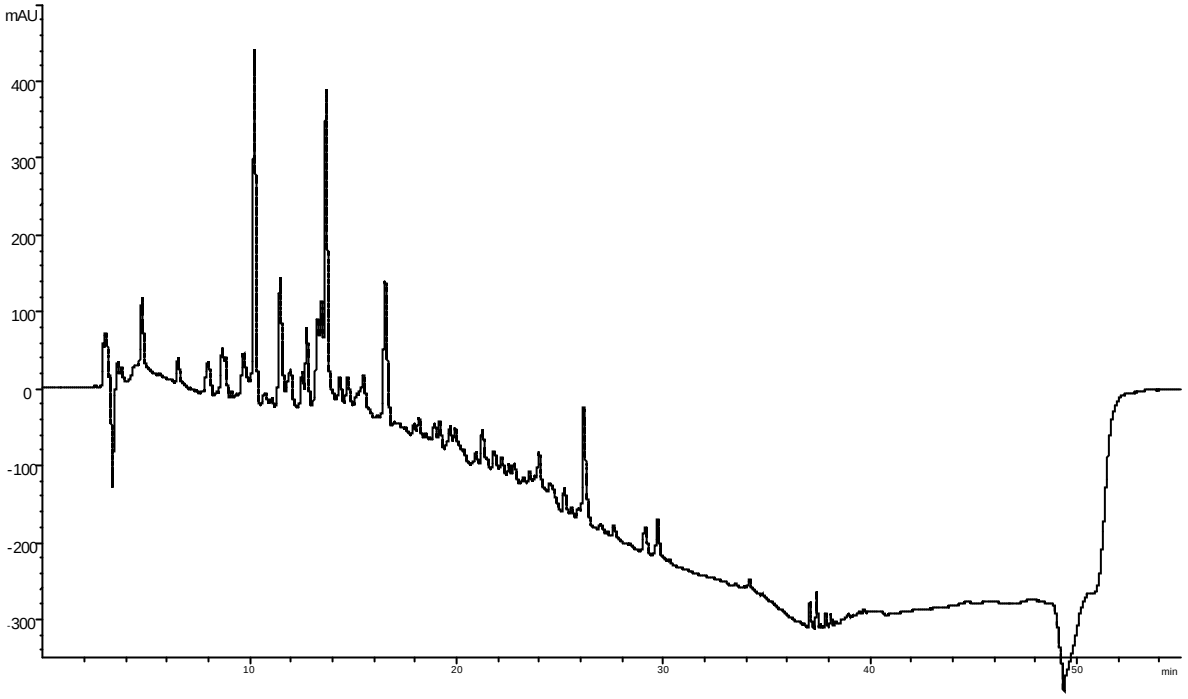


Fig. 13: Chromatogram of grape pomace extract without forced precipitation; concentration 15 mg.ml-1

Mass distribution in the purification process

Fig. 14 displays the distribution of the dry mass extract in the different phases used during the polyphenol extraction process optimized at GAT. This process was carried out through an extraction of the grape pomace with an ethanolic solution, which was submitted to a liquid-liquid extraction with ethyl acetate. The organic phases were collected and the polyphenols forced to precipitation. All phases were evaporated to dryness and weighted. The results were expressed in weight-% of the total mass of the dry extract.

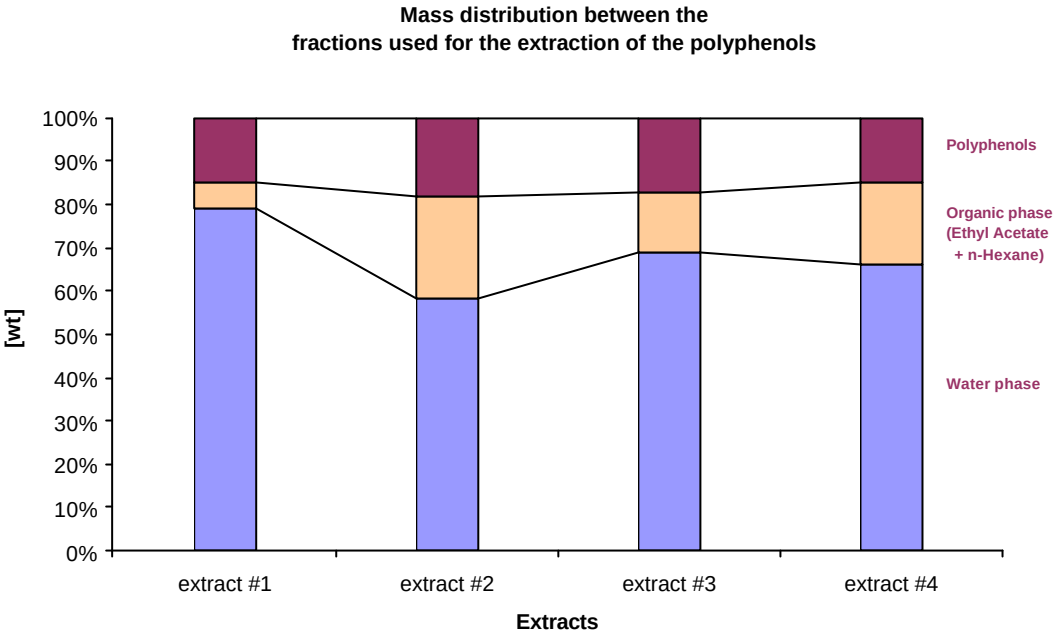


Fig. 14: Mass distribution [wt% solid] in the alcoholic extract in four grape pomaces

Fig. 14 shows that the alcoholic extracts contain a large fraction of non-phenolic compounds. This fraction dilutes the polyphenols and thereby gives a low response to the Folin-Ciocalteu assay.

Step No.4/5/6: Development of an extraction/ purification/ scale up protocol (WP #2 & 3)

Extraction: scale up

The concern at this point of the work was to minimize the use of solvents used in the extraction scheme developed in the laboratory (Fig. 11).

To minimize the content of ethyl acetate still present with the grape pomace after the extraction process, a new approach was designed. An extraction aiming to withdraw all possible material from the grape pomace was performed before the selective extraction with ethyl acetate. Therefore, this extraction had a threefold goal:

- Extraction with a non-toxic solvent widely accepted in food industry
- Reduction of the volume of material to be extracted by ethyl acetate and reducing solvent consumption
- Preventing the treatment of soaked pomace in order to retrieve residual organic solvent

CO_2 is an effective agent to induce the precipitation of the polyphenols. The peak pattern shown by both of the extracts obtained via the precipitation of n-hexane or CO_2 is the same (Fig.15). The selectivity of the precipitation is therefore identical for these two reagents. CO_2 can still be used in replacement of n-hexane for the purification step of polyphenols in the ethyl acetate extracts.

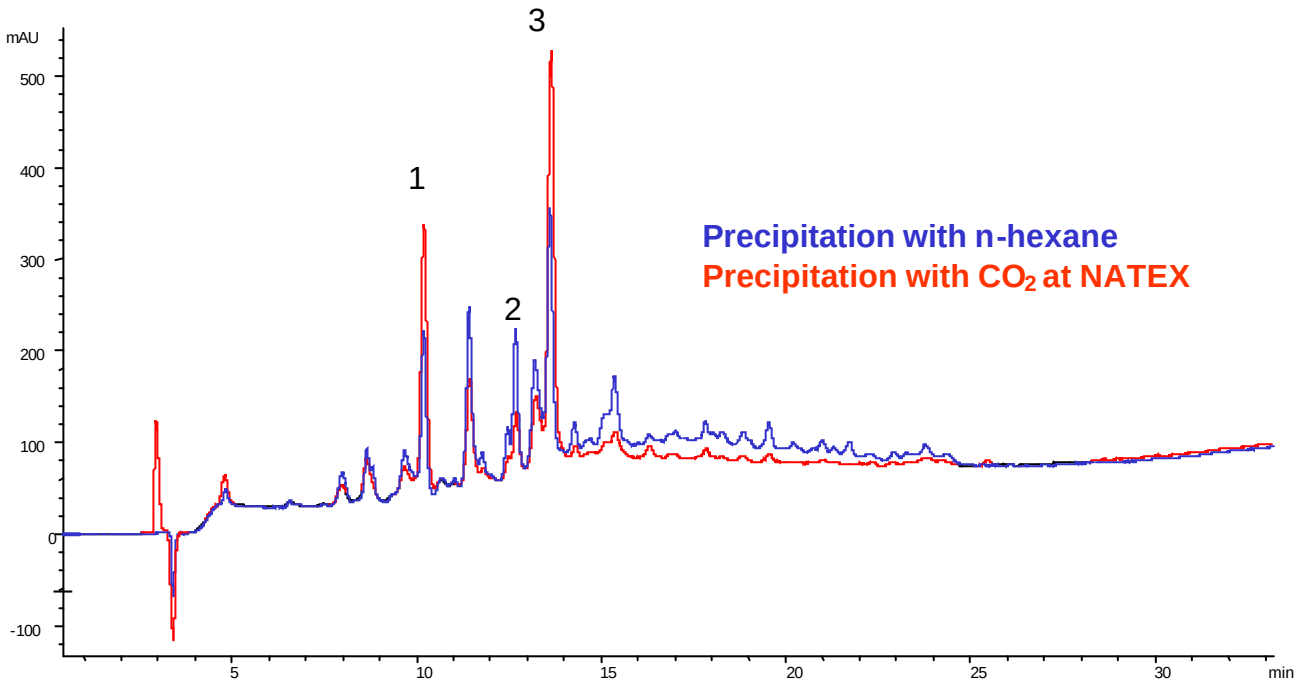


Fig. 15: Chromatograms of the grape pomace extract after precipitation with n-hexane or supercritical CO_2. (1) catechin; (2) procyanidin B_2; (3) epicatechin

Fig. 15 shows two chromatograms made at the same concentration. The condition of pressure and temperature required to maintain the CO_2 supercritical seemed to have an effect upon the concentration of procyanidin B_2 (peak 2). Under such conditions, procyanidin B_2 appears to be partially cleaved into its constitutive monomers, respectively catechin (peak 1) and epicatechin (peak 2). Such harsh conditions are not present with n-hexane, where the procyanidin B_2 peak was bigger than the catechin and epicatechin peaks.

Polyphenol extracts can be purified by CO_2 in replacement of n-hexane. This allows an easy upscale of the extraction technique and minimize its impact on the material. The precipitation of the polyphenols by CO_2 will be therefore employed in the production line of the extracts (Fig. 16).

Fig. 16: Pilot plant for the precipitation of the polyphenols by supercritical CO_2; picture taken at NATEX Prozesstechnologie GesmbH (Ternitz, Austria)

The alcoholic extracts were evaporated to dryness for storage. These extracts were then reconstituted prior to use by dissolution in a 25 times volume equivalent of a 10 % [v] acetic

acid solution in water. The resulting liquid was extracted with 3 x 1 volume equivalent of ethyl acetate. The organic phases were collected and the selective precipitation of the polyphenols was triggered by the addition of one volume equivalent of n-hexane.

Publishable executive summary

Description of the research work of NATEX

All tests for the PARADOX Project were carried out on the 5 litre/1000bar R&D plant in the workshop of NATEX (Fig. 1).

Fig. 1 5 litre/1000 bar R&D plant (NATEX laboratory)

The tests of the second report period were arranged in the following way:

Table 1 Arrangement of tests for the second report period of the PARADOX project

4th series	**extraction of grape seeds with ethanol, extraction of the ethanol extract with ethyl acetate followed by antisolvent SCE**
5th series	**extraction of grape seeds with ethyl acetate followed by antisolvent SCE**
6th series	**extraction of grape seeds with acetone followed by antisolvent SCE**

Fourth Series of Tests

In the fourth series of tests Hungarian grape seeds were used as raw material. The company GAT extracted the uncrushed seeds with ethanol in their laboratory and supplied NATEX with the raw extract.

In the first step 3kg of raw extract were mixed 3 times with 2,8l ethyl acetate to transfer the polyphenols into the ethyl acetate phase. Ethanol solves a lot of protein and sugar substances out of the wet seeds. During mixing with ethyl acetate the ethanol solution solves a lot of ethyl acetate and becomes a stable gel. It is very difficult to separate these two solvents into two phases. However, part of the ethyl acetate with the polyphenols can be separated. Then the mixture of ethanol extract and ethyl acetate was mixed twice with ethyl acetate (1:2) in the second step to make sure that all the polyphenols are transferred into the ethyl acetate phase. In total 42 l ethyl acetate were mixed stepwise with 6 kg ethanol extract. This resulted in 32 l ethyl acetate extract and 10 l gel. In the following step the 32 l ethyl acetate extract was treated with the antisolvent SCE process to produce a polyphenol concentrate according to the flow-sheet shown in Figure 2.

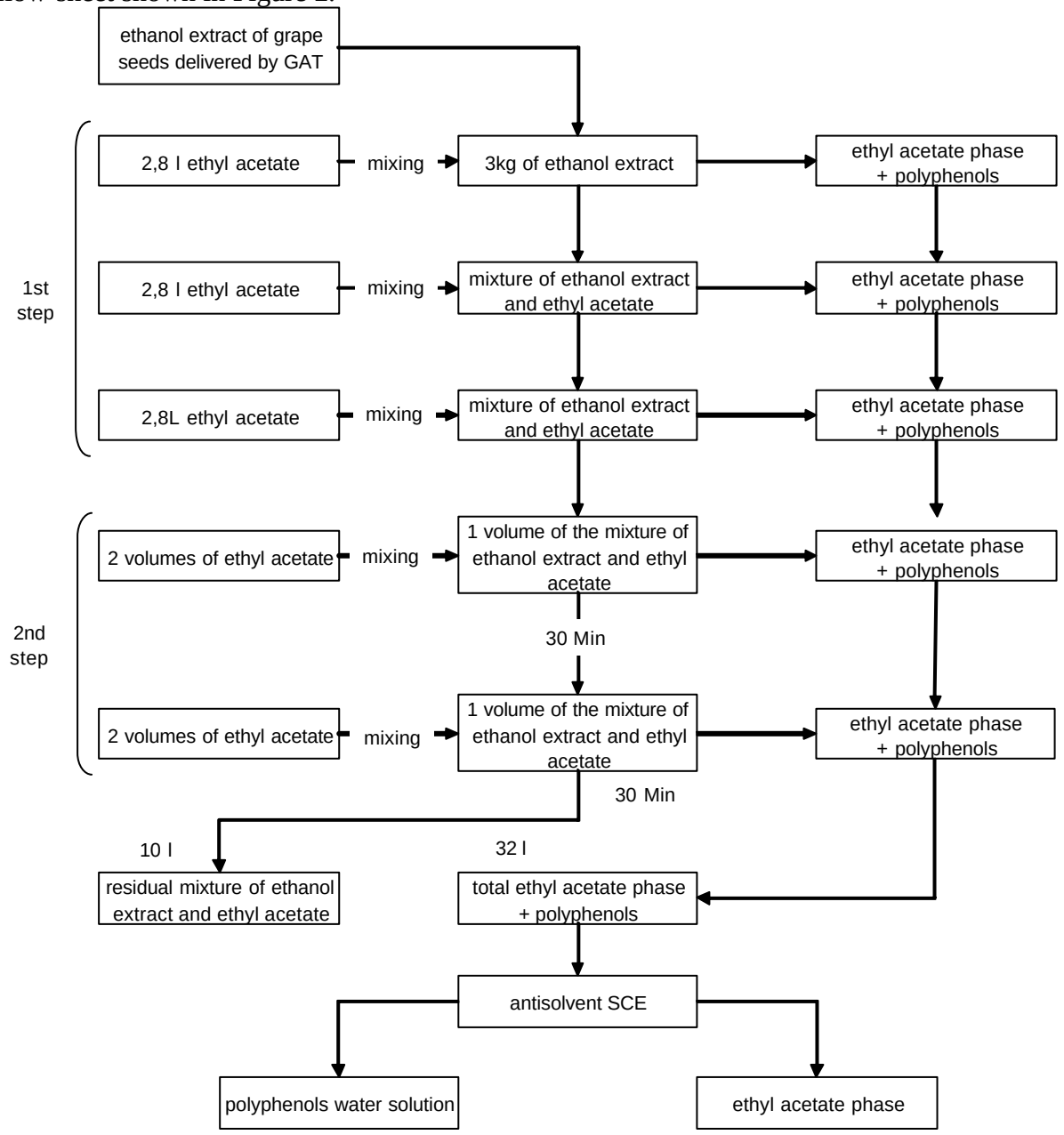


Fig. 2 Schematic overview of the 4th series of tests

13 SCE tests were executed in the 4th test series. In the first test with 2,5 l ethyl acetate extract an aqueous phase at the bottom of the extractor was separated during the static pressurization up to 225 bar. This phase included all polyphenols solved in ethyl acetate and could be directly drained out of the bottom of the extractor. In the following tests water was added to the ethyl acetate extract to improve the separation of the polyphenols out of the solvent CO_2 phase. About 50 – 100 ml distilled water were added to the 2,5 l ethyl acetate extract. As a result of these tests 1 l dark red water solution with all the polyphenols was gained, which was sent to GAT for analysis and further treatment.

Fifth Series of Tests

In the 5th test series Hungarian grape seeds were directly extracted with ethyl acetate. Then the ethyl acetate solution was dried with sodium sulphate to get a complete water-free ethyl acetate extract. Afterwards the solution was extracted with CO_2 till a dry polyphenol powder remained in the extractor. As the polyphenol powder stuck at the wall of the extractor it was necessary to remove the powder manually from the extractor. The polyphenol powder was sent to GAT for analysis and formulation. The production procedure is shown in the flow-sheet in Figure 3.

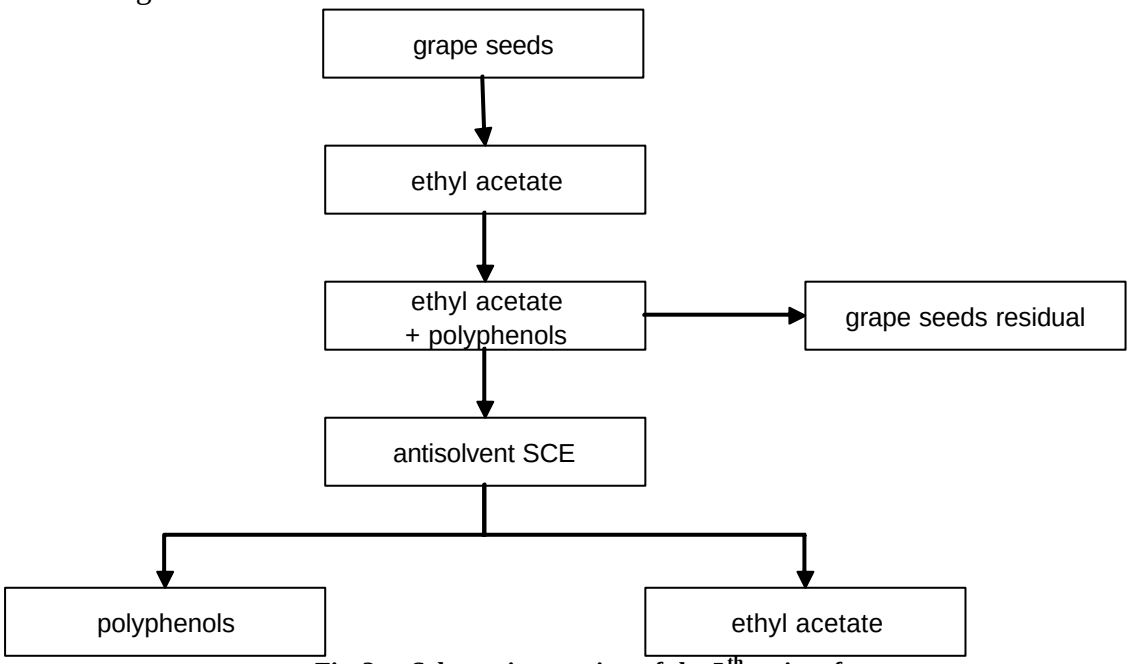


Fig. 3 Schematic overview of the 5th series of tests

With this procedure 14 l ethyl acetate extract was produced, which yielded a polyphenol quantity of 30 g powder.

Sixth Series of Tests

In the last test series Hungarian grape seed were extracted with an acetone-water-solution (9:1) and with pure acetone. The produced extract was treated with supercritical CO_2 at 250 bar and 50°C. Feed quantity was 2,5 l for each test. The extraction process was continued until the whole solvent and the water were extracted and separated in the first separator. The dry powder with the polyphenols remained in the extractor. Totally 19 l acetone extract were treated with a total yield of 152 g dark red powder, which should contain all the polyphenols.

This powder was sent to GAT for analysis and further treatment. The extraction process and the following antisolvent process is shown in Figure 4.

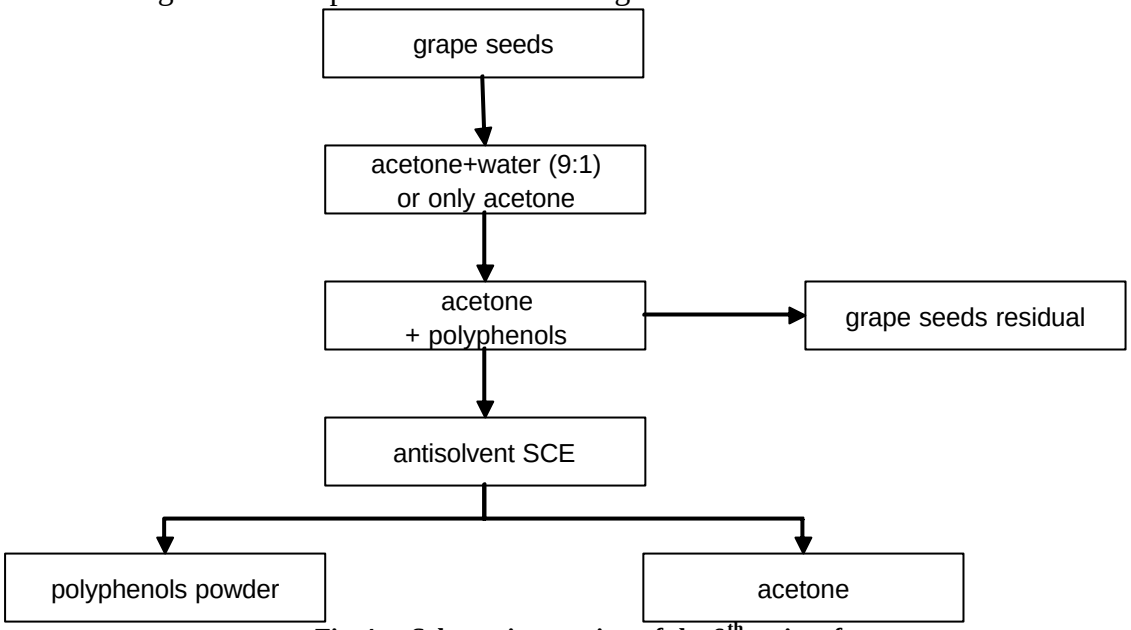


Fig. 4 Schematic overview of the 6th series of tests

Summary

As polyphenols from grape seeds are very polar a primary extraction with supercritical CO_2 is not feasible. A primary extraction with ethanol, ethyl acetate and acetone is possible. For concentration and removal of the solvent the CO_2 antisolvent process is very suitable and yields a highly concentrated polyphenol powder. The extraction of water with CO_2 needs longer extraction times because of the low loading of water in supercritical CO_2. Therefore the polyphenol solution to be treated with the CO_2 process should have a low water content.

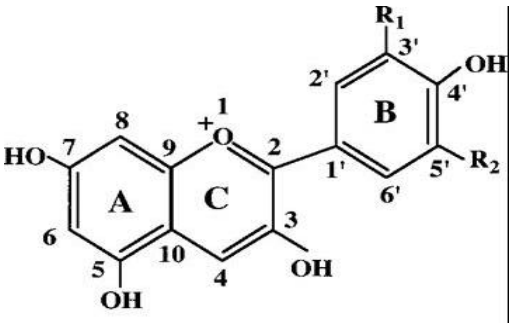
Executive summary

Description of the research work of C.I.N.S.

1. INTRODUCTION

There is an increasing interest in the substitution of synthetic food colorants and antioxidants by natural ones. Recently articles published on natural antioxidants and colorats reported that anthocyanins, which are major plant colorants have antiradical and antioxidant activities, so they have potentional nutritional and therapeutic effect depending on their bioavability and metabolism. Compared to other flavonoids in food, anthocyanines exist in abundance, especially in dark berries and grapes. Special attention is focused on their extraction from inexpensive or residual sources from agricultural industries. Very rich sources are grape skins, which during wine and juice making remain as husks and are usually made into compost.

Phenolic compounds are plant secondary metabolites, which are important determinants in sensory and nutritional quality of fruits and vegetables. Antho cyanins are natural colorants belonging to the flavonoid family. The anthocyanins are glycosides and acylglycosides of anthocyanidins. Some common anthocyanidins with different hydroxyl and methoxyl substitutions in their basic structure, flavylium (2-phenylbenzonzopyrilium) are shown in Fig. 1. There are over 250 naturally occuring anthocyanins and all are O-glycosylated with different sugar substitutes (glucose, rhamnose, xylose, galactose, arabinose, fructose).



Name	R1	R2
Delphinidin	OH	OH
Petunidin	OCH3	OH
Cyanidin	OH	H
Pelargonidin	H	H
Peonidin	OCH3	H
Malvidin	OCH3	OCH3

Fig. 1: Chemical structure of anthocyanidins.

Anthocyanins, as well as other phenolics, can act as antioxidant by donating hydrogen to highly reactive radicals, thereby preventing further radical formation. Their antioxidant potentional is dependent on the number and arragement of the hydroxyl groups and the extent of structural conjugation, as well as the presence of electron-donating and electron-withdrawing substituents in the ring structure.

1.1 Grapes

Grapes are the fruits of vine (Vitis vinifera) a plant whose origin is located by the area of the Middle East, but nowadays it is extended in many regions of warm Mediterranean climate, since this plant needs a mild climate to be able to live appropriately. Wine is obtained from the fermented grapes. It is a drink that enjoyed great tradition everywhere in Classical times.

Many grape types exist that are classified fundamentally in two varieties: white and black grapes; inside each one of them there are different classes.

Fig. 2: Picture of black and white grapes.

Pressing the grape a juice is obtained, called must. By fermenting it, we produce wine. We can avoid its fermentation by introducing it in a hermetic recipient and cooking it in a water bath or double boiler, during half an hour to destroy organisms that cause fermentation. In this way we obtain grape juice which possesses the same properties than the fresh grape, without any alcoholic content.

Grapes constitute one of the main detoxifying foods, ideal to carry out cures where they are the only food eaten. By practicing the diet rich in grapes it is possible to lose the superfluous weight, at the same time that our organism is purified. Therefore this diet is not only recommended to obese people but to all those who need a special depurative diet: people affected by rheumatic illnesses such as gout or arthritis; people with kidney problems that

need help to eliminate toxins or those with problems in the circulatory system: hypertension, arteriosclerosis or bad circulation in general. All of them will benefit from this treatment. The reason of all this has to be attributed fundamentally to its wealth in potassium (especially in raisins) that controls the balance of the liquids in the organism, and also to its low levels of sodium (in fresh grapes). Equally the presence of vitamin B that intervenes in the metabolism of the fats and carbohydrates.

It is necessary to consider the grape an alkalizer, reason why it purifies the blood. Its consumption can inhibit the growth of cancerous cells. Numerous studies have been carried out to check in which way the presence of tannins and caffeic acid, besides constituting stupendous bactericides, could reduce the probabilities of acquiring this illness. The extract of grape seeds prevents the appearance of some cancers, as breast, prostate or colon cancers. The main component responsible for this property is a flavonoid that appears in the skin of black grapes or in black wine, called resveratrol. Flavonoids are plant pigments responsible for the vibrant red, blue and purple colours in fruit and vegetables and many times more potent than vitamin C. Resveratrol is both water and fat-soluble and therefore even more versatile than vitamins at protecting body tissues.

Grapes, used externally, constitute a very interesting cosmetic help for the protection and embellishment of skin, since they are one of the best skin humidifiers, so that they hydrate and recover it from the effects of dryness.

This fruit, for its wealth in sugars, is not very appropriate for diabetics or those that present intestinal problems because it increases fermentation and flatulence.

Composition of grapes per 100 g.

	Raw	Raisins	Must
Water	80,5 g	16,57 g	85 g
Energy	71 Kcal	296 Kcal	40 Kcal
Fat	0, 58 g	0,54 g	0,1 g
Protein	0, 66 g	2, 52 g	2,5 g
Carbohydrates	17, 7 g	78, 47 g	8 g
Fiber	1 g	6,8 g	0
Potassium	185 mg	825 mg	110 mg
Sodium	2 mg	28 mg	0,8 mg
Phosphorous	13 mg	75 mg	10 mg
Calcium	11 mg	28 mg	10 mg
Magnesium	3 mg	30 mg	12 mg
Iron	0, 26 mg	2,59 mg	0,3 mg
Zinc	0,05 mg	0,18 mg	0,05 mg
Vitamin C	10, 8 mg	5,4 mg	5,4 mg
Vitamin B1	0,092 mg	0,112 mg	0,09 mg
Vitamin B2	0, 057 mg	0,182 mg	0, 2 mg
Vitamin B6	0, 110 mg	0,188 mg	0,08 mg
Vitamin A	73 IU	--	--
Vitamin E	0, 700 mg	0,700 mg	0,700 mg
Folacin	4 mcg	3 mcg	--
Niacin	0. 300 mg	0, 5 mg	0,2 mg

1.2 Scope of study

Agricultural and industrial residues are attractive sources of natural antioxidants. By-products, which remain after processing of fruit and vegetable in food processing industry still contain a huge amount of phenolic compounds. Some studies have already been done on by-products, which could be potential sources of antioxidants. Furthermore, there were many researches done on the polyphenols acquiring from grape marc.

In Slovenia there is produced a lot of grape for wine making. After fermentation (maceration) and pressing, seeds and skins remain as marc, which still content some anthocyanins and polyphenols. Due to this fact we investigated the possibilities of obtaining natural antioxidant compounds (mainly anthocyanins) from inexpensive residual sources.

In the present work the efficiency of conventional and supercritical extraction techniques for isolation of extract from marc (grapeskins) were examined and compared. The main purpose was to obtain the highest yield of extractions. The conventional extractions were first made in small quantities to determine the influence of ratio material : solvents, temperature and addition of acids on the yield of extraction. Afterwards, large scale conventional and supercritical extractions using two different types of solvents in two separeted batches (with different material) were performed. At the end, pilot and industrial scale convencional extractions of marc were on the basis of our previous research investigated, partly in laboratory and partly in industry.

2. METHODS

2.1 Conventional extractions

The prelimenary extraction experiments with conventional solvents were performed in a batch extractor, a 100 mL round-bottomed flask, which was filled with solvent and extracting material. The content was left in the ultra-sound bath or water bath when using higher temperature. After two hours of extraction, the extraction mixture was filtered, solvent evaporated and the mass of the extract was determined. The yield of extraction was calculated by the formula:

$$\text{Yield (\%)} = \frac{m_{\text{extract}}}{m_{\text{raw material}}} \cdot 100 \quad (1)$$

where m_{extract} is the mass of the extract and $m_{\text{raw material}}$ is the mass of the raw material (marc) extracted.

The large scale extraction experiments using two solvents (ethanol with different amount of added water) were also performed in batch reactor. But the batch extraction system used in this part of study composed of a 2 L round-bottomed flask connected to the condenser, a magnetic stirrer and a boiler. The batch extractor was first filled with a solvent and raw material. The content was heated to the desired temperature (60 °C) and mixed for three hours. The yield of extraction was determined on the same way as described above.

In order to scale up the process that showed the highest extraction yield, we performed two experiments, in pilot and industrial scale. The first one was performed in a 20 L laboratory reactor and the second in a 2500 L industry reactor. In both cases, the reactor was first filled with the material (marc plus solvent) and then during the mixing heated to the set

temperature of 60 °C. After reaching the desired temperature we continued mixing for another three hours. The extraction mixture was squeezed or filtered above the vacuum, solvent evaporated to the residue and dried in the vacuum dryer over the night to get the fine powder of the crystals. With the determined mass of the extract, the yield of extraction was calculated by the formula (1).

2.2 High-pressure extractions

The extraction experiments with dense CO_2 at pressure 200 bar and temperature 60 °C were performed in pilot high-pressure extraction plant (UHDE), designed for maximal operating pressure 500 bar and temperature 120 °C. The apparatus consist of two extractors (V = 4 L and V = 0,5 L), equipped with heating jackets, high-pressure pump and heat exchanger. Approximately 300 g of material and 3 L of co-solvent were charged into the extractor. The liquefied CO_2 was pumped into the extractor and when reached the desired level the conditions were held constant for three hours. The principle of the supercritical extracting process is the same as for conventional described above.

3. RESULTS

3.1 Preliminary conventional extractions

3.1.1 Effect of ratio material : solvent and its composition on extraction yield

For the preliminary extraction experiments, two extraction mixtures of ethanol and water in three different ratios with raw material were taken (Table 1; Fig. 3). The best result of extraction yield was obtained, when 50 % EtOH in the ratio of 1:50 (material : solvent) was used (4,22 %).

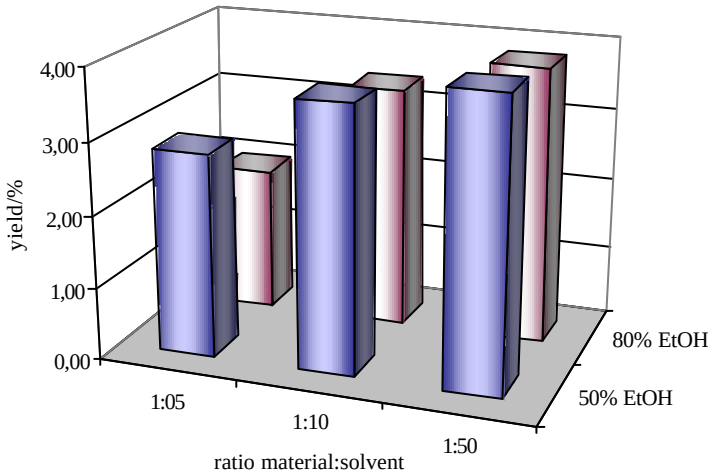


Fig. 3. Influence of the solvent mixtures and ratios of raw material per solvent on the

extraction yield (%).

Table 1: Results of extraction yield using two different types of solvents at three different ratios material : solvent.

solvent	50 % ethanol		80 % ethanol	
ratio	sample label	η / %	sample label	η / %
1:5	G1	2,80	G4	2,00
1:10	G2	3,68	G5	3,35
1:50	G3	4,22	G6	3,82

3.1.2 Effect of temperature on extraction yield

Furthermore, for the experiment, when the highest extraction yield was obtained (50 % EtOH in the ratio with the raw material = 50:1), the influence of temperature was investigated. From the Table 2 and Fig. 4 it can be observed that the yield decreases with the temperature from 4,22 % at 20 °C to 4,00 % at 60 °C.

Table 2: Results of extraction yield using 50 % EtOH (ratio material : solvent = 1:50) at higher temperatures.

temperature / °C	sample label	η / %
20	G3	4,22
40	G7	4,05
60	G8	4,00

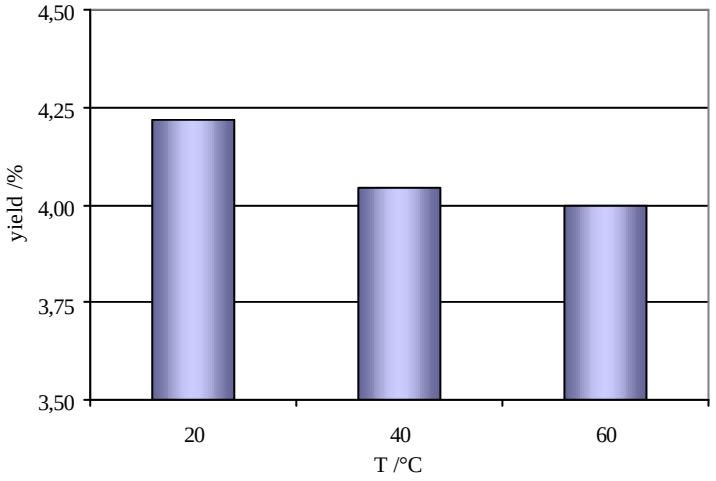


Fig. 4. Influence of the temperature on the extraction yield (%).

3.1.3 Effect of acidification on the extraction yield

In this investigations extractions were performed in bigger scale with 50 g of raw material and 50 % EtOH as a solvent in economical more acceptable ratio 1:10. Before mixing and heating to the desired temperature (60 °C) the chosen acid (acetic or phosphoric) was slowly added to the solvent till the level of pH decreased to ~ 2 or 4. Because of using the new batch of the raw material (batch 3) for this series of experiments, two samples without added acid and different way of mixing were made for the comparison. The first one (G17) was mixed for three hours with the magnetic stirrer and the second one (G22) with the mechanical mixer. The obtained results are presented in Table 3 and on the diagram in Fig. 5.

Table 3: Results of extraction yield using 50 % EtOH (ratio material : solvent = 1:10) at temperature 60 °C in addition of two different acids at two pH level.

added acid	sample label	pH	η / %
without	G17	4,6	6,27
	G22	4,6	5,78
acetic acid	G18	2,4	8,87
	G19	3,6	5,98
phosphoric acid	G20	2,1	27,68
	G21	3,6	6,05

It can be observed that the acidification of the solvent till reaching the pH ≅ 3,6 has no significant influence on the extraction yield for both acids, while the pH ≅ 2,4 (or 2,1) of the solvent mixture increases the extraction yield from 6,27 % (no acid added) to 8,87 % or 27.68 %, when used acetic or phosphoric acid, respectively. Generally, all the obtained extracts are in form of fine powder, accept of the very viscous but still liquid extract (sample G20) with the highest yield of extraction.

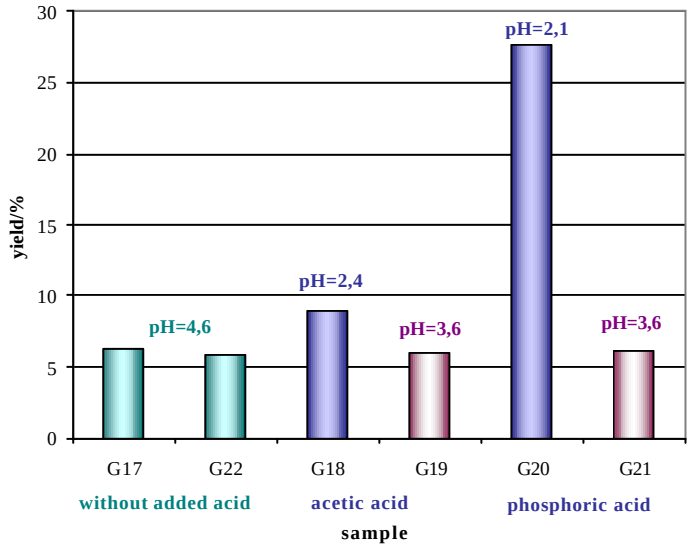
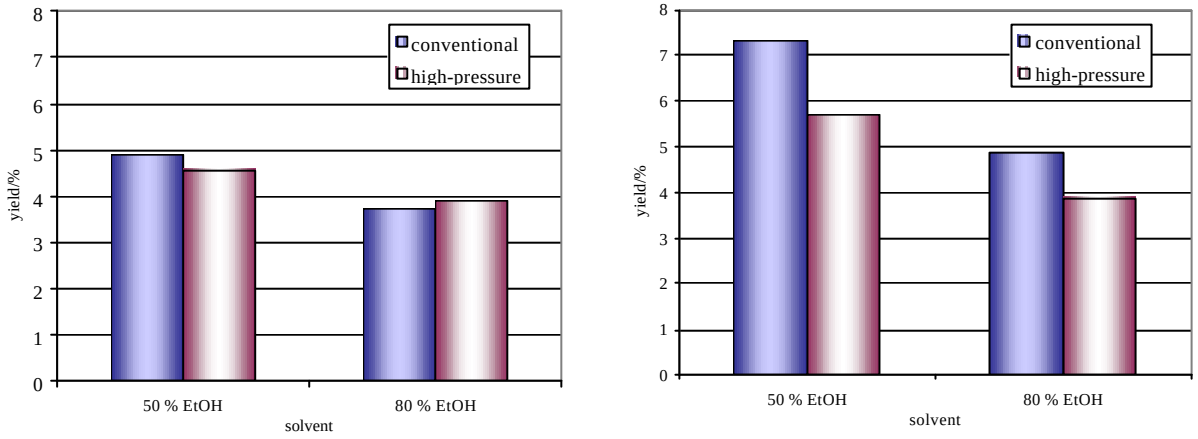


Fig. 5. Influence of the acidification on the extraction yield (%).

3.2 Large scale extraction experiments

In order to compare the results of extraction yield obtained with conventional and supercritical extraction in larger scale, the same working conditions except of the pressure were taken and repeated in two separate batches (batch 1 and batch 2). The ratio (material : solvent) was set to 1 : 10, temperature on 60 °C and time of extraction on three hours.

The results, presented in Fig. 6 and Table 4, shows that extractions performed with the raw material from the batch 2 give much higher results of extraction yield for both types of extraction as well as for both solvents. Furthermore, extraction yield obtained with 50 % ethanol is better than in case of using 80 % ethanol as a conventional extraction solvent and also as supercritical extraction co-solvent. Generally, results, achieved with the conventional extraction of marc, are higher than with high-pressure extraction, especially when using 50 % ethanol as a solvent (4,88 % for batch 1 and 7,32 % for batch 2).



a) Batch 1 b) Batch 2

Fig. 6. Conventional and high-pressure extractions using two different mixtures of ethanol and water.

Table 4: Results of conventional and supercritical extraction of marc with mixture of ethanol and water (ratio material : solvent = 1:10) and temperature 60 °C.

Extraction	Yield (%) with solvents			
	50 % Ethanol		80 % Ethanol	
	Batch 1/sample	Batch 2/sample	Batch 1/sample	Batch 2/sample
Conventional	4,88 / G9	7,32 / G13	3,73 / G10	4,86 / G14
Supercritical (200 bar)	4,58 / G11	5,71 / G15	3,90 / G12	3,87 / G16

Extracts obtained with 50 % ethanol were in the shape of small crystals and were darker when

performing extraction in supercritical media. In the case of 80 % ethanol, the obtained extracts were very sticky, a little less at conventional conditions.

3.3 Pilot and industrial scale extractions

The results of our previous work indicated that the total yield depends on extraction pressure, temperature, ratio material : solvent, solvent composition and its acidification. Designed experiments were carried out to map quantitative effects of these parameters.

In order to reduce the costs of producing the powdery extract of grape marc with the fine antioxidant effects in pilot and industrial scale, some operating conditions were modified. This two experiments (pilot and industrial scale) were performed in conventional way with organic solvent (50 % ethanol) without any added acid. The raw material of marc was taken from the batch 3 and mixed for three hours at temperature 60 °C. The results of extraction yield are presented in the Table 5.

Table 5: Results of pilot and industrial scale extractions of grape marc using 50 % ethanol as a conventional solvent at temperature 60 °C.

Extraction scale	sample label	ratio mat:solv	$m_{mat.}$ (kg)	$m_{extr.}$ (kg)	Yield (%)
Pilot	G23	1:5	3,05	0,110	3,61
Industrial	G24	1:6	95	0,800	0,84

Some technical problems occurred during the extraction procedure in industrial scale and consequently the extraction yield is much lower than expected. Therefore additional industrial scale experiment with the same operating conditions but using the new raw material from the batch 4 was performed. Moreover, the filtrate obtained after extraction was not evaporated till dryness but till the compact but still liquid residue. For calculating the yield of the extraction, a small part of the obtained residue was additionally evaporated till dryness in laboratory scale. The results presented in the Table 6 show much higher value for extraction yield which is comparable to the extraction yield obtained in a pilot scale.

Table 6: Results additional industrial scale extractions of grape marc using 50 % ethanol as a conventional solvent at temperature 60 °C.

Extraction scale	sample label	ratio mat:solv	$m_{mat.}$ (kg)	$m_{res.}$ (kg)	$m_{extr.}$ (kg)	Yield (%)
Industrial	G25	1:6	100	6,6	3,58	3,58

4. CONCLUSION

In conclusion, the data obtained in the present work have demonstrated that by-products obtained after pressing grape obviously still contain large amounts of phenolic, especially anthocyanin compounds with antioxidant activity and are resulted in fine extracts. The aim was to produce the extracts from the grape marc with the highest yield on anthocyanins. Between investigated types of extraction and their operating parameters, conventional extraction using 50 % ethanol without added acid in ratio with raw material 1:10 at temperature 60 °C was established to give the highest yield of extraction. Due to this investigations, pilot and industrial scale experiments with modified ratio material : solvent (1:6) were performed and gave promising and comparable results on the yield of grape marc extraction.

Step No.5: Analysis (WP #3)

Characterization of the precipitate

The precipitate was characterized by

- Colour change of the red precipitate/ polyphenols depending on pH: red under acidic, blue under basic conditions
- Chromatography HPLC/ DAD vs. standards (Fig. 12 and 13)
- Total polyphenol content (Folin-Ciocalteu assay)

Compared with the initial method, the optimized extraction method including forced precipitation revealed a much higher total polyphenol content by applying the Folin-Ciocalteu assay (Tab. 3).

Tab. 3: Folin-Ciocalteu-assay for grape pomace extracts

Extracts (1 mg dry mass)	[wt-%] gallic acid equivalent
EtAc* – Austria	8
EtAc – Spain	5.2
EtAc – Hungary	12.26
CINS G9	12.44
CINS G10	14.80
CINS G11	15.32
CINS G12	12.57
Natex 1070 A – Austria	0.7**
Natex 1071 A – Austria	1.32**
Natex 1072 A – Austria	0.6**
EtAc – Hungary – forced precipitation	**67.65**
BREKO OPC40	77.32

* ethyl acetate; **in turbid solution

HPLC results of the extract were inconclusive.
ChiroBlock supports the HPLC experiments by the complementary analytical method capillary zone electrophoresis (CZE). This method is known to exhibit a much higher resolution power compared to HPLC. On the other hand, CZE is less robust and normally requires more efforts for method development due to the higher number of variable parameters.

The CZE experiments were structured as follows:
a) method development for the pomace analysis aiming to a high resolution
b) qualitative analysis using commercially available reference compounds

a) Development of a ‘high-resolution’ CZE method

Standard conditions:

Beckmann-capillary electrophoresis PACE 5510
capillary: 67cm fused silica, 75 µm diameter, migration length 60 cm, length of the detection window 500 µm
wavelength 200nm
T = 20°C
Equilibration steps (daily prior measurement):
15 min rinsing with 1N NaOH (HPCE grade, Fluka)
15 min rinsing with HPCE-water (Fluka)
5min rinsing with separation buffer
5min 30 kV

Using this conditions, a borate buffer system was selected for the first experiments. According to the literature, borate buffers are preferred for related separation problems because of their ability to form associates with the analyte molecules. The results of these first experiments are summed up in the following table:

conditions	result
borate, pH = 8.0	migration time too long, broad signals
borate, pH = 10.0	poor resolution
borate, pH = 9.0 ... 9.5	poor resolution, fast migration
borate, pH = 9.3, addition of SDS	reasonable resolution and migration time, but high current, resulting in a higher internal temperature with negative consequences for the resolution power
borate, pH = 9.3, addition of SDS, thinner capillary (50 µm)	improved resolution, but still insufficient for the intended purpose – see the following picture

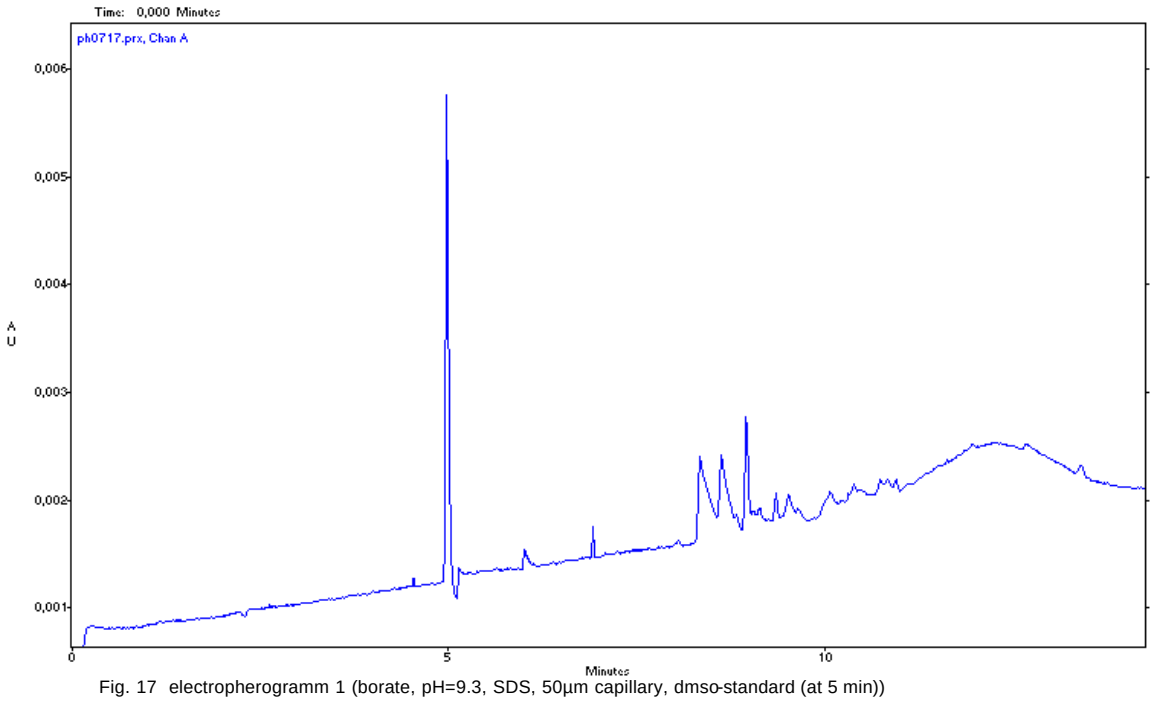


Fig. 17 electropherogramm 1 (borate, pH=9.3, SDS, 50µm capillary, dmso-standard (at 5 min))

In order to improve the hitherto unsatisfying results, a completely different separation system was tested. The separation of polyphenolic compounds, to which many of the pomace

ingredients belong, is known to proceed better under CZE conditions where the electro osmotic flow is reversed. This often is accomplished by using tetra alkyl ammonium salts:

conditions	**result**
150 mmol tetra ethyl ammonium tetra fluoro borate	reasonable resolution, but migration times > 30 min, see below:

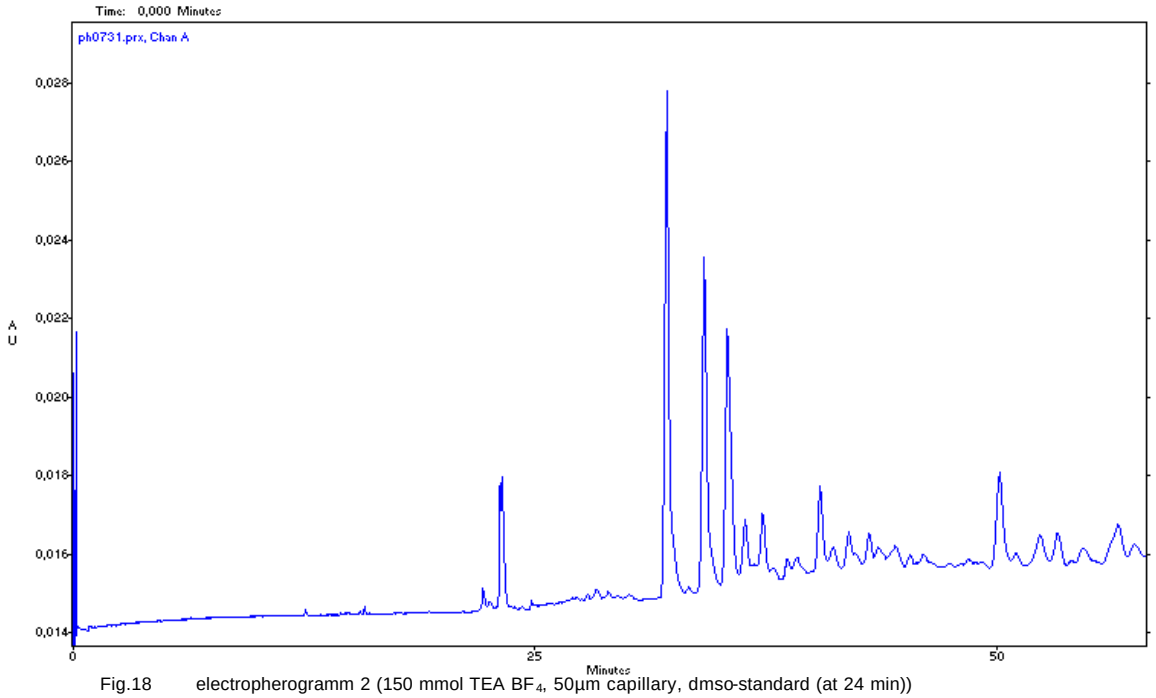


Fig.18 electropherogramm 2 (150 mmol TEA BF_4, 50µm capillary, dmso-standard (at 24 min))

conditions	**result**
150 mmol tetra ammonium tetra fluoro borate + SDS	no resolution at all
200 mmol tetra ethyl ammonium tetra fluoro borate	poor baseline stability due to high current resulting in high internal Joule-heating
200 mmol tetra ethyl ammonium tetra fluoro borate, reduced voltage	better resolution - see below

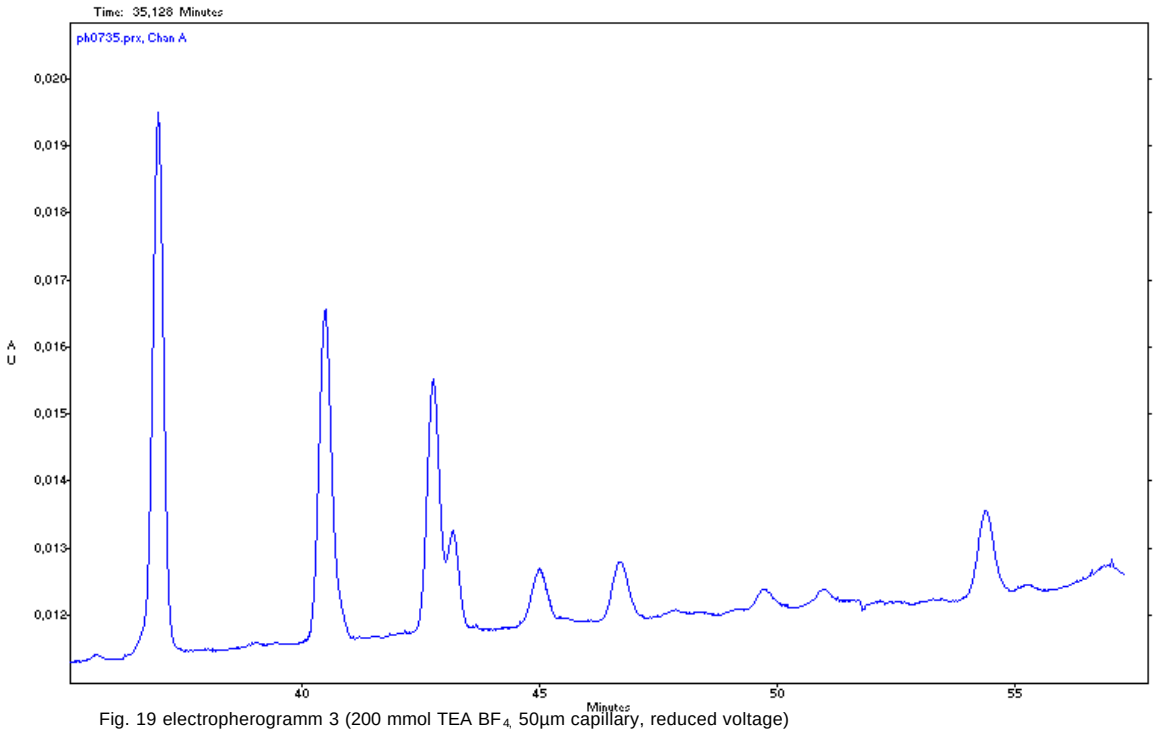


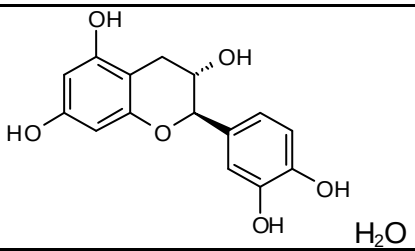
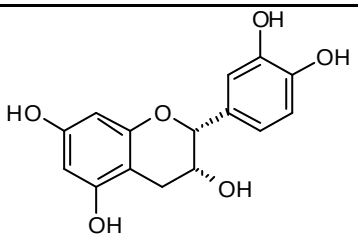
Fig. 19 electropherogramm 3 (200 mmol TEA BF_4, 50µm capillary, reduced voltage)

conditions	**result**
140 mmol tetra ethyl ammonium tetra fluoro borate + 10 mmol tetra butyl[1] ammonium tetra fluoro borate	under investigation, first promising results (even better resolution)

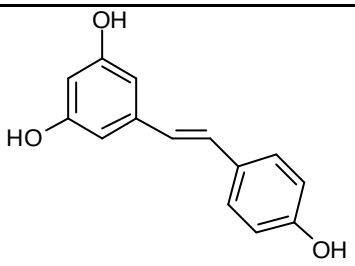
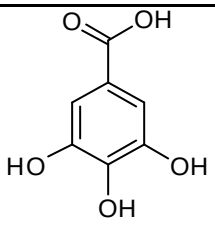
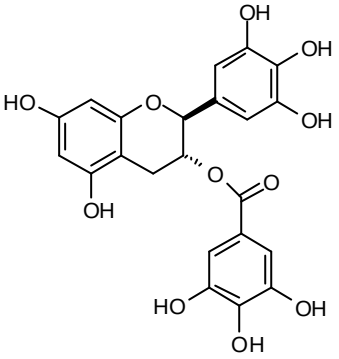
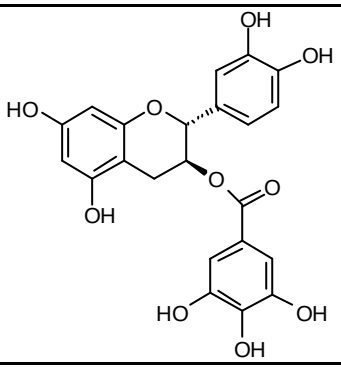
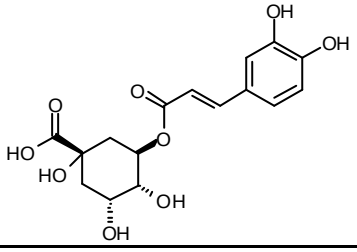
Attempts to increase the amount of the obviously beneficial TBA BF_4 by adding acetonitrile as organic solvent failed. The migration time is increased with the resolution collapsing. Currently, further experiments aiming at an even better resolution are in progress. Independently, the separation conditions stated above were used for the first qualitative / quantitative analyses.

b) Qualitative Analyses

Our initial qualitative analysis experiments made use of the same commercially available reference compounds as were used in the GAT-HPLC analyses. These compounds are:

No.	**structure**	**name**
1		(+) – catechine hydrate
2		(-) – epicatechin

[1] longer alkyl chains are known to improve resolution, but TBA BF_4 is only poorly soluble in water

3		trans resveratrol
4		gallic acid
5		(-) – epigallocatechin gallate
6		(-) – epicatechin gallate
7		chlorogenic acid, predominantly trans

Up to now, the first signal in electropherogramm 3 could unambiguously be identified as (-) epicatechin. Experiments for the assignment of the remaining six reference compounds, followed by the respective quantitative analyses will be carried out after final optimisation of the separation conditions using the new tetra ethyl ammonium tetra fluoro borate / tetra butyl ammonium tetra fluoro borate system.

Step No.6: Synthesis of analytical references (WP #3)

Synthesis of pomace compounds

As stated above, the availability of reference compounds – which are the pivotal prerequisite for any kind of analytical characterisation – is limited. The detection of the pomace ingredients for which such standards can be purchased, certainly is important for the quality control of this product, but it will not be sufficient, especially, if these compounds do not prove to be the most active components related to the 'French Paradoxon'.

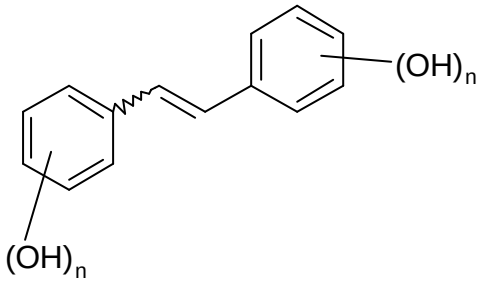
Therefore, one main contribution of ChiroBlock to this project is the development / improvement of synthesis strategies towards commercially not available reference compounds. At the beginning of the project it was not yet clear, which reference compounds would be needed. It was clear, however, that these molecules would also belong to the class of polyphenols as the available reference compounds do. The syntheses of such substances is not easy in most cases due to a number of (undesirable) properties:

- many of these molecules are chiral; the stereogenic centres have to be formed in a clearly defined way
- the polyphenols are closely related to each other, showing similar properties and hence are difficultly to separate, especially on preparative scale
- sophisticated protecting group chemistry is required in order to address the different hydroxyl groups
- the compounds tend to polymerise
- the polyphenols are prone to oxidation (by air)

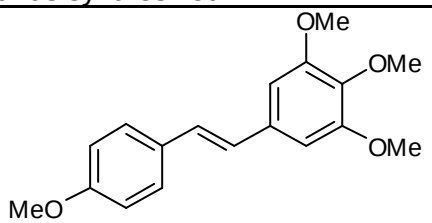
In order to explore and to improve the synthesis of monomer polyphenolic compounds, ChiroBlock had to focus on a sub-class which on the one hand is known for being abundant in pomace and on the other hand consists of building blocks for a number of more complex polyphenols.

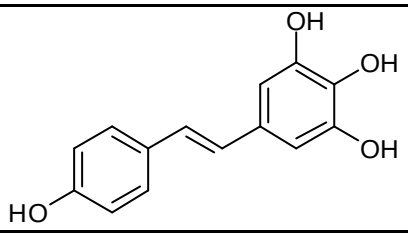
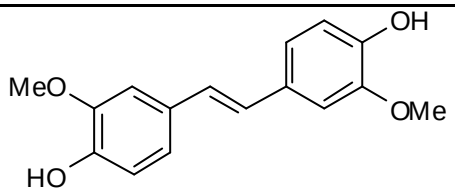
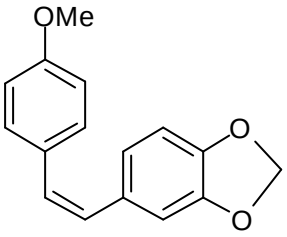
In the course of our first synthesis experiments, we explored the synthesis of molecules bearing the basic structural elements of resveratrol (reference compound No. 3), which in turn represents a basic structural motive of many naturally occurring polyphenols.

Basic structural motive of the compounds synthesized:



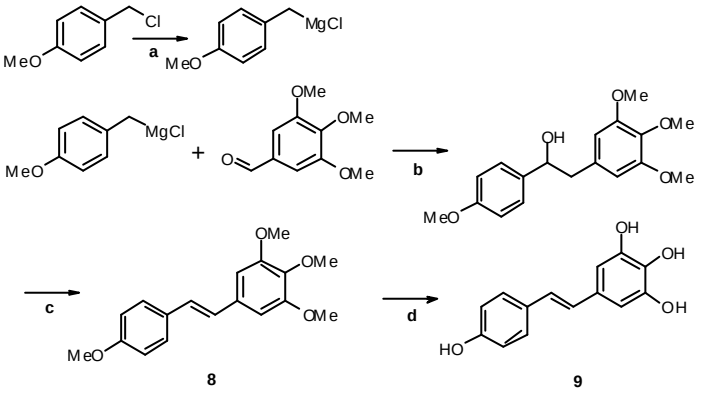
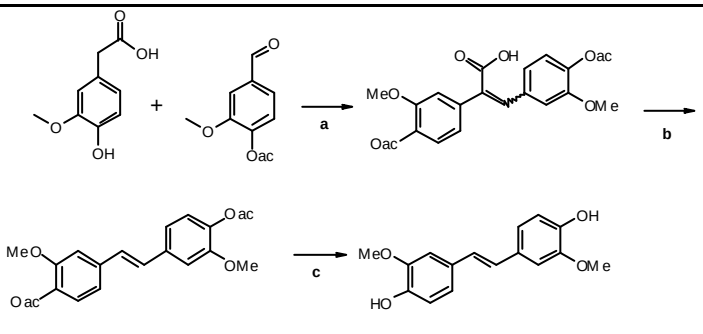
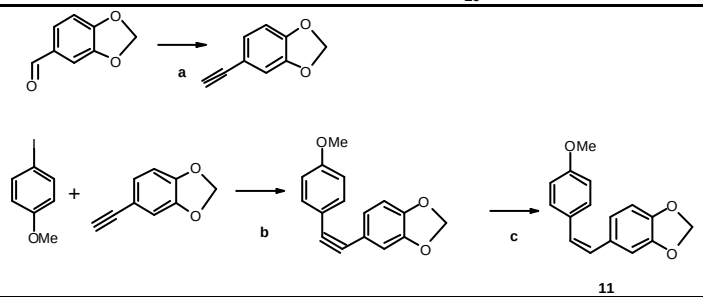
Compounds synthesized:

8		trans-3,4,4',5-tetra methoxy stilbene

9		trans-3,4,4',5-tetra hydroxy stilbene
10		trans-4,4'-dihydroxy-3,3'-dimethoxy stilbene
11		cis-gnetin

While 8, 10 and 11 are still protected 'storage forms' of polyphenolic compounds, substance No. 9 represents the 'deprotected' molecule 8 and hence a final polyphenol, likely to be found in pomace extracts.

The synthesis routes to these molecules are summarized below:

8, 9		a+b: 20% total yield c: T+HCl: many byproducts; Tonly(200°C): 35%, byproducts; d: TMS-Iodide: no product pyridinium chloride: 65%
10		a: TEA, acetic acid anhydride, DMAP, 57% b: chinoline, Cu, acetic acid anhydride, 21% trans (8% cis as byproduct) c) pyrrolidine, DCM, 45%
11		a: TPP, Zn, CBr_4, DCM, 66% b: $PdCl_2$, TPP, CuI, diethyl amine, 61% (the reversed conversion of ethinyl anisole and 5-bromo-1,3-benzodioxol failed completely) c: not yet completed

All these synthesis steps are based on known procedures which needed to be adapted to the target compounds, especially with respect to improvements of yields and purity of the products. Critical steps are c and d for 8 / 9 and b for compound 10. The yields of these reactions are still unsatisfactory. As with comparable polyphenolic compounds, the substances 9 and 10 are air sensitive and have to be stored under an inert atmosphere at low temperatures.

Now, the pomace extract can be spiked with these new reference compounds in order to verify their occurrence.
With this synthesis know-how in hand, it should be possible to make a variety of related compounds on demand, which – together with optimised analytical procedures - considerably extend the possibilities of the analytical characterisation of the pomace extract.

Step No.7: Development of an innovative formulation (WP #4)

The goal of the project consists in the extraction, the identification and the formulation of potent antioxidants from red wines pomace.

The micro-encapsulation technique allows the protection of the oxygen- and light-sensitive antioxidants by an encapsulation of the grape pomace extracts within a complex polysaccharide membrane. The resulting microcapsules serve as carrier for the antioxidants, which can be thereafter released by an acidity shift of their media, i.e. the pH of the stomach. These microcapsules are palatable and hide the bitterness of polyphenols, which allow their use as functional food additives.

The formulation technique was developed with commercially available grape pomace extracts having a high polyphenol content as well as a standardized composition.

Formulation

A "water in oil in water" formulation, also known as W/O/W was chosen to obtain the maximum efficiency from both the lipophilic and hydrophilic antioxidants from the grape pomace extracts. This W/O/W formulation is based on an interfacial polymerization process.

Composition

The formulation consists of a water phase and an oil phase.

The composition of the two phases is given below:

Microcapsule compartment	**Ingredient**	**[wt %]**
Oil phase	Grape seed oil	33.00
	Emulsifier	0.85
	Stabilizer type 1	0.1
	Stabilizer type 2	0.025
	Aromatic plant extract	0.025
	Water	19.15
Water phase	Aromatic plant extract	0.025
	Polyphenol powder	5.0
Wall forming agents	Wall monomers	25.0
	Wall polymers	4.82
	Stabilizer	7.0

Procedure

The W/O/W formulation of grape pomace extract is based on a polymerization in suspension. A primary stabilized emulsion is formed where hydrophilic droplets containing the grape pomace extract are suspended in oil. This emulsion is used to form a secondary emulsion by simultaneously coating and suspending the primary emulsion in water.
The formulation steps:

- Homogenize the water phase
- Homogenize the oil phase
- Form the primary emulsion (W/O) by mixing the water and the oil phase under permanent agitation

- Formation of the secondary emulsion in water (under permanent agitation): add successively the wall mono- and polymers, then the electrostatic stabilizer
- Polymerization of the wall-forming elements by a heat treatment
- Curing of the microcapsules

Composition of the extract

Polyphenol powder	**Specification [wt %]**	**Method**
Water content	< 7%	105°C
Total polyphenols	> 92 %	Absorption 280 nm eq. catechin
Proanthocyanidins	>50 %	Butanolyze from Bate-Smith: Absorption 550 nm Eq. Procyanidin B2
Oligomers Procyanidins	>15 %	Fractionation on Sep-Pak cartridge - Vanilin method (Eq. Catechin)
Resveratrol	>100 ppm	HPLC
Anthocyanes	> 2%	Ribéreau-Gayon Absorption 520 nm

Grape seed oil	**Specification [wt %]**	**Method**
Density	0.923-0.925	Densimeter
Viscosity	53-58 cp	Viscosimeter
Fatty acid content	98.8-99.8%	GC
Linoleic acid	58-78%	GC
Oleic acid	12-28%	GC
Vitamin E	330-480 $mg.kg^{-1}$	GC
Peroxides (meq O_2 . kg^{-1})	<1	Rancimat

Physico-chemical determination of the formulation

Parameter	**Unit**	**Measured value**
Acidity (at 20°C)	pH	5,76
Particle size (50%) (see Fig. 2, 3)	µm	2,95
Particle size (90%) (see Fig. 2,3)	µm	5,51
Density $_{(20°C)}$	$g.cm^{-3}$	1,00
Viscosity ? $_{T = 10\ (25°C)}$	$Pa.s^{-1}$	0,433
Yield point T_0	Pa	3,58
at shear rate ?	$[s^{-1}]$	9,55
Polyphenol powder	[wt.%]	4.672
Palmitate	[wt.%]	2.64
Stearate	[wt.%]	3.43
Oleate	[wt.%]	6.35
Linoleate	[wt.%]	22.39
Linolenate	[wt.%]	0.121

Fig. 20 & 21 represent the size distribution and the appearance of the microcapsules, respectively.

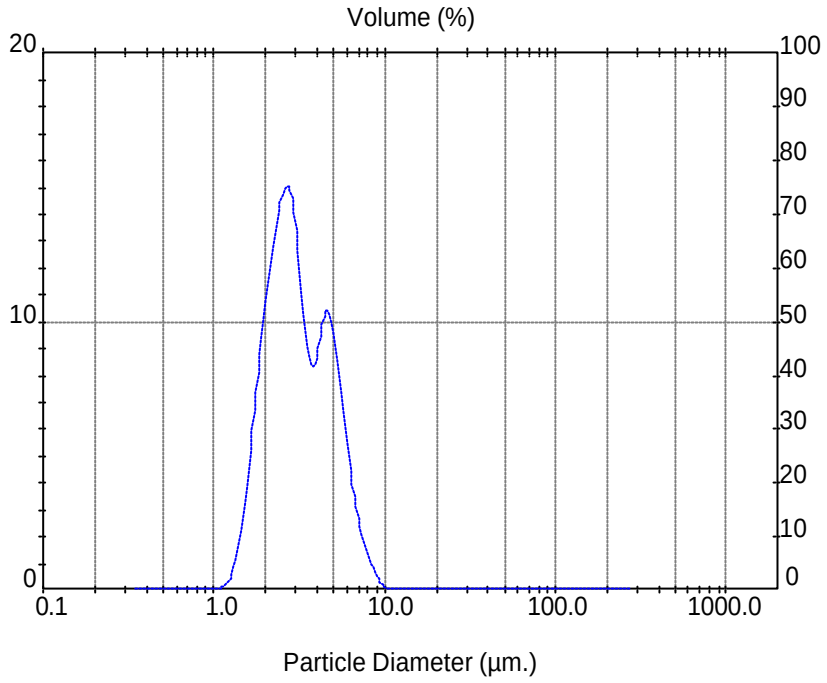


Fig. 20: Particle size distribution of the microcapsules.

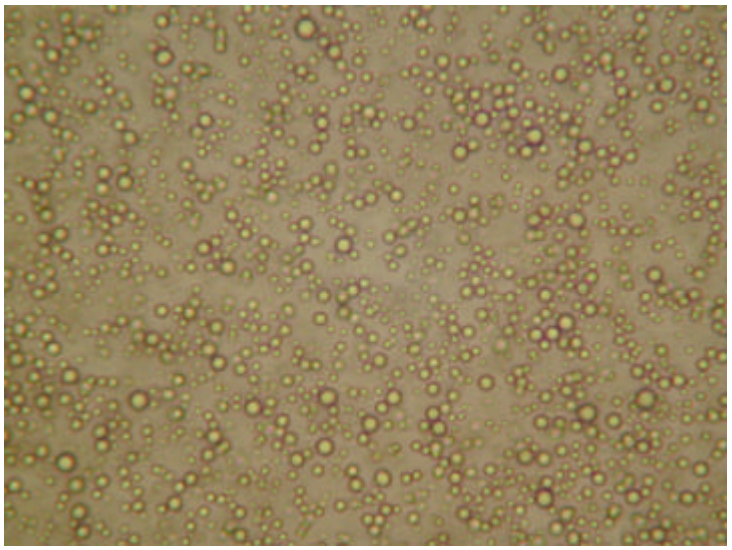


Fig. 21: Microphotograph of the microcapsules; water in oil emulsion within the capsules

Several techniques (chromatography, Folin-Ciocalteu assay, ORAC assay) were applied to characterize the extracts having the highest antioxidative properties.

The most potent extracts were sent to Prof. Elias Castanas (University of Crete), Prof. Roger Corder (Queen Mary University of London) and Prof. Antonio Ceriello (University of Udine) to perform the bio-evaluation with different cell lines.

In vitro efficacy of red wine residue extract and formulation on human prostate cancer cell lines (LNCaP, PC3, DU145) and human breast cancer cell lines (MCF7, T47D-hormone sensitive; MDA-MB-231-hormone resistant.

In vitro efficacy of red wine residue extract and formulation on the inhibition of Endothelin-1 synthesis (ET-1) by bovine aortic endothelial cells (BAECs). ET-1 is seen as a key factor in the development of vascular disease and atherosclerosis. Modification of NOS isoenzymes synthesis. Activity of NOS and NO production in different cell lines (breast and prostate cancer, vascular endothelial cells). Interaction of residue extracts with the Ah receptor-effector CYP activity.

In vitro efficacy of red wine residue extract and formulation on decreasing hyperglycemia induced oxidative stress, apoptosis in human endothelial cells in culture.Oxidative stress will be evaluated by nitrotyrosine staining, NADPH subunits expression and 8-OhdG formation, apoptosis by Bcl-2 and caspase-3 activity and expression.

In vivo observation study on volunteers consuming regulary red wine residue formulations on the alterations of HDLs(high density lipoproteins), fibrinolytic activity, and platelet aggregation. (Verum group versus placebo group in a standardised population sample).

The question regarding the involvement of systematic genetic factors is actually tested by Prof.J-F Rossi, in Montpellier, using gene chips. The aim of the investigation is the possible gene activation or silensing after antioxidant treatment of cells.

Depending on the outcome of these studies, the test system developed by Prof.Rossi will be included or not into the in vitro procedures of work package 5. The method will be transferred to one of the RTD partners and run for the formulations of wine food additives developed under the PARADOX project.

Process Development (WP # 6)

Scale up and process optimisation work.
Standardisation of process steps and process conditions.
Compilation of a process data matrix.
Elaboration of SOPs for product handling.
Patent writing and definition of claims.
Process Manual.
Product specifications.
Quality control manual.
Draft patent.
Product samples and technical documentation.

Training (WP # 7)

Training seminars for selected sales staff of dissemination partners in the laboratory of GAT.

Demonstration of the technological approach and the analytical results of the product.

Q & A for technical sales calls to customers in the food industry.

Elaboration of technical sales brochure for the red wine food additive. (printed, CD, WEB page).

Creation of a product brand name.

Training seminar for the technical sales staff of dissemination partners 3-5 days at GAT Laboratories in Austria.

Technical sales brochures (printed and electronic).

Sales presentations.Product brand name®

Dissemination (WP # 8)

Publication of the product brand name®.

Scheduled presentations and meetings with development managers of the food manufacturing industry in the territories and areas of activity of each dissemination partner.

Product development assistance to partners in the food industry.

Dissemination Activities Overview

Dates actual planned	Type Description	Type of audience	Countries addressed	Partner Responsible/ Involved
April 2004	Establish Marketing Department			GAT
Mai 2004	Investigation food industry – functional food			GAT, KUK, Campi y Jove
June 2004	Negotiation distribution contracts	Agents as potential distribution partners	Spain, Portugal, Austria	GAT, KUK, Campi y Jove
July 2004	Business trips	Distribution Partners	Spain, Portugal	Campi y Jove
August 2004	Further negotiations, distribution contracts	Agents as potential distribution partners	Austria, Central and Eastern Europe	GAT
August 2004	Distribution contract conclusion	Two distribution partners	Austria, Central and Eastern Europe	GAT, KUK
August 2004	Work out technical product information			GAT
September 2004	Preparation Marketing- sales material			GAT
September 2004	Launch Web Site gat-foodessentials.com	General public	worldwide	GAT
September 2004	Distribution contract conclusion	Two distribution partners	Northwest Europe Asia Pacific	GAT
October 2004	Improvement sample order,- shipment	Distribution partners, Administration GAT		GAT, KUK , Campi y Jove
October 2004	Product launch	Distribution partners,	Spain, South Europe	GAT, KUK, Campi y Jove
October 2004	Implementation Product portfolio and improvement offer procedure for food industry			GAT
October 2004	Product Sample Management and implementation of quality control			GAT
November 2004	Trade Fair “Hi Europe” in Amsterdam	Food industry	Europe	GAT, KUK, Campi y Jove
November 2004	Conception GAT Food Essentials “Corporate and Brand Identity”			GAT
November 2004	Completion Product catalog, announcement and dispatch	Distribution partners, clients	worldwide	GAT

Dates actual Planned	Type Description	Type of audience	Countries addressed	Partner Responsible/ Involved
November 2004	Drafting Labeling & Nutritional nformation			GAT
November 2004	Distribution contract conclusion	Distribution partner	Scandinavia	GAT
December 2004	New design concept – Corporate Identity			GAT
January 2005	Improvement pilot production			GAT
February 2005	HACCP-Certification planning			GAT
March 2005	Negotiations and conclusion Distribution contracts	Distribution partners	Latin America	GAT
April 2005	Implementation of first critical points HACCP			GAT
May 2005	Business trips	Distribution partners and clients	Southern Europe	GAT
June 2005	Business Trips	Distribution partners, clients	BENELUX, Scandinavia, Italy	GAT
July 2005	Business Trip	Searching Distribution partners, clients	USA	GAT
September 2005	Production and dispatch promotion food products	Distribution partner, clients	Worldwide	GAT
October 2005	GAT-Marketing Seminars	Distribution partners, clients	Austria, Germany, Serbia	GAT
November 2005	Business development Planning 2006, forecast	Distribution Partners, clients	Worldwide	GAT
December 2005	Promotion Material for X-Mas	Distribution Partners, clients	Worldwide	GAT
January 2006	HACCP next steps, pre-work Kosher Certification U.S. Business Trips U.K., Norway	Distribution Partners, clients		GAT
February 2006	Pre-work Exhibition U.S., Print Material Organization			GAT
March 2006	Co-Exhibitor at Nutrial Products Expo West U.S.A.	Food Industry U.S.A. 45.000 Visitors	U.S.A-	GAT

Project Management (WP # 9)

Implementation of decision-making boards.
Build up of communication tools and structures. (PARADOX Web page)
Follow up and controlling.
Reporting.
Management of knowledge and patent drafting.
Brand protection.

Deliverables

Reports accessible on-line on the PARADOX Web page
Reports to the EU commission at every 6 months.
Patent draft.
Milestone 4: Project closure meeting.
Expected results:
Patent filed.
Brand registered.
Final project report.

Project : COOP-CT-2003-508649 **PARADOX**

Workpackage No.:	Workpackage Title	Participant Short Name	Actual full (duration of project) Resources - working hours deployed	BUDGET (full duration of project)	
				Person-Months	hours
WP 1	Raw material / red wine residues	RODA	736	3	510
		HEINRICH	876	3	510
		TILIA		3	510
		WINECELLER	652	3	510
		CHAPOUTIER	12	3	510
WP 2	Extraction	CINS	1.140	9	1.530
		NATEX	1.067	9	1.530
WP 3	Analytical Characteriation	GAT	2.237	13	2.210
		CHIROBLOCK	1.206	12	2.040
WP 4	Formulation	GAT	2.201	12	2.040
WP 5	Efficacy Testing	CASTANAS	4.701	31	5.270
		CORDER	1.049	21	3.570
		CERIELLO	5.062	17	2.890
WP 6	Process Development	GAT	1.130	6	1.020
WP 7	Training	GAT	702	4,5	765
WP 8	Dissemination	CAMPI Y JOVÉ	432	1,5	255
		VALMAR	0	1,5	255
		KUK	131	1,5	255
		ATYS	0	1,5	255
WP 9	Project Management	GAT	1.042	4,5	765
TOTAL			**24.376**		**27.200**

Project no. : COOP-CT-2003-508649

Acronym : PARADOX

Project title : French Paradox
Red wine extract food additives

Instrument : Special Research Project for SME`s
Cooperative Research (CRAFT)

Deliverable No : D1

Deliverable Title : Raw material /Red wine production residues

Due date of deliverable : September2004/2005

Actual submission date : October 2004/2005

Start date of project : 15.02.2004 Duration : 24 months

Organisation name of lead contractor for this deliverable :

Bodegas Roda S.A.

Project co-founded by the European Commission within the Sixth Framework Programme (2002-2006)		
Dissemination Level		
PU	Public	
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	**X**

Project no. : COOP-CT-2003-508649

Acronym : PARADOX

Project title : French Paradox
Red wine extract food additives

Instrument : Special Research Project for SME`s
Cooperative Research (CRAFT)

Deliverable No : D2

Deliverable Title : Extraction – water based, Oil based compounds

Due date of deliverable : Juli 2004

Actual submission date :

Start date of project : 15.02.2004 Duration : 24 months

Organisation name of lead contractor for this deliverable :

NATEX Prozesstechnologie GmbH

Project co-founded by the European Commission within the Sixth Framework Programme (2002-2006)		
Dissemination Level		
PU	Public	
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	**X**

PARADOX

FRENCH PARADOX
RED WINE EXTRACT FOOD ADDITIVES

Instrument:

Thematic priority:

Periodic activity report

from

NATEX Prozesstechnologie GesmbH

Period covered: from 1.1.2005 to 15.2.2006 Date of preparation: 07.03.2006

Start date of project: 1.2.2004 Duration: 24 months

Project coordinator name:

Revision: draft1

Publishable executive summary

Description of the research work of NATEX

All tests for the PARADOX Project were carried out on the 5 litre/1000bar R&D plant in the workshop of NATEX (Fig. 1).

Fig. 1 5 litre/1000 bar R&D plant (NATEX laboratory)

The tests of the second report period were arranged in the following way:

Table 1 Arrangement of tests for the second report period of the PARADOX project

4th series	**extraction of grape seeds with ethanol, extraction of the ethanol extract with ethyl acetate followed by antisolvent SCE**
5th series	**extraction of grape seeds with ethyl acetate followed by antisolvent SCE**
6th series	**extraction of grape seeds with acetone followed by antisolvent SCE**

Fourth Series of Tests

In the fourth series of tests Hungarian grape seeds were used as raw material. The company GAT extracted the uncrushed seeds with ethanol in their laboratory and supplied NATEX with the raw extract.

In the first step 3kg of raw extract were mixed 3 times with 2,8l ethyl acetate to transfer the polyphenols into the ethyl acetate phase. Ethanol solves a lot of protein and sugar substances out of the wet seeds. During mixing with ethyl acetate the ethanol solution solves a lot of ethyl acetate and becomes a stable gel. It is very difficult to separate these two solvents into two phases. However, part of the ethyl acetate with the polyphenols can be separated. Then the mixture of ethanol extract and ethyl acetate was mixed twice with ethyl acetate (1:2) in the second step to make sure that all the polyphenols are transferred into the ethyl acetate phase. In total 42 l ethyl acetate were mixed stepwise with 6 kg ethanol extract. This resulted in 32 l ethyl acetate extract and 10 l gel. In the following step the 32 l ethyl acetate extract was treated with the antisolvent SCE process to produce a polyphenol concentrate according to the flow-sheet shown in Figure 2.

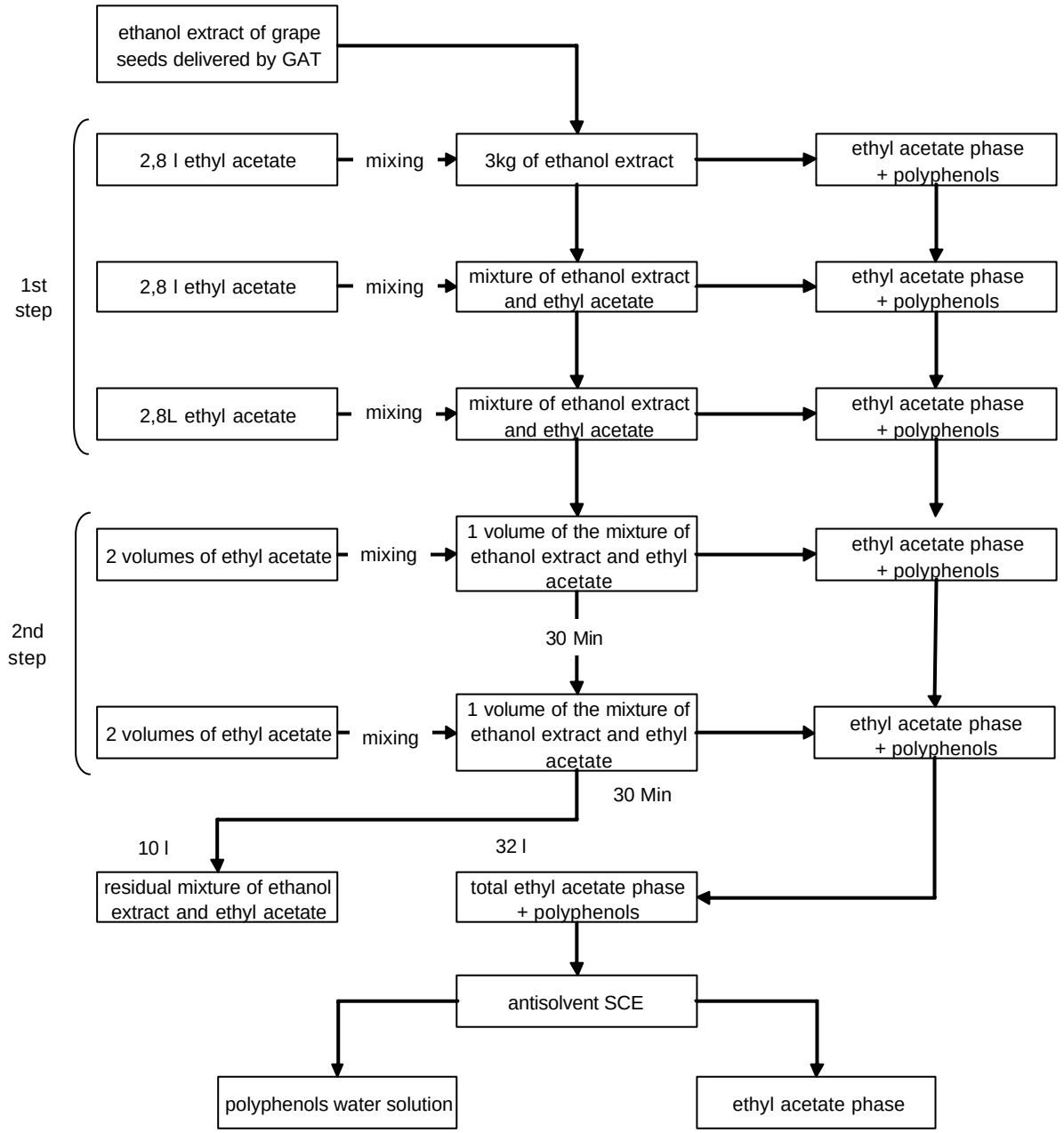


Fig. 2 Schematic overview of the 4th series of tests

13 SCE tests were executed in the 4th test series. In the first test with 2,5 l ethyl acetate extract an aqueous phase at the bottom of the extractor was separated during the static pressurization up to 225 bar. This phase included all polyphenols solved in ethyl acetate and could be directly drained out of the bottom of the extractor. In the following tests water was added to the ethyl acetate extract to improve the separation of the polyphenols out of the solvent CO_2 phase. About 50 – 100 ml distilled water were added to the 2,5 l ethyl acetate extract. As a result of these tests 1 l dark red water solution with all the polyphenols was gained, which was sent to GAT for analysis and further treatment.

Fifth Series of Tests

In the 5th test series Hungarian grape seeds were directly extracted with ethyl acetate. Then the ethyl acetate solution was dried with sodium sulphate to get a complete water-free ethyl acetate extract. Afterwards the solution was extracted with CO_2 till a dry polyphenol powder remained in the extractor. As the polyphenol powder stuck at the wall of the extractor it was necessary to remove the powder manually from the extractor. The polyphenol powder was sent to GAT for analysis and formulation. The production procedure is shown in the flow-sheet in Figure 3.

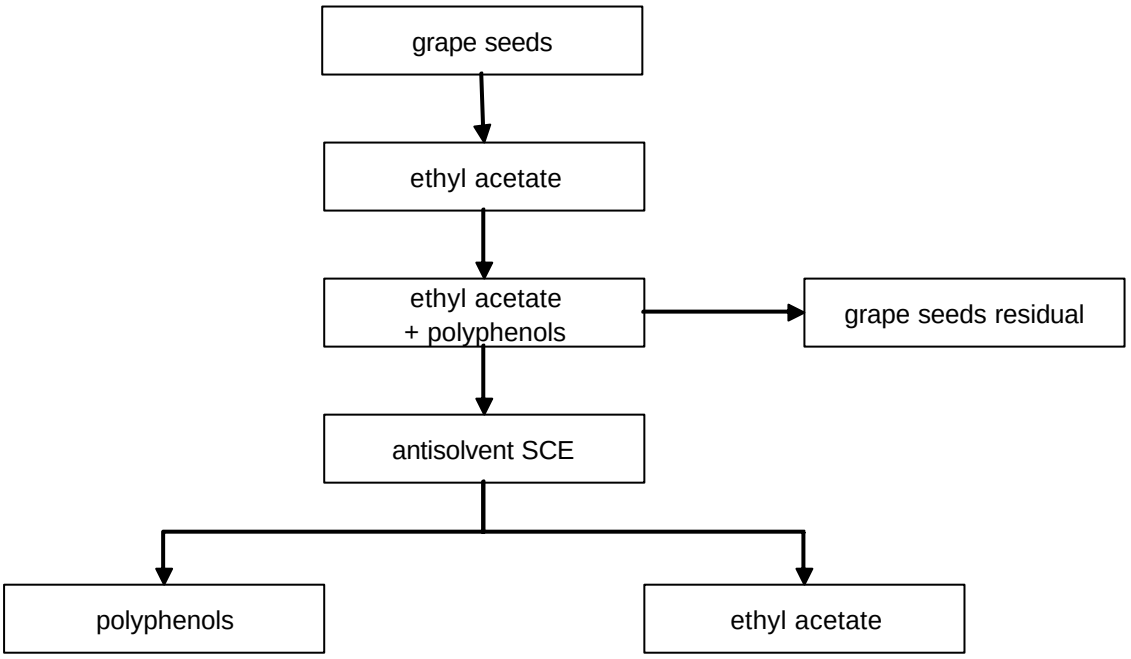


Fig. 3 Schematic overview of the 5th series of tests

With this procedure 14 l ethyl acetate extract was produced, which yielded a polyphenol quantity of 30 g powder.

Sixth Series of Tests

In the last test series Hungarian grape seed were extracted with an acetone-water-solution (9:1) and with pure acetone. The produced extract was treated with supercritical CO_2 at 250 bar and 50°C. Feed quantity was 2,5 l for each test. The extraction process was continued until the whole solvent and the water were extracted and separated in the first separator. The dry powder with the polyphenols remained in the extractor. Totally 19 l acetone extract were treated with a total yield of 152 g dark red powder, which should contain all the polyphenols.

This powder was sent to GAT for analysis and further treatment. The extraction process and the following antisolvent process is shown in Figure 4.

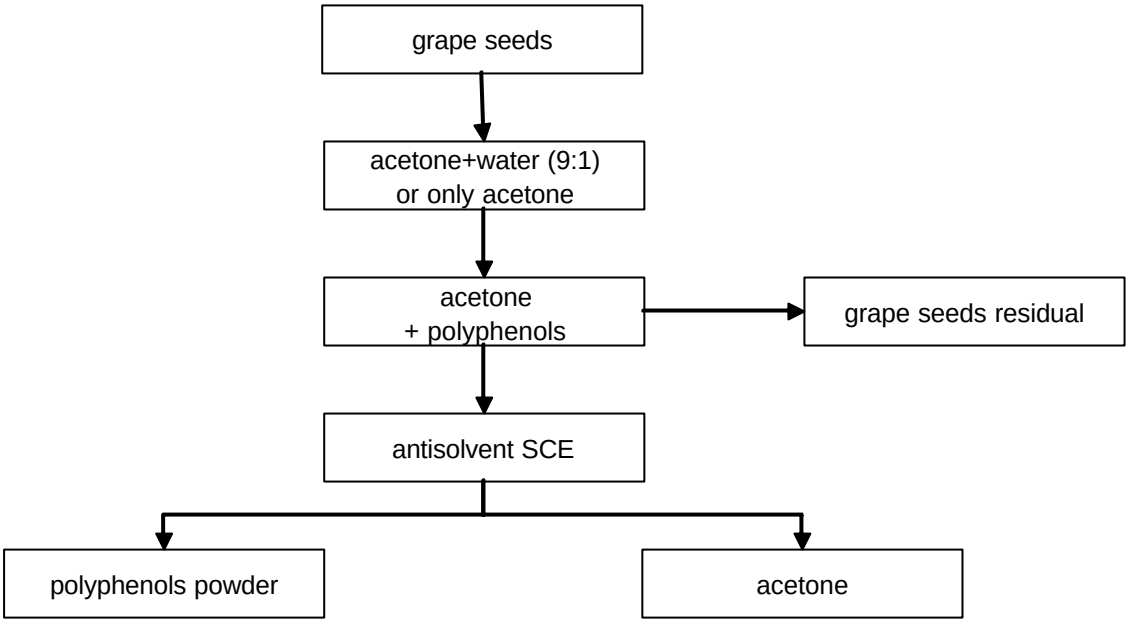


Fig. 4 Schematic overview of the 6th series of tests

Summary

As polyphenols from grape seeds are very polar a primary extraction with supercritical CO_2 is not feasible. A primary extraction with ethanol, ethyl acetate and acetone is possible. For concentration and removal of the solvent the CO_2 antisolvent process is very suitable and yields a highly concentrated polyphenol powder. The extraction of water with CO_2 needs longer extraction times because of the low loading of water in supercritical CO_2. Therefore the polyphenol solution to be treated with the CO_2 process should have a low water content.

Project no. : COOP-CT-2003-508649

Acronym : PARADOX

Project title : French Paradox
Red wine extract food additives

Instrument : Special Research Project for SME`s
Cooperative Research (CRAFT)

Deliverable No : D3

Deliverable Title : Analytical characterisation

Due date of deliverable : January 2005

Actual submission date :

Start date of project : 15.02.2004 Duration : 24 months

Organisation name of lead contractor for this deliverable :

CHIROBLOCK GmbH

Project co-founded by the European Commission within the Sixth Framework Programme (2002-2006)		
Dissemination Level		
PU	Public	
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	**X**

Project no. : COOP-CT-2003-508649

Acronym : PARADOX

Project title : French Paradox
Red wine extract food additives

Instrument : Special Research Project for SME`s
Cooperative Research (CRAFT)

Deliverable No : D4

Deliverable Title : Formulation, Microencapsulation

Due date of deliverable : January 2005

Actual submission date : April 2005

Start date of project : 15.02.2004 Duration : 24 months

Organisation name of lead contractor for this deliverable :

GAT Formulation GmbH

Project co-founded by the European Commission within the Sixth Framework Programme (2002-2006)		
Dissemination Level		
PU	Public	
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	**X**

WP 4 has been completed.

Formulation work developed a novel, stable and easily reproducible microencapsulation process suitable to manufacture plant extracts and oils in the format of liquid microcapsule suspensions in water. The procedure has been submitted to the Spanish patent office and subsequently to the European patent office. The patent was published under

WO2005058476

2005-06-30

The patent application text is attached to this report as WP 6 process development.

Project no. : COOP-CT-2003-508649

Acronym : PARADOX

Project title : French Paradox
Red wine extract food additives

Instrument : Special Research Project for SME`s
Cooperative Research (CRAFT)

Deliverable No : D5

Deliverable Title : Efficacy testing – in vitro, in vivo

Due date of deliverable : June 2005

Actual submission date : February 2006

Start date of project : 15.02.2004 Duration : 24 months

Organisation name of lead contractor for this deliverable :

University of Crete, School of Medicine

Project co-founded by the European Commission within the Sixth Framework Programme (2002-2006)		
Dissemination Level		
PU	Public	**X**
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	

Final Activity Report of the proposal COOP- CT-2003-508649
Project PARADOX

Participant: Laboratory of Experimental Endocrinology, University of Crete, School of Medicine, Heraklion, Greece

Artemissia-Phoebe Nifli, Vassiliki Pelekanou, Elias Castanas

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Heraklion, March 2006

Table of Contents

Introduction

The Laboratory of Experimental Endocrinology was involved in WorkPackage 5 of the PARADOX project. Its main activity was:

1. To test the vine pomace extracts, provided by the coordinator, on a number of cell systems *in vitro*.
2. To validate and provide accurate assays for the *in vivo* tracing of the extracts
3. To perform an initial *in vivo* validation of formulated extracts.

Additionally, the Laboratory tested either the vine extracts of isolated substances for biological activity in a number of *in vitro* cellular systems, in order to provide the scientific basis of their action.

First Reporting Period (Febryary 2004-February 2005)

During the first year of the proposal, we have addressed two types of questions:

Which are the mechanisms of interaction of wine polyphenols with basic cellular mechanisms?

In order to address this question, we continued our previous work on the effect of polyphenols on cell proliferation. Our previous results have shown that breast and prostate cancer cell lines respond to wine extract or isolated polyphenols by decreasing cell growth. The antioxidant action of these substances and their interaction with steroid receptors as well as their interaction with the NO/NOS system seem to be the major mechanisms involved. We have therefore continued on this line of data, addressing the question of a possible interaction of wine polyphenols with the hepatocellular cancer cell line HepG2. This line presents some interesting characteristics: (1) It retains the majority of the normal hepatocyte functions. (2) It responds to the same stimuli as the normal

hepatocyte. Therefore, it represents a useful alternative to study normal hepatic function. Polyphenols on the other hand, transit from the liver, after their absorption from the intestine. Therefore, the hepatocyte represents a good target to study their possible cellular action. Our results indicate that wine polyphenols can inhibit the proliferation of HepG2 cells but only resveratrol and catechin (IC_{50}s $3.11x10^{-11}$ and $9.91x10^{-13}$) inhibit cell growth at nano/picomolar concentrations. These two polyphenols also inhibit significantly the production of reactive oxygen species. We further report that resveratrol but not catechin, may act by modulation of the NO/NOS system, via increasing the expression of iNOS and eNOS transcripts, NOS activity and NO production. Inhibition of NOS enzymes attenuates the antiproliferative effect of resveratrol.

We have further investigated the effect of four wine polyphenols (resveratrol, and the flavonoids quercetin, catechin and epicatechin) in the hormone-sensitive human cancer cell line T47D, at concentrations compatible with their calculated plasma concentrations after ingestion of moderate quantity of wine (nM or pM). Our results indicate that cell growth was decreased, with cells being arrested at the S phase of the cycle. In addition, we provide evidences about a bimodal modulation of the NO/NOS system, affecting its activity and transcription. We show that modulation of this system is sufficient for the explanation of polyphenol action on this cell line. This result, suggests a potential importance of wine polyphenols and possibly the consumption of other polyphenol-rich dietary foods and drinks in the control of breast cancer cell growth.

On the other hand, in addition to monomeric flavanols, wine or grape extracts contain their oligo- or polymerized forms, which are mainly found in the pomace extract. We have concentrated on two dimmers, namely B2 and B5, found in wine seed extracts. We have reported that these substances induce equally apoptosis of breast cancer cells, acting on cell membranes, and targeting membrane androgen receptors. These receptors, studied extensively by our group for the last 3 years, are preferentially expressed on different cancer cells (prostate, breast, leukemias/lymphomas, pheochromocytomas, etc), being preferentially expressed on cancer cells. They may present a sign of de-differentiation of the cell, while their activation induces actin cytoskeleton rearrangements, and apoptosis.

B2 and B5, and in a lesser extend catechin and epicatechin, bind to membrane androgen receptors and modify the actin cytoskeleton in a similar way as testosterone-BSA, the prototype ligand of these receptors. In addition, the pattern of apoptosis obtained with flavanols is similar to that obtained by testosterone-BSA. These results indicate a new mode of action of polyphenols, which could explain their experimental and epidemiologically relevant anticancer effects.

How could one monitor early antioxidant action in biological fluids?

In 2002, we have introduced a new automated assay for the measurement of total antioxidant capacity in biological fluids (TAC assay). We have reported that the TAC assay may record changes in the antioxidant status after dietary interventions (antioxidant-rich diet) and medical manipulations (renal dialysis patients), or inflammatory bowel disease. Here we report that after chemotherapy or in patients with hepatic diseases (especially billiary cirrhosis) the TAC assay may record significant alterations of the plasma antioxidant status.

As the final product of the PARADOX project will be a micro-encapsulated concentrate of wine polyphenols, we have tested the effect of champagne wine (rich in different categories of antioxidants) on the modification of the antioxidant status in the serum of normal volunteers. Here, we have introduced another assay (the TOC assay) measuring directly the totality of circulating oxidative substances. Furthermore, we have introduced a new index, based on the concomitant measurement of antioxidants and oxidants (TAC and TOC), which was found to be a more sensitive index of a person's redox status. Our results indicate that as early as one hour after champagne ingestion (250 ml, two glasses) there is a significant reduction of oxidants and an increase of antioxidants in the blood of normal humans. This was not an effect of the alcohol, as this interference (very slight and not significant) was always subtracted from our values. The above results indicate that TAC and TOC can be used for the monitoring of the redox status in humans, after consumption of the PARADOX deliverable.

Second Reporting Period (February 2005-February 2006)

Test of the biological role of the pomace extracts

During the second reporting period, we were provided by the coordinator with pomace extracts. According to WP description, we have tested them *in vitro* and *in vivo*.

In vitro results

Initial formulated extracts

Introduction

According to previous discussions of the PARADOX steering committee, it was decided to extract pomace in such a way to increase its OPC content. In this respect, and in accord with the previous results of the group, tests are modified as compared to the initial ones on the WP, in order to test the biological activity of the extract. As a control, two OPC commercially available products have been assayed: OPC 40, provided by GAT, and OPC 80, provided by R. Corder. Both powders were dissolved in 20% absolute ethanol in 10 mM HCl, up to a concentration of 120 mg/mg, and thereafter in culture medium.

Pomace extraction was made by GAT. We had two different products:

1. GAT extract, as a sterile liquid, at a concentration of 120 mg/ml.
2. A formulated product containing 5% of the extract (v/v).

In a common meeting at May 16, it was agreed that the method of extraction of the formulated product will be the following:

a. Add 50 mM HCl
b. Mix
c. Let to stand at 4°C overnight
d. Use the lower phase in cultures, dissolved in culture medium

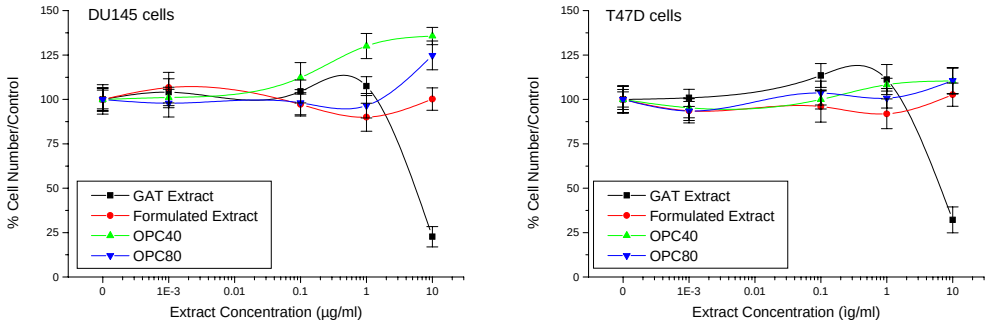
We have tested the action of the four products in three cell lines, as follows:
i. Human breast cancer T47D cells
ii. Human prostate cancer DU145 cells
iii. Human hepatocellular carcinoma HepG2 cells

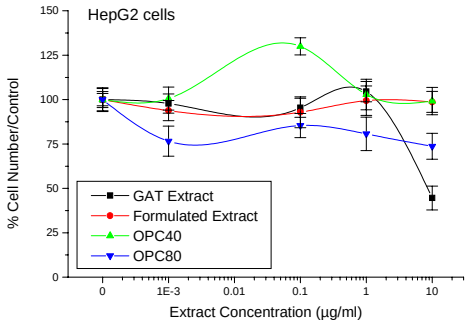
The choice of these cell lines was made according to previous results of our group (see results).

Results

Cell proliferation

Cells were incubated for 3 days (1.5 cell cycles) in the absence (control) or in the presence of different concentrations of the agents, ranging from 0.001 to 10 μg/ml (w/v). Cell number was assayed by the MTT method. Results are normalized to the corresponding control.



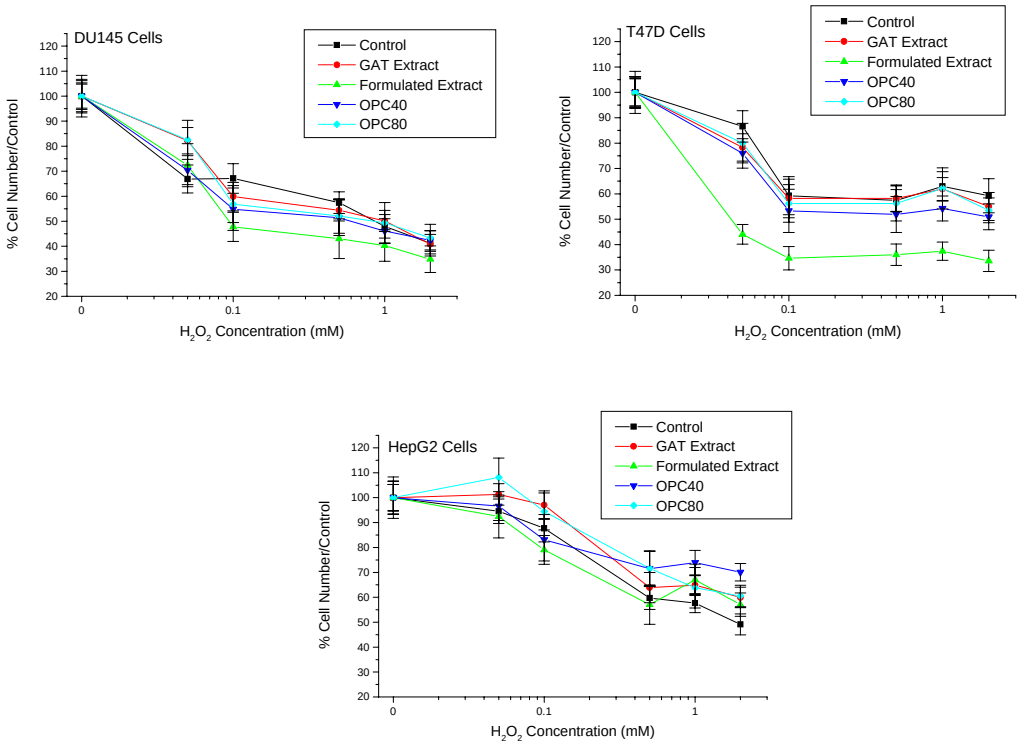


As shown, the effect of the four extracts on cell proliferation varied, depending on the cell line studied. In general, GAT extract was inducing (at concentrations > 1 µg/ml) a decrease of cell growth in the three cell lines studied. DU145 cells were more sensitive, while HepG2 cells were the most resistant to the action of the extract. In contrast, the formulared product was ineffective, at the same concentrations.

OPC40 and OPC80, were ineffective in T47D cells, stimulatory (at concentrations ≥ 1 µg/ml) in DU145 cells, while only OPC40 showed a transient stimulatory effect in HepG2 cells. Similar stimulatory effects have been observed with a number of other wine-derived purified substances, and in order to explain the results, a detailed analysis of constituents should be made.

Antioxidant effects

As the major constituent of wine and vine extracts is phenolic substances, which may act as antioxidants, we have tested the effect of extracts on their ability to protect cells from a major oxidative injury, mimicked by H_2O_2 treatment. The interpretation of the results of this assay depend on the antioxidant capacity of the substance used: if it acts as an antioxidant, is shifts the curve of H_2O_2 to the right, meaning that cells can survive in the presence of higher concentrations of H_2O_2. In order to investigate whether the extracts can protect cells from the oxidative injury, we have pre-incubated cells with reagents for 24 hours, before H_2O_2 treatment. In all cases, we have used a fixed concentration of each extract equal to 10 µg/ml, which induced the maximum effect on cell growth/survival.

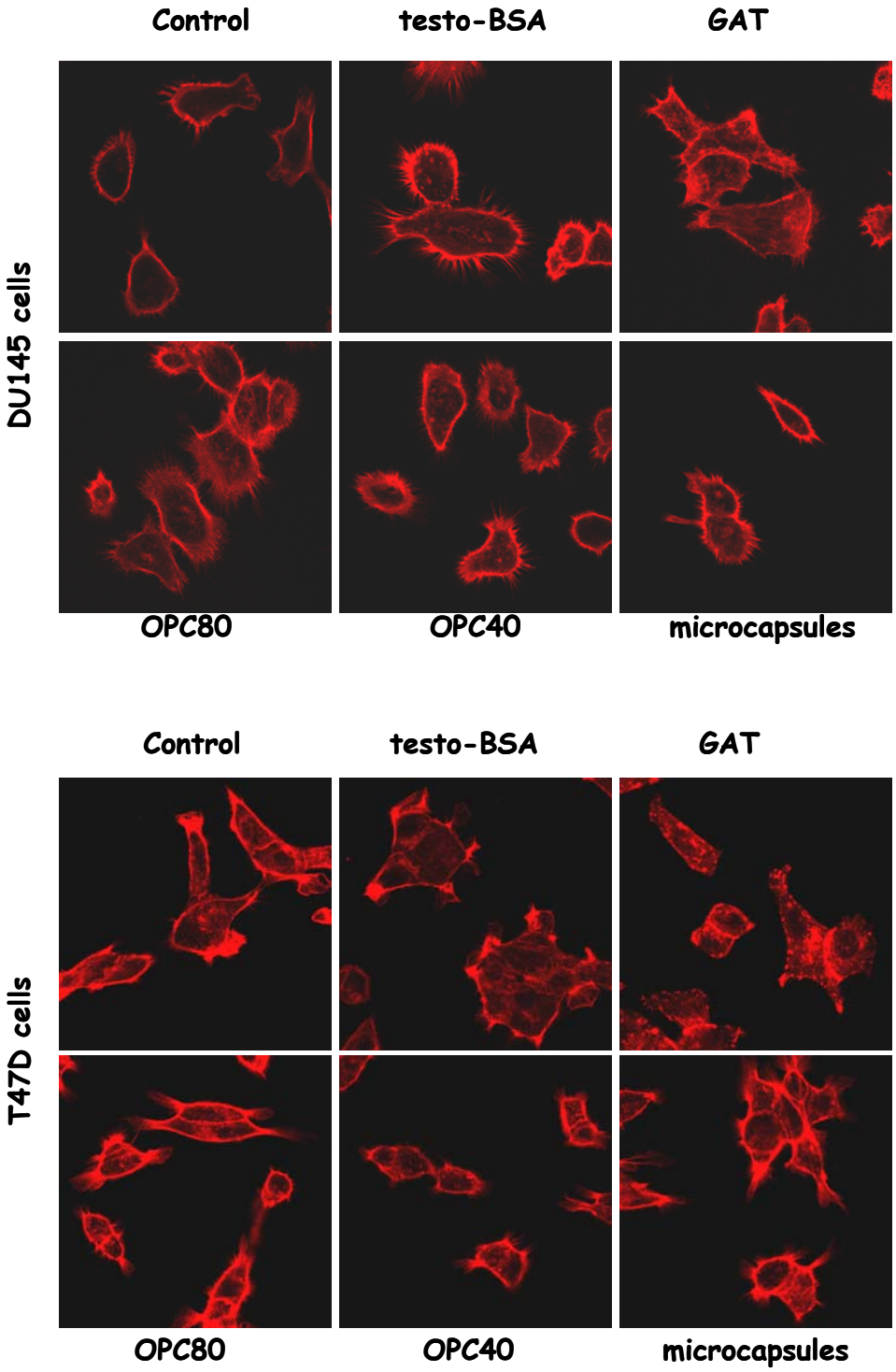


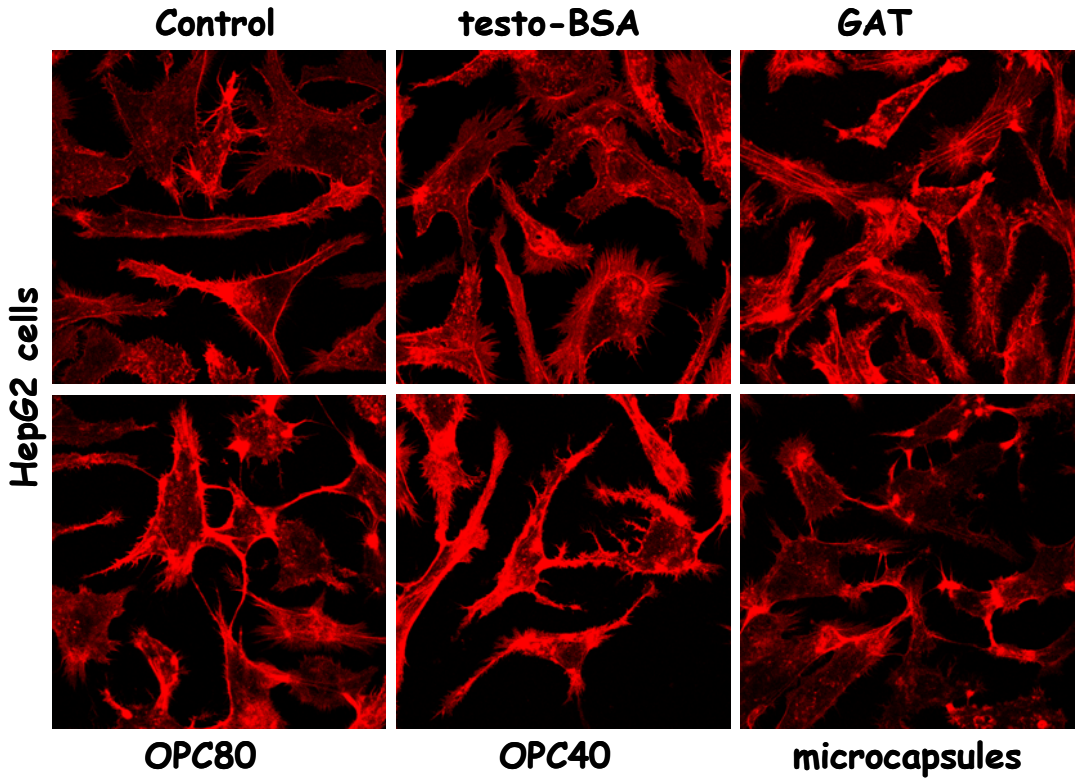
As depicted in the above graphs, in breast (T47D) and prostate cancer cells (DU145) no particular protection by any extract occurred. In addition, formulated extract is rather pro-oxidant, sensitizing T47D cells to H_2O_2. In contrast, in HepG2 cells, a slight protection could be observed by all extracts especially at high H_2O_2 concentrations. The differential effect could be attributed to the different nature of cell lines. HepG2 is a hepatocarcinoma cell line, expressing many characteristics of normal hepatocytes. It is therefore plausible that these cells could metabolize proanthocyanins or viniferins to their simpler building blocks (epicatechin or resveratrol) which have a well characterized antioxidant activity.

Modification of actin cytoskeleton

Our previous results (Nifli AP, Bosson-Kouamé A., Papadopoulou N, Kogia C, Kampa M, Castagnino C, Stournaras C, Vercauteren J, Castanas E. Monomeric and oligomeric flavanols are agonists of membrane androgen receptors. Exp. Cell Research, 2005, *in press*) showed that procyanidins, a possible major constituent of the different extracts, are able to bind to cell membranes and induce actin cytoskeleton rearrangement. In order to

verify this possible effect, we have incubated cells with extracts (10 µg/ml) for 1 hour, and observed the induced modifications in actin cytoskeleton using confocal microscopy.





In this experimental series, a differential effect of extracts has been also observed. GAT extract was the only one that induced a radical actin rearrangement, in all cell lines. The formation of stress fibers was prominent in DU-145 and HepG2 cells. Small foci of polymerized actin have been observed in all cell types, indicating an extensive and progressive induction of actin cytoskeleton remodeling. These data correlate with the inhibitory effect of GAT on cell growth, as cell death is linked with changes in cell shape and possible detachment of the substrate matrix, mainly effected through cytoskeleton. However, the observed modifications were not comparable with those induced by testosterone. This effect could be due to the concentrations of the applied substances: testosterone has been used at micromolar concentration (10^{-7} M), whereas GAT concentration was approximately $2*10^{-5}$ M, if considered as a monomeric flavonoid mixture. Another possibility could involve an enhancement of flavonoid activity, due to synergistic effects of individual compounds, or the polymeric flavonoid content.

Nevertheless, testosterone effect seemed to be comparable to the action of OPC80, OPC40 and microcapsules' extract. In all cases, an increased induction of peripheral actin polymerization has been observed, including actin mesh, lamelipodia, filopodia and focal adhesion formation. Testosterone produced mainly a filopodia wreath in DU-145 and HepG2 cells, while in T47D, as previously shown, it induced a massive induction of lamelipodia and more concrete actin localization. Application of OPC80, OPC40 and microcapsules' extract on T47D and DU-145 cells resulted in thin arrays of filopodia and to some extent in polymerization of actin mesh in cell periphery. Among the tested extracts, OPC80 was more effective than OPC40. Microcapsule formulation treatment had no effect on T47D cells, whereas in HepG2 cells, it induced a global actin depolymerization. In the latter case, no specific actin related structures were formed. Contrariwise, OPC80 and OPC40 induced first a robust formation of lamelipodia and subsequently their ramification in smaller protrusions. In this case too, the composition of the different extracts could account for the observed actions.

In conclusion, our data indicate that the formulated extract maintains the capacity to induce the same cellular changes as the initial product. This effect relays mainly to the modification of the actin cytoskeleton of the cell, as expected by the extraction method, promoting the extraction of OPC rather than of monomeric polyphenols.

Test of different extraction methods on a number of in vitro parameters

At a second stage, we have tested a number of total extracts, produced with different methods of extraction. The obtained results are as follows:

Introduction

According to previous discussions of the PARADOX steering committee, it was decided to extract pomace in such a way to increase its OPC content. In this respect, and in line with results presented above the biological activity of the extract was assayed in vitro. The following samples have been received:

1 Crude extract ethanol 80 % / water 20%, 5 mg/ml (3 ml)
2 Crude extract acetone 90% / water 10%, 6.9 mg/ml (1 ml)
3 Crude extract ethyl acetate 90% / water 10%, 3.9 mg/ml (9 ml)
4 Hexane precipitated ethyl acetate 90% / water 10% extract ("polyphenol extract"), 23.2 mg/ml (0.2 ml)
5 CO_2 precipitate of the ethyl acetate / water 10% extract (industrial production), 100 mg
6 Blank Sephadex fraction 5
7 Sephadex fraction 6 (epicatechin fraction - approx. 90%), 0.25 mg/ml (4 ml)
8 Sephadex fraction 8 (catechin & epicatechin fraction), 0.88 mg/ml (3 ml)
9 Sephadex fraction 9 (procyanidins & catechin - approx. 3:1; not confirmed by HPLC-NP), 0.48 mg/ml (2 ml)
10 Sephadex fraction 10 (procyanidins - approx. >80%, not confirmed with HPLC-NP), 0.25 mg/ml (8 ml)

According to preparation report, all Sephadex fractions were eluted by an ethyl acetate/water 9/1 mixture.

As the solvents of each preparation were different, and some of them toxic for cells, all extracts were evaporated in a rotor evaporator, and dissolved in acidified ethanol at a concentration of 12 mg/ml. Thereafter, they were dissolved in culture medium, in order to be tested on a number of cellular parameters.

We have tested the action of the four products in three cell lines, as follows:
i. Human breast cancer T47D cells
ii. Human prostate cancer DU145 cells
iii. Human hepatocellular carcinoma HepG2 cells

The choice of these cell lines was made according to previous results of our group (see results).

Methods

Cell lines and culture conditions

The human DU-145 (hormone-independent prostate cancer), T47D (hormone-dependent breast cancer) and HepG2 (hepatocellular carcinoma) cell lines were purchased from the European Collection of Cell Cultures (Salisbury, UK) or DSMZ (Braunschweig, Germany). They were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), in a humidified atmosphere of 5% CO_2 in air. All culture media and serum were from Gibco BRL (Life Technologies, Paisley, UK). The serum batches used were assayed, prior to use, for the presence of polyphenol oxidase (seruloplasmin) and transferrin, by conventional nefelometric techniques. In no case, measurable levels of either substance were found.

Measurement of cell prolifetation

For the estimation of cell proliferation, cells were cultured in 24-well plates at an initial density of $2 \cdot 10^4$ cells/well. One day after seeding, designated as day 0, fresh medium was provided, containing the different substances and the cells were grown for 3 days. Cell growth and viability were measured by the tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT, Sigma, St-Louis, MO)] assay.

Measurement of antioxidant activity

H_2O_2 viability

Cells were seeded in 24-well plates in an initial density of 150 000 cells/well. After 24 hours, medium was replaced, FBS was omitted, and the different samples, dissolved in culture medium (10 μg/ml) were introduced. Twenty four hours later, medium containing different concentrations of H_2O_2 (0.05-2.0 mM) were provided. After three hours at 37°C, cells were washed in PBS, and their viability was determined by the MTT method. Although preliminary experiments did not show any interference of the serum with H_2O_2, serum was eliminated from all the experiments. Cell viability was not influenced, for the short periods of the experiment by the absence of serum.

In another set of experiments, the different extracts were added to cells at the same time with H_2O_2. Cell viability was assayed after 3 hours of co-incubation.

Production of ROS

Reactive Oxygen Species (ROS) production after a short PMA stimulation was assayed by flow cytometry. Briefly, one million of cells, treated or not with the different extracts (10 μg/ml) for 24 hours, were removed from dishes, loaded with 10 μl of a 100 μM dihydroxyrhodamine 123 solution (Molecular Probes, Leiden, The Netherlands) in a total volume of 1 ml and incubated for 7 min at room temperature. Thereafter, 10 μl of a solution of 10 μM Phorbol-12 myristate 13-acetate (PMA, Sigma Chemicals St. Louis, MO) was added, incubated for another 5 min, counted in a a Beckton-Dickinson FACSArray apparatus (Beckton-Dickinson, Franklin Lakes, NJ) and analyzed with the CELLQuest (Beckton Dickinson) and ModFit LT (Verify Software, Topsham, MN) software. In the presence of intracellular ROS, dihydroxyrhodamine 123 is transformed to green-fluorescent rhodamine 123, trapped intracellularly. Measurements were repeated at determined time intervals for one hour.

Actin cytoskeleton modifications

Cytoskeleton was visualised with direct fluorescence staining of actin microfilaments by rhodamine-phalloidin (Molecular Probes, Leiden, NL). Cells (3×10^5) were seeded at poly-L-lysine coated cover slips, placed in 6-well plates. One day after seeding, cells were pre-incubated with serum free medium for 3 hours and subsequently treated with the different extracts (2 μg/ml, dissolved in culture medium) for one hour. After fixation with 3.7% formaldehyde and ice-cold acetone, cells were incubated for 40 min at room temperature with rhodamine-phalloidin (1 unit/well) to stain the filamentous actin. Slides were mounted with Mowiol anti-fading medium. The cover slips were analyzed using a confocal laser scanning module (Leica Lasertechnik, Heidelberg, Germany) equipped with an Ar-Kr laser.

Results

Cell proliferation

The antiproliferative activities of the extracts are presented in Figures 1A-C, for the three cell lines tested.

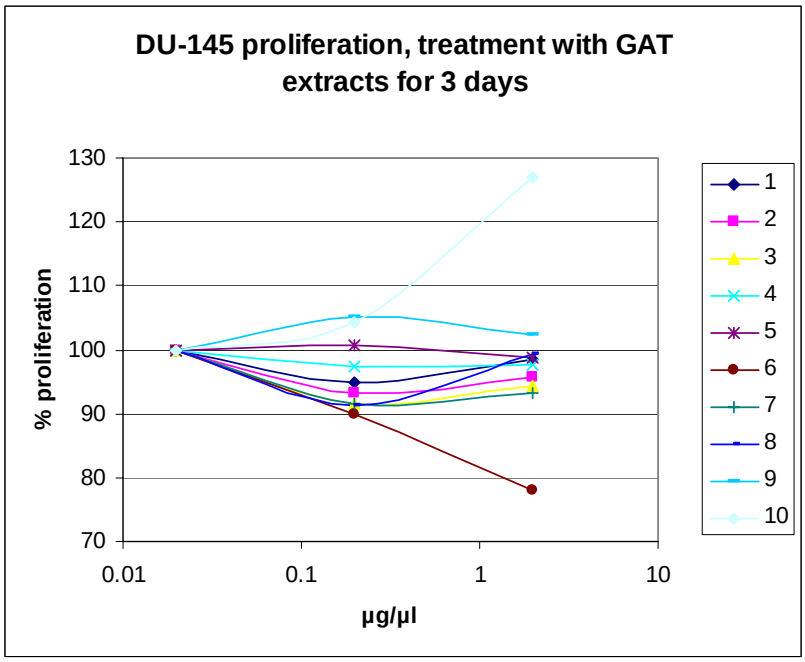


Figure 1 A

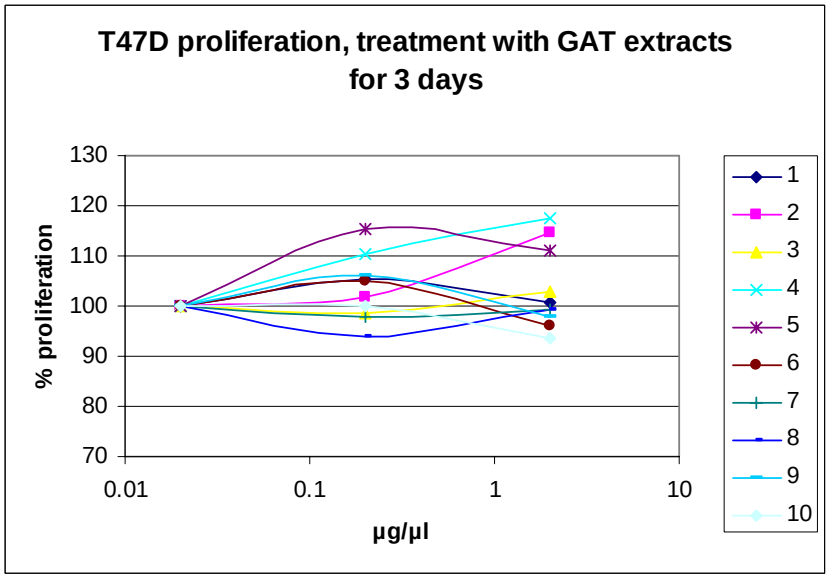


Figure 1B

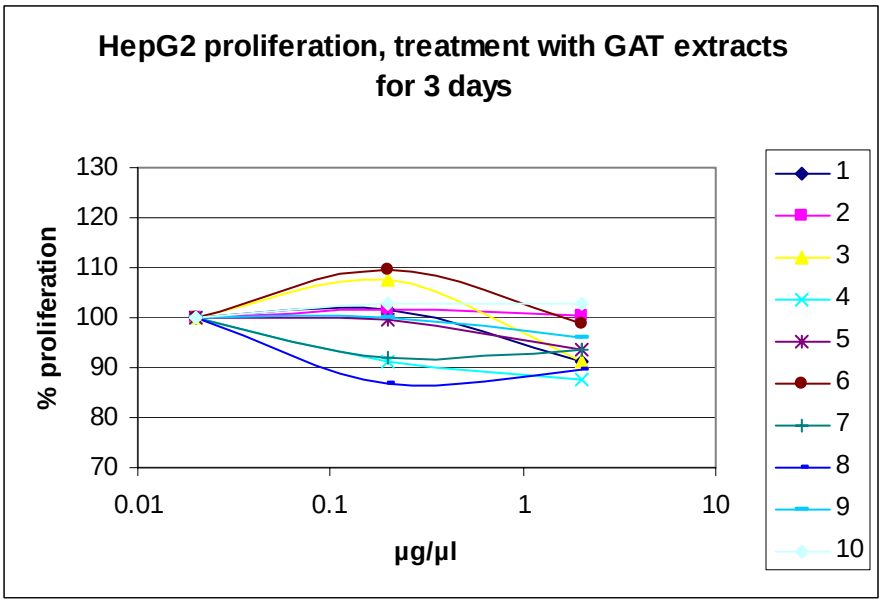


Figure 1C

The legend in these three figures referes to the different extracts as presented above.

As presented, the majority of the extracts do not presents any significant antiproliferative activity in the three cell lines. In particular, the antiproliferative effects are presented in the following Table:

Table 1: Percent inhibition of cell proliferation in the three tested cell lines. NS=non-significant

Extract Number	DU-145	T47D	HepG2
1	NS	NS	NS
2	NS	NS	NS
3	6	NS	9
4	NS	NS	13
5	NS	NS	7
6	22	5	NS
7	7	NS	7
8	NS	NS	11
9	NS	NS	NS
10	NS	NS	NS

As presented, extracts show a differential effect on each cell line. Extracts 1 and 2 (ethanolic or acetonic extracts) do not have any effect on cell growth. The same is also true for the procyanidin fractions 9 and 10. The most competent total extract seems to be the ethyl-acetate extract (Fraction 3) and its hexane precipitate (Fraction 4). Crude extracts inhibit cell growth by 9 and 13% in HepG2 cells, while only by 6% in DU-145 prostate cells. In contrast, they have no effect on the growth of breast cancer T47D cells. CO_2 precipitated extract, in contrast seems to have a slight antiproliferative activity on T47D cells.

Fractionation of the total polyphenolic extract (Fractions 3-4) on Sephadex LH20, resulted in fractions 6-10, with a different phenolic content. Regarding cell proliferation, primary Sephadex fractions, containing monomeric catechin and epicatechin, present the most potent inhibitory activity. Indeed, fraction 6, containing mainly phenolic acids, potently inhibits DU-145 cells (by 22%) and weakly T47D breast cancer cells (by 5%). Fraction 7 (containing mainly epicatechin) weakly inhibits the growth of DU-145 and HepG2 cells (by 7%), while the catechin fraction (#8) is an intermediate inhibitor of HepG2 cell growth (by 11%).

We concluded that the ethyl acetate total extract could be an valuable product to work on, taking into consideration its ease in production and formulation.

Reactive species scavenging

H_2O_2 protection

Figure 3 presents the long-term protection of the three cell lines from H2O2 toxicity, tested after a 24-hour preincubation with 10 μg/ml of the different extracts. As shown, a slight protection is induced by different extracts (with the exception of # 1, 3, 6 and 7) in

DU-145 cells, for H_2O_2 concentrations ≤ 0.1 mM. Thereafter, no protective effect was

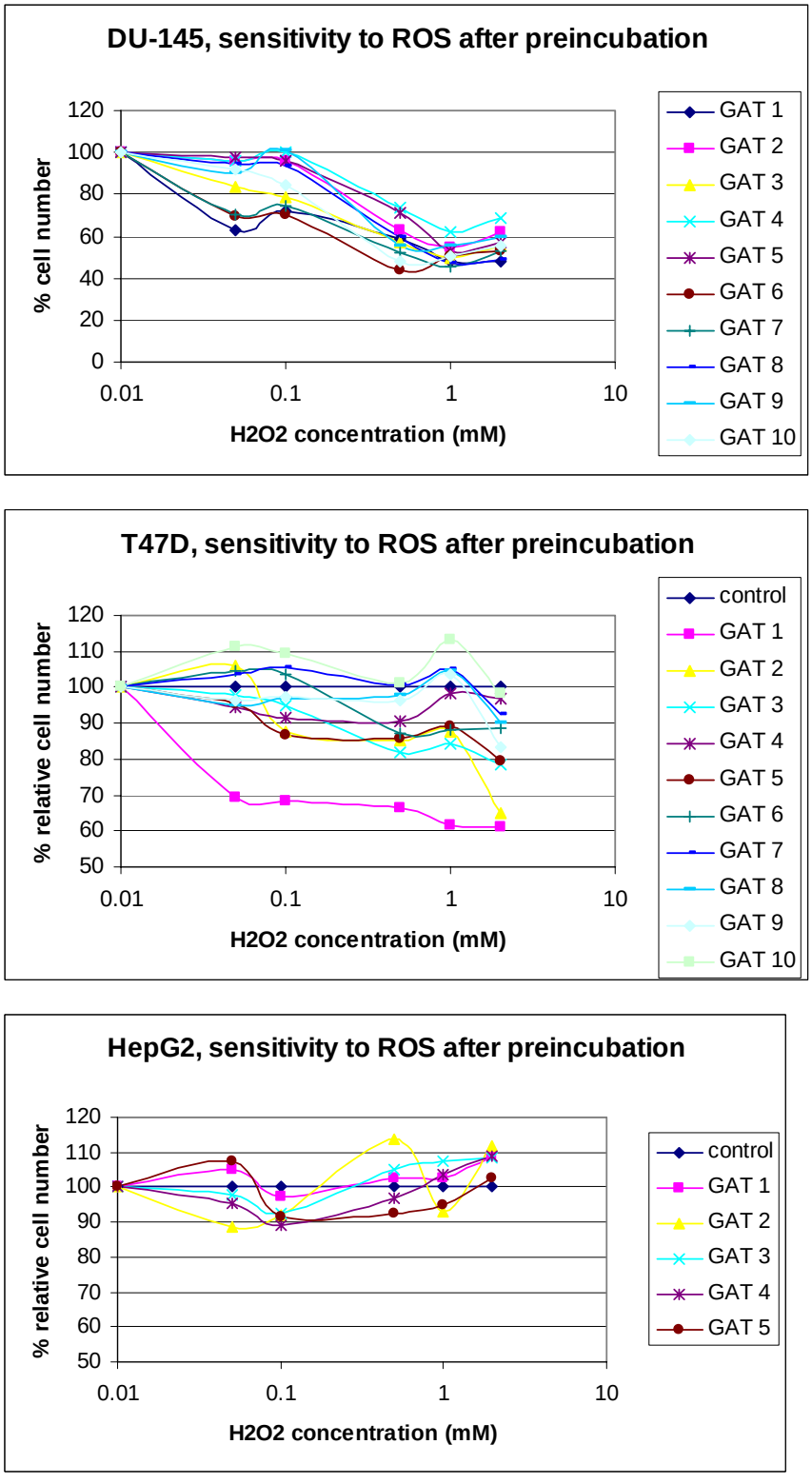
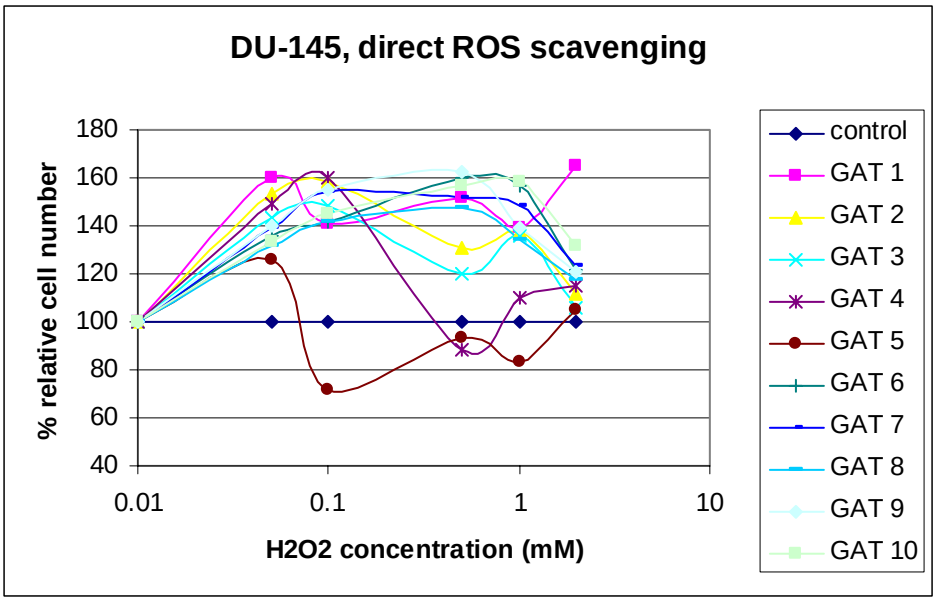
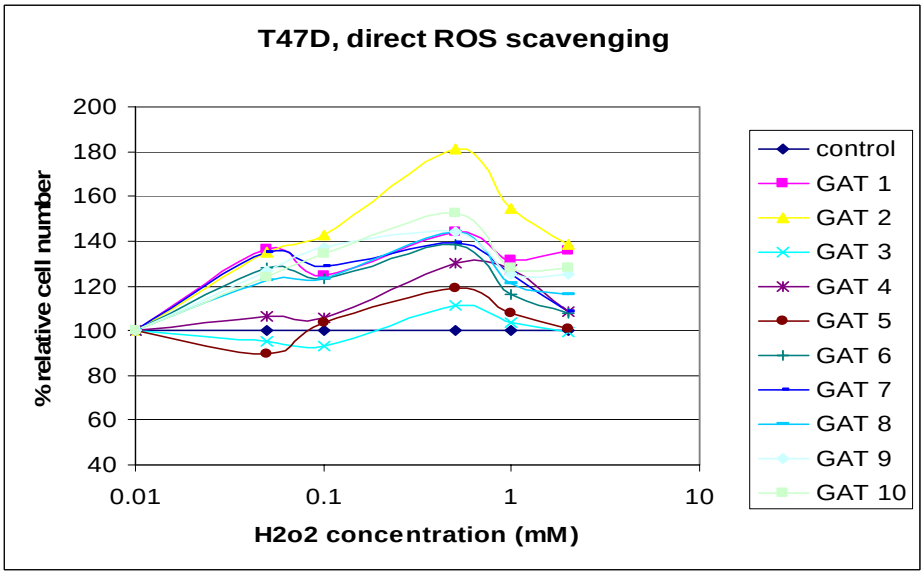


Figure 2. Effect of different extracts on H_2O_2 protection of cells. Cells were pre-incubated with the different extracts (10 µg/ml) for 24 hours. Thereafter, the indicated concentrations of H_2O_2 were added,

incunation was proceeded for 3 hours, and cell viability was assayed by the MTT method. Results are expressed as a percentage of the corresponding control at the same time-point. A value >100 indicates protection.

observed. In T47D cells, in contrast, extracts #2, 3, 4, 6-10 protected in a different degree cells from H_2O_2 toxicity, and all extracts were active in HepG2 cells. These results are in line with the greater sensitivity of DU-145 cells to H_2O_2, reflecting the different metabolism of each cell line. However, as shown in Figure 3, all extracts (with the





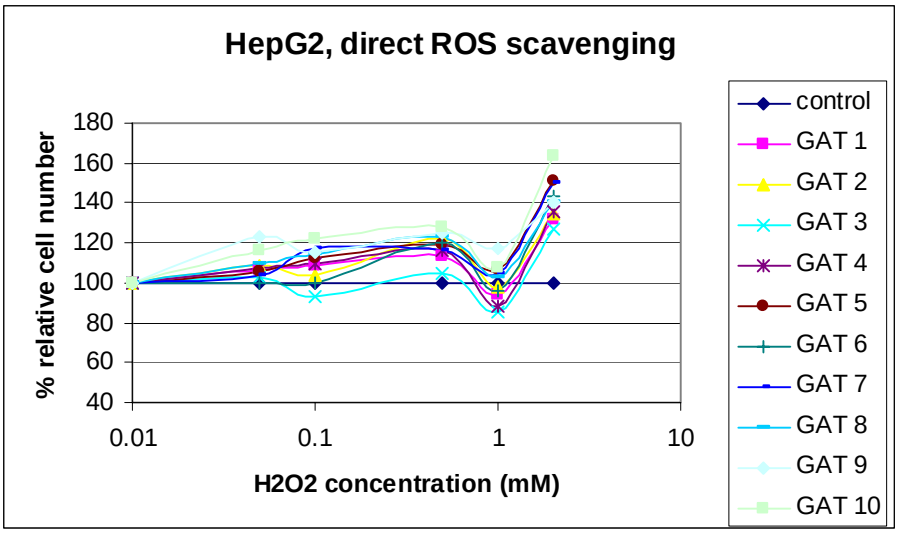


Figure 3. Effect of different extracts on H_2O_2 acute protection of cells. Cells were co-incubated with the different extracts (10 µg/ml) and the indicated concentrations of H_2O_2 for 3 hours. Cell viability was assayed by the MTT method. Results are expressed as a percentage of the corresponding control at the same time-point. A value >100 indicates protection.

exception of (#4 and 5) potently potentiate acute H_2O_2 toxicity in DU-145 cells. In addition, all extracts could protect HepG2 and T47D cells from a direct ROS burst.

ROS scavenging

Figure 4 presents the ROS-scavenging activity of all products (10 µg/ml) tested on the three cell lines. As shown, the effect of each fraction is different, depending on the cell line tested.

In general, extracts 1-3 show an enhancement of ROS production, in all three cell lines. Similar results were also found in hepatocellular carcinoma and prostate cells treated with Sephadex fractions. Contrariwise, Sephadex fractions inhibit ROS production in breast cancer cells. Finally, extract 4 presents a slight (but significant) decrease of ROS production in the three tested cell lines.

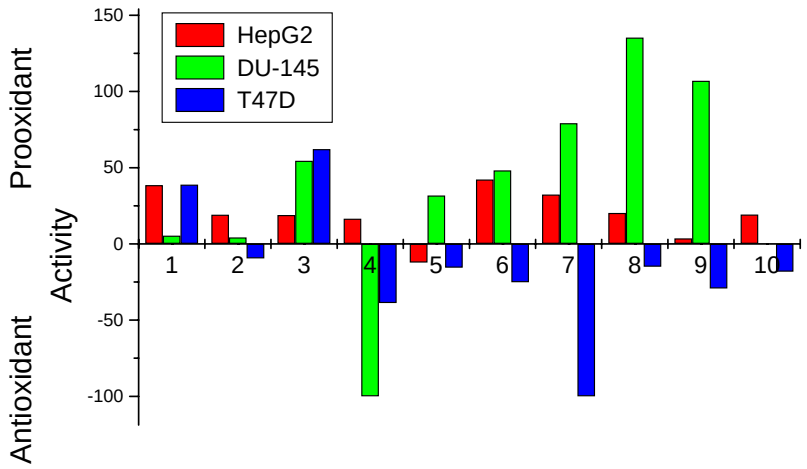


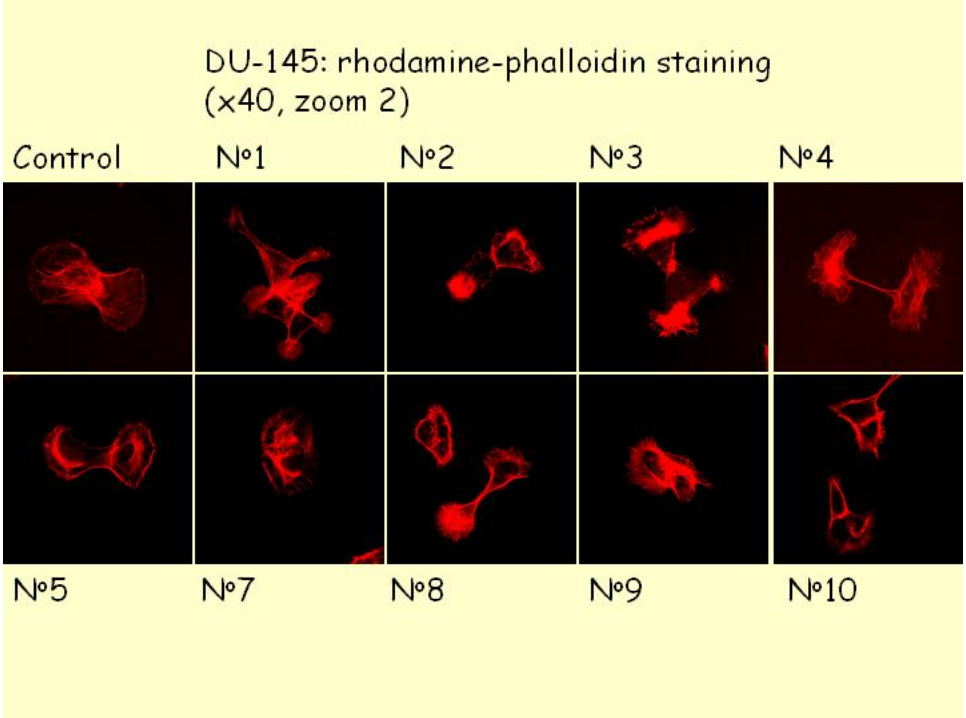
Figure 4 : ROS inhibition (negative values) or enhancement (positive values) after 24-hour preincubation with extracts and stimulation of cells with PMA. Cells were incubated for 24 hours with extracts, loaded with dihydrorhodamine123, stimulated with PMA and analyzed at different time-frames by flow cytometry. The results at 20 minutes are presented, when ROS production has reached a plateau, in all three cell lines. Figure presents the % change in ROS production, as compared to the control, taken as zero.

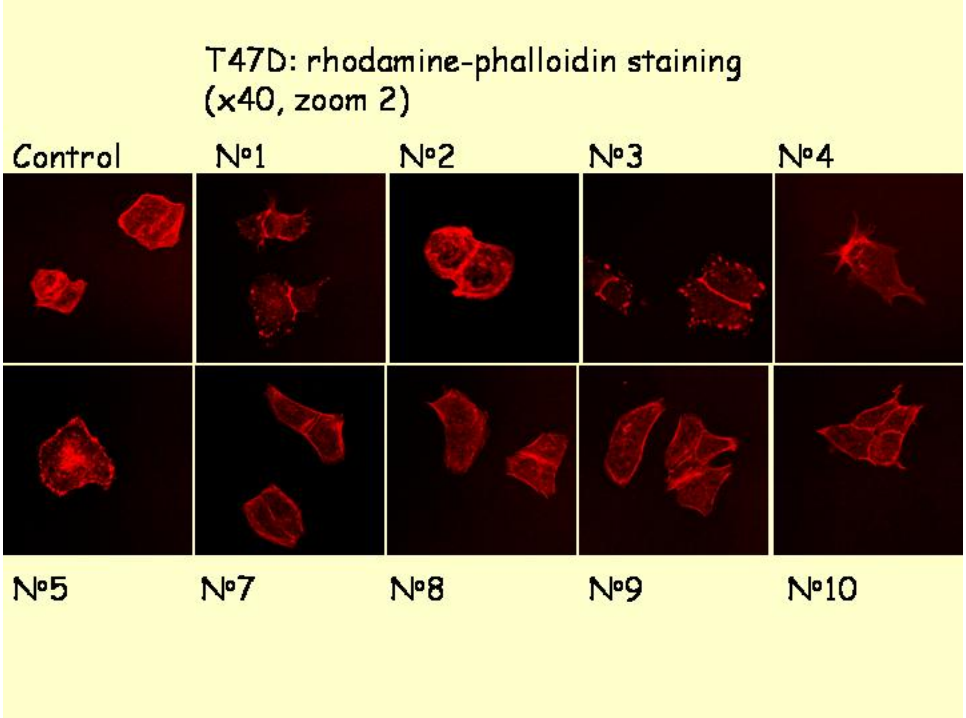
The design of this experiment (PMA stimulation) and its interpretation is different than the H_2O_2 protection experiment: the former represent the ability of the fractions to modify cell metabolism in order to decrease long- or short-term ROS production; the latter shows the potential of each fraction to scavenge the existing ROS in the cell.

Modification of actin cytoskeleton

A recently reported effect of catechin/epicatechin (Nifli AP, Bosson-Kouamé A., Papadopoulou N, Kogia C, Kampa M, Castagnino C, Stournaras C, Vercauteren J, Castanas E. Monomeric and oligomeric flavanols are agonists of membrane androgen receptors. *Exp. Cell Research*, 2005, 329-339) is that monomeric and especially dimeric conjugates (OPC) are able to bind to cell membranes and induce rapid actin cytoskeleton rearrangement. In order to verify this possible effect, we have incubated cells with extracts (10 μg/ml) for 1 hour, and observed the induced modifications in actin cytoskeleton using confocal microscopy. Sephadex blank fraction (extract #6) was used

as negative control for the subsequent LH20 fractions. As expected its addition had no impact on actin rearrangement. Results are presented in the following Figure:





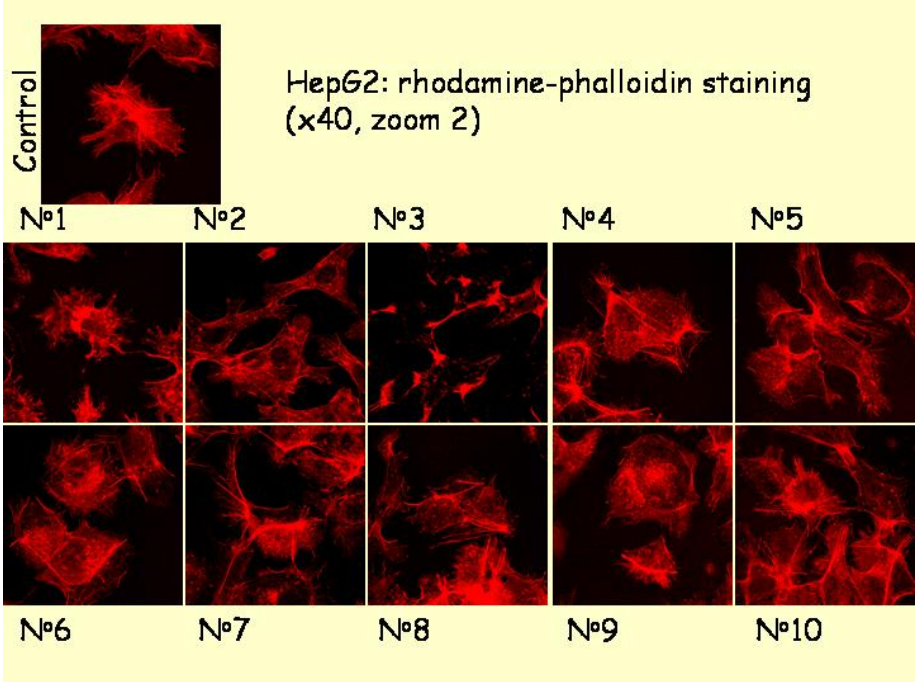


Figure 5: Modification of the actin cytoskeleton of DU-145, T47D and HepG2 cells, after one hour incubation with the different extracts. Filamentous actin was stained with rhodamine-falloidin.

As shown, in DU-145 cells, extracts 1,3 and 5 induced significant changes on actin cytoskeleton, with a peripheral bundling of actin, and a decreased or completely abolished intracellular actin network. Sephadex LH20 fractions induced minor peripheral actin redistribution, especially filipodia formation.

In breast cancer T47D cells, extracts 1 and 3 exerted an extended actin depolymerization, while they induced focal peripheral actin polymerization. Moreover, extracts 1, 3 and to a lesser extend extract 5 induced the formation of lamellipodia and intercellular actin scaffold. In addition, when extract 4 was applied on breast cancer cells, the formation of filopodia-like structures was prominent. In contrast, Sephadex fractions induced minor cytoskeletal changes: no specific structures were observed, while there was a intercellular redistribution of actin. This effect was minimal, and non-compatible with the OPC concentration of the different fractions.

Finally, in hepatocellular carcinoma HepG2 cells, the major effect was obtained by fraction #3, which induced a complete peripheral redistribution and condensation of

polymerized actin with filopodia formation. In addition, fractions 1 and 4, and to a lesser extend #5 induced a lamellipodia-like redistribution of actin. Sephadex fractions 7 and 8, and to a lesser degree #10 equally induced lamellipodia formation. However, their effect was weaker, compared to the one exerted from crude fractions.

The above results indicate that:

1. Actin cytoskeleton changes is a more sensitive method in assaying the cell initial actions of phenolics.
2. Sephadex LH20 might not contain the reported concentration of monomeric (catechin/epicatechin) or dimerized flavanols, as their primary effect (on a ponderal basis) on actin polymerization and redistribution state is not observed in the three studied cell lines.
3. The effect of the crude extracts is more prominent in T47D breast cancer than on DU-145 or HepG2 cells, indicating probably a differential biological effect of the extracts.

Our results indicate that perhaps, in view of an industrial exploitation of vine pomace extracts, an ethyl acetate/ethanol extraction method might be a more interesting one, as compared to a further fractionation or other manipulation of the product.

In vivo results

After the decision taken by the project steering committee, one of the extracts was microencapsulated and indroduced in a commercial fruit juice. This additioned juice, after a written and signed informed concent, was administered in nine (9) healthy volunteers, (5 male, 4 female) aged 25-28+54y. Half-a-liter of juice was intaken per day. The life-style and habbits of the individuals was not modified during the study.

Blood was withdrawn at the onset, 1 and 2 weeks after. In the serum, a number of biochemical parameters was measured. The antioxidant capacity of serum was measured by an automated kit (TAC assay, Medicon Hellas, Gerakas, GR). Biochemical parameters were measured by reagents from Olympus. Corrected TAC (cTAC) was estimated by

substracting the interference of urate, albumin and billirubin from TAC, according to Kampa et al (BMC C Chemical Pathology 2: 3, 2002) and Malliaraki et al (BMC Nephrology 4:4, 2003). TOC was assayed by a modification of the method by Tatzber et al (Anal. Biochem. 316, 147-153, 2003).

Results

Biochemical parameters

Biochemical parameters did not show any significant variation, as presented in the following table. The slight lipid changes were attributed to the diet followed, and was not correlated with the intake of the juice. In this respect, we have concluded that formulated juice intake does not modify, in any measure the biochemical profile in serum.

	tot Bilir mg/dl	conj Bil	non-conj Bil	tot Chol	HDL	TG	LDL	Alb	urate	Fe
1.1	0.68	0.12	0.56	189	46	55	132	4.6	6.6	66
1.2	0.89	0.14	0.75	193	48	92	127	4.8	6.4	108
1.3	0.68	0.11	0.57	170	46	136	97	6.1	7.2	
2.1	0.43	0.08	0.35	152	50	96	83	4.4	4.3	61
2.2	0.58	0.11	0.47	170	60	143	81	4.3	4.4	119
2.3	0.75	0.14	0.61	174	61	76	98	4.4	4.8	145
3.1	0.57	0.08	0.49	160	57	52	93	4.8	3.8	103
3.2	0.65	0.11	0.54	152	57	43	86	4.7	4.4	60
3.3	0.87	0.18	0.69	142	56	45	77	4.6	5.1	
4.1	0.55	0.07	0.48	264	52	232	166	4.8	5.6	101
4.2	0.57	0.08	0.49	245	48	153	166	4.8	6.5	99
4.3	0.67	0.07	0.6	262	50	181	176	4.6	6	147
5.1	0.62	0.1	0.52	222	67	140	127	4.2	5.3	138
5.2	0.42	0.07	0.35	201	64	128	111	3.9	4.8	76
5.3	0.48	0.08	0.4	216	66	150	120	4	4.7	93
5.1	0.5	0.08	0.42	226	61	84	148	4.6	4.1	109
6.2	0.39	0.07	0.32	225	61	92	146	4.5	4.1	93
6.3	0.45	0.08	0.37	201	54	100	127	4.3	4.4	120
7.1	0.23	0.04	0.19	195	76	161	87	4.2	3.6	81
7.2	0.23	0.04	0.19	227	81	90	128	4	3.3	34
7.3	0.19	0.05	0.14	210	73	68	123	3.7	4.1	53
8.1	0.6	0.1	0.5	184	68	113	93	4.3	2.8	65
8.2	0.41	0.07	0.34	201	71	147	101	4.6	2.3	39
8.3	0.87	0.13	0.74	191	66	73	110	4.4	2.7	141
9.1	0.41	0.07	0.34	176	61	60	103	4.1	3.2	51
9.2	0.32	0.04	0.28	177	57	170	86	4.1	3.1	17
9.3	0.25	0.05	0.2	175	66	47	100	4.1	3.1	35

Plasma redox status

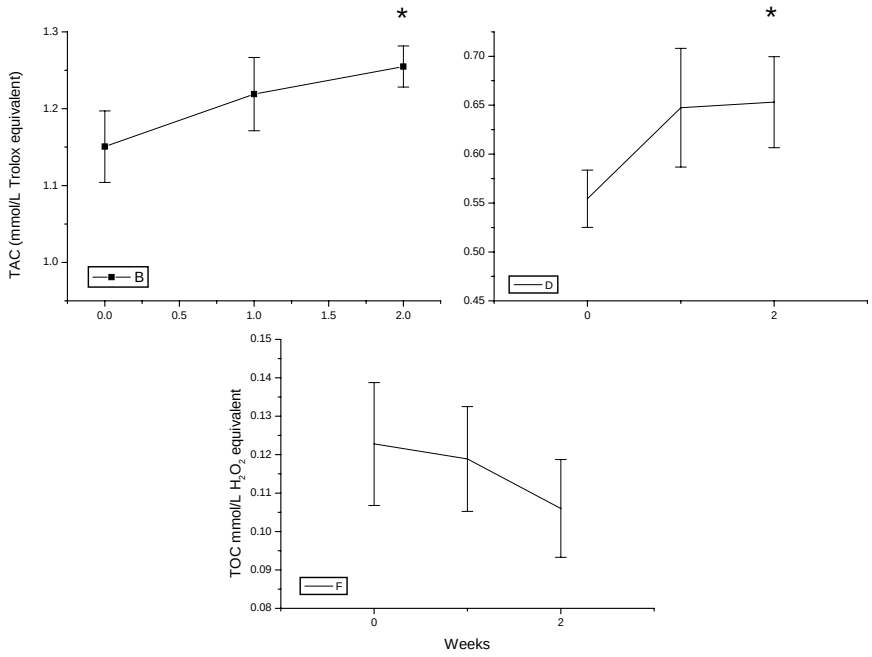
Reactive oxygen species (ROS) are produced as a consequence of normal aerobic metabolism in animal species. These “free radicals” are removed and/or inactivated in vivo by a battery of antioxidants. A biological antioxidant is defined as a substance, which, at low concentrations compared to that of the oxidisable substrate, significantly delays or prevents this oxidation. Individual members of the antioxidant defense team are employed to prevent the generation of free ROS, to destroy potential antioxidants and to scavenge ROS. However, the relative sufficiency of the organism antioxidant defenses is critical in the development of oxidative stress in patients affected by a number of diseases, including infections, neurodegeneration, diabetes, angina, certain forms of cancer and ageing. These diseases are characterized by an overproduction of free radicals, i.e. when the antioxidant defense of an organism is overwhelmed or are established when a deficit of defenses of the organism against oxidation occurs.

The primary defense against oxidative stress in extracellular fluids results from a number of low molecular weight antioxidant molecules either water- (ex. ascorbic acid) or lipid-soluble (ex. Vitamin E). These antioxidants can also be generated during normal metabolism (ex. uric acid, bilirubin, albumin, thiols) or introduced in the body by the consumption of dietary products rich in antioxidants (olive oil, fruits and vegetables, tea, wine, etc). The sum of endogenous and food-derived antioxidants represents the **total antioxidant capacity** of the extracellular fluid, while the sum of all ROS and related substances circulating at a given moment represent the **total oxidant capacity** in circulating fluids. In addition, the levels of these antioxidants are suitable not only as a protection against oxidation, but could also reflect their consumption during acute oxidative stress states. The cooperation among different antioxidants provides a greater protection against attack by reactive oxygen or nitrogen radicals, than any single compound alone. Thus, the overall antioxidant capacity may give more relevant biological information compared to that obtained by the measurement of individual parameters, as it considers the cumulative effect of all antioxidants present in plasma and body fluids. Nevertheless, due to the participation of diverse metabolites to the

antioxidant capacity of human plasma, its increase may not be necessarily a desirable condition. Indeed, in some cases, such as renal failure (uric acid), icteric status (bilirubin), hepatic damage (hypoalbuminemia) the increase or decrease of several metabolites falsely modifies the plasma antioxidant capacity, a situation returning to normal values after correction of the underlying disease.

In order to palliate to this draw-back, we have recently introduced the concept of the **corrected Total Antioxidant Capacity.** We have assayed the participation of each major metabolite on the antioxidant capacity, measuring their antioxidant effect of single substances and in serum. We have found that uric acid, bilirubin, lipoproteins and albumin accounted for 0.11 mmol/mg, 0.14 mmol/mg, 0.18 and 0.01 mmol/100 mg respectively. Taking into account the normal concentrations of these analytes, it was concluded that about 1 mmol/L (i.e.about 85% of the TAC) is due to endogenous analytes, and only 15% of the observed TAC might be due to exogenously provided antioxidants. This corrected value, representing food-derived antioxidants is the corrected TAC.

In further validation of the assays, we have observed that TAC, as well as cTAC presents slow variations in human plasma, as a chained reaction among all circulating antioxidants in a biological fluid at a given moment, results in a chained reaction, with oxido-reductive reactions, according to their redox potential. In addition, endogenous antioxidant substances buffer and attenuate abrupt changes of antioxidants in the blood. Therefore, TAC, in addition of being a functional indicator of the antioxidant status in a given moment, represents a medium-term integrator of the total status of the organism.



Measurement of the TAC and cTAC, before (0) and during two weeks of formulated juice intake, resulted in a significant enhancement of circulating antioxidants. This is better reflected by cTAC, being an indicator of exogenous antioxidants. In contrast, a significant decrease of TOC was observed, at the same time-interval, confirming the antioxidant capacity of the formulated juice.

We have therefore concluded that a significant improvement of the plasma redox state was obtained in the tested group. As the nutritional status of the individuals was not modified, during the test period, the observed changes were attributed to the intake of the formulated juice. It is to note that the juice itself did not modify significantly the redox status of the subjects.

Biological role of polyphenols

During the second year of the PARADOX proposal, we continued to advance the same scientific questions addressed during the first year. Therefore, the same headings will be used for the advancements we have made during this year.

How could one monitor early antioxidant action in biological fluids?

We have further advanced this question, by measuring the effect of a drastic weight loss in morbidely heavy patients. We have concluded that weight loss significantly improves the antioxidant status of the individuals. Actually, we are exploring different methods used for madically-driven weight reduction, in order to explore the possible benefit of a given treatment.

In addition, we have investigated patients with major vascular problems. Vascular insufficiency of a big vessel results in a decreased blood (and therefore oxygen) supply to the affected member. As a consequence, an increased ROS production occurs, with an antiparallel decrease of the redox state. Initial measurements in the general circulation did not show a significant modification of the redox status. In contrast, measurements in the affected member revealed the expected changes, which return to normal after a successful operation.

Which are the mechanisms of interaction of wine polyphenols with basic cellular mechanisms?

In the current year, we continued to investigate the cellular and molecular effects of polyphenol actions in cells. In a first step, we heve found that selective flavonoids (quercetin) is very quickly internalized in cells, enters the nucleus, and concentrate tho

the nucleolus. There, it induces specific enzymatic modifications, which, at a later stage, influence the transcription of specific genes, and the modulation of cell fate, towards surcival or apoptosis. This is actually investigated by gene-chip analysis.

In addition, we have found that incubation of immune cells with polyphenols results in a specific modulation of cell activation, with changes of selective cytokines, and a decrease of the in vitro inflammatory response. This action of polyphenols could be of interest in the case of PARADOX, as the specific actions of the isolated substances could provide, in addition to the improvement of the redox state, an anti-inflamatory action, beneficial action in cases of immune-related inflammatory diseases.

Finally, we have found that OPC administration (in which the PARADOX-derived products are very rich) have an anti-tumor activity in human tumor-bearing mice. This could also provide a supplementary health indication of the PARAFDOX-derived products.

Scientific production of the group in relation to the PARADOX project (papers in bold acknowledge the financial support of PARADOX)

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3. Koutroubakis I.E., Malliaraki N., Dimoulios P., Karmiris K., Castanas E., Kouroumalis E. Decreased total and corrected antioxidant capacity in patients with inflammatory bowel disease. Dig. Dis. Sci. 49, 1433-1437, 2004

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Total Scientific production of the group (2004-2006)

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EVALUATION OF INITIAL EXTRACTS OF RED WINE POMACE ON ENDOTHELIN-1 SYNTHESIS BY CULTURED ENDOTHELIAL CELLS

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AIMS OF THESE TESTS:

To compare the relative inhibition of endothelin-1 synthesis by four extracts prepared by CINS for the Paradox project (sample codes G9, G10, G11 and G12). To correlate this biological activity to specific phenolic fractions prepared by Sephadex LH20 chromatography. To provide insight for CINS and GAT on the relative of effectiveness of the different extraction procedures.

METHODS:

Extracts were reconstituted in 75% ethanol containing 5 mM HCl. Samples were resuspended by gentle mixing at room temperature for 10 min. Insoluble material was removed by centrifugation at 3000 rpm for 5 min at 4ºC. Extracts were analysed for total phenolics using Folin and Ciocalteu's reagent with dilutions of (+)-catechin as a reference standard. A representative sample of each extract was fractionated using Sephadex LH20 to estimate the relative amounts of oligomeric procyanidins (acetone eluate).

Experiments to assess the effect of each extract on endothelin-1 synthesis were carried out as previously described using bovine aortic endothelial cells (*Corder et al., Nature 2001; 414: 863-864*). A red wine polyphenol extract used in these earlier studies was included as a reference.

After an initial set of experiments to determine which extract was the most potent a further set of experiments was undertaken to compare the biological activity of this extract (G11) with fractions prepared by Sephadex LH20 chromatography of low molecular weight phenolics (methanol eluate) and oligomeric procyanidins (acetone extract).

RESULTS:

Analysis of the samples by measurement of total phenolics indicated a low level of purity (<0.2 mg phenolics/mg extract). (See Table of Results below). The acetone eluates from Sephadex LH20 fractionation showed that of the total phenolics in each extract approximately 60% were oligomeric procyanidins).

TABLE OF RESULTS FOR SEPHADEX LH20 ANALYSIS OF EXTRACTS (CATECHIN EQUIVALENTS µG/MG EXTRACT)

Sample:	G9	G10	G11	G12
IC_{50} µg/ml	19.6	13.7	12.5	17.0
Total phenolics	131.5	189.3	195.0	153.0
Unadsorbed phenolics	8.9	15.6	13.4	16.2
Methanol Eluate	28.2	44.7	39.2	45.7
Acetone Eluate	80.4	110.6	117.8	81.1
LH20 recovered phenolics	117.5	170.7	170.4	142.9

Figure 1 (below) shows the relative potency of the four extracts compared to a red wine polyphenol extract.

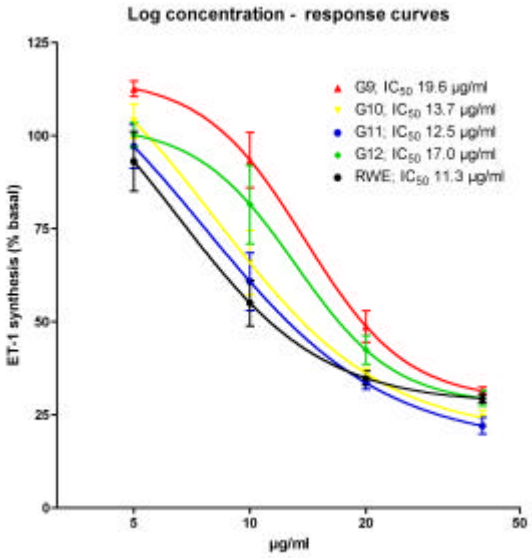

Figure 1 – Concentration dependent inhibition of endothelin-1 synthesis by red wine pomace extracts and a representative red wine polyphenol extract.

Correlation of the different phenolic fractions (Table of Results) with the biological activity of each extract gave the following:

Total phenolics $r^2 = 0.99$, P = 0.004
Unadsorbed phenolics $r^2 = 0.33$, P = 0.428
Methanol eluate $r^2 = 0.34$, P = 0.414
Acetone eluate $r^2 = 0.90$, P = 0.05

These results indicate that the inhibition of ET-1 synthesis is dependent on the level of oligomeric procyanidins in each extract. This was confirmed by comparing acetone and methanol eluates prepared from G11 using Sephadex LH20 with the crude extract (Figure 2).

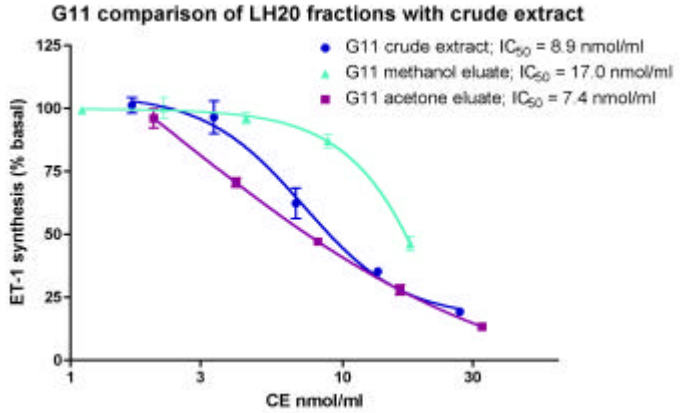

Figure 2 – Comparison of the inhibitory effect of G11 with methanol and acetone fractions prepared from G11 using Sephadex LH20

CONCLUSIONS: These initial extracts had a fairly low level of purity based on the high level of insoluble particulate material and low percentage of total phenolics per extract. Consistent with previous work biological activity was correlated with the oligomeric procyanidin content of each extract.

EVALUATION OF NEW EXTRACT OF RED WINE POMACE ON ENDOTHELIN-1 SYNTHESIS BY CULTURED ENDOTHELIAL CELLS: COMPARISON WITH COMMERCIAL GRAPE SEED EXTRACTS

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AIMS OF THESE TESTS:

To compare the relative inhibition of endothelin-1 synthesis by the new Paradox Project extract (delivered 16-5-05) and two samples of commercial grape seed extract (one provide by GAT, the other from Polyphenolics, California, USA). To correlate this biological activity to specific phenolic fractions prepared by Sephadex LH20 chromatography.

METHODS:

Extracts were reconstituted in 20% ethanol containing 10 mM HCl. Extracts were analysed for total phenolics using Folin and Ciocalteu's reagent with dilutions of (+)-catechin as a reference standard. A sample of each extract was fractionated using Sephadex LH20 to estimate the relative amounts of oligomeric procyanidins (acetone eluate). Results for unadsorbed phenolics, low molecular weight phenolics (methanol eluate), and oligomeric procyanidins (acetone extract) are shown below.

Experiments to assess the effect of each extract on endothelin-1 synthesis were carried out as previously described using bovine aortic endothelial cells (*Corder et al., Nature 2001; 414: 863-864*).

RESULTS:

Analysis of the Paradox Extract by measurement of total phenolics and biological activity showed a lower level of purity than the samples of grape seed extract. (See Table of Results and Figures below).

TABLE OF RESULTS FOR SEPHADEX LH20 ANALYSIS OF EXTRACTS (CATECHIN EQUIVALENTS µG/MG EXTRACT)

Sample:	Paradox Extract	GAT GSE	Polyphenolics GSE
IC_{50} µg/ml	15.0	3.4	3.9
Total phenolics	401.0	663.0	644.0
Unadsorbed phenolics	15.1	11.9	19.6
Methanol Eluate	244.8	308.6	333.7
Acetone Eluate	150.5	369.9	294.0
LH20 recovered phenolics	410.0	690.4	647.3

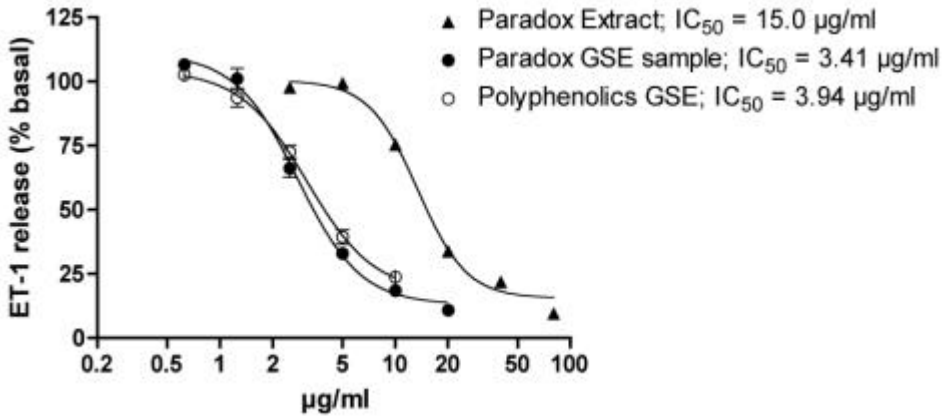

Figure 1 – Concentration dependent inhibition of endothelin-1 synthesis by red wine pomace extract: comparison with commercial grape seed extracts.

These results show the two commercial extracts to be approximately 4 fold more potent. However if the results are expressed relative to total phenolics as catechin equivalents (Figure 2), or acetone eluted oligomeric procyanidins (Figure 3), then the grape seed extracts are only 2.5 and 1.8 fold more potent respectively.

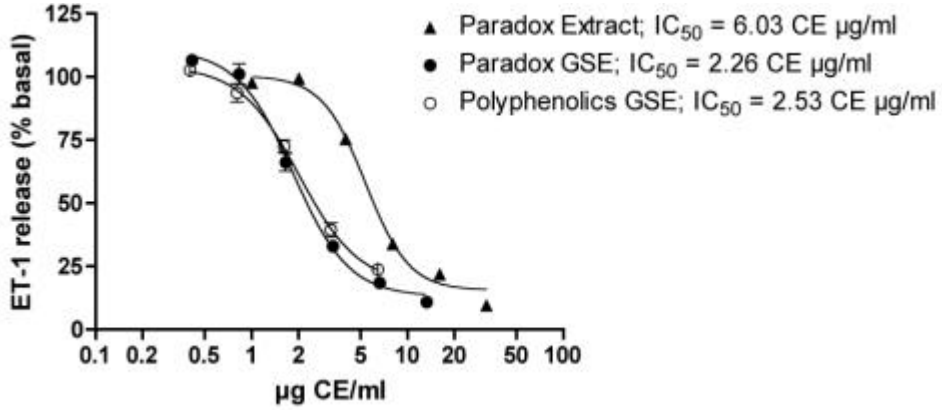

Figure 2 – Comparison of Paradox Extract with grape seed extracts expressed relative to total phenolics (CE µg/ml)

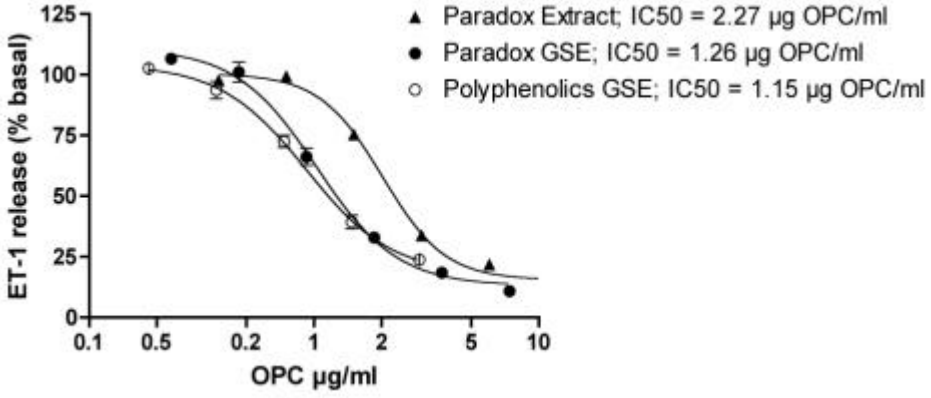

Figure 3 – Comparison of Paradox Extract with grape seed extracts expressed relative to the amount of oligomeric procyanidins (µg/ml)

CONCLUSIONS: The red wine pomace extract tested in these studies is still relatively impure compared to commercial grape seed extracts. It seems unlikely that this is of sufficient purity to undertake clinical studies.

EVALUATION OF NEW EXTRACTS OF RED WINE POMACE ON ENDOTHELIN-1 SYNTHESIS BY CULTURED ENDOTHELIAL CELLS

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AIMS OF THESE TESTS:

1. To compare the relative inhibition of endothelin-1 synthesis by the new Paradox Project samples numbered 1 – 10 (delivered 16-11-05).
2. To analyse the samples for total phenolics using the Folin and Ciocalteu's method.

DESCRIPTION OF SAMPLES

#1 Crude extract ethanol 80 % / water 20%;
concentration by weight-%: 5 mg/mL volume: 3 mL

#2 Crude extract acetone 90% / water 10%
concentration by weight-%: 6.9 mg/mL volume: 1 mL

#3 Crude extract ethyl acetate 90% / water 10%
concentration by weight-%: 3.9 mg/mL volume: 9 mL

#4 Hexane precipitated ethyl acetate 90% / water 10% extract ("polyphenol extract")
concentration by weight-%: 23.2 mg/mL volume: 0.2 mL

#5 CO_2 precipitated "Polyphenol extract" weight: 100mg

#6 Blank Sephadex fraction 5
concentration by weight-%: 0.35 mg/mL volume: 7 mL

#7 Sephadex fraction 6 (epicatechin fraction - approx. 90%)
concentration by weight-%: 0.25 mg/mL volume: 4 mL

#8 Sephadex fraction 8 (catechin & epicatechin fraction)
concentration by weight-%: 0.88 mg/mL volume: 3mL

#9 Sephadex fraction 9 (procyanidins & catechin – approx. 3:1; not confirmed by HPLC-NP)
conc. by weight-%: 0.48 mg/mL volume: 2 mL

#10 Sephadex fraction 10 (procyanidins – approx. >80%, not confirmed with HPLC-NP)
conc. by weight-%: 0.25 mg/mL volume: 8 mL

METHODS:

Aliquots of each sample (#1 - #4 and #6 - #10) were dried down under nitrogen to remove solvents and then reconstituted in 20% ethanol containing 10 mM HCl for testing on bovine aortic endothelial cells. A portion of sample 5 was weighed and reconstituted in the same way ready for testing on endothelial cells.

The reconstituted samples were analysed for total phenolics using Folin and Ciocalteu's reagent with dilutions of (+)-catechin as a reference standard.

Experiments to assess the effect of each extract on endothelin-1 synthesis were carried out as previously described using bovine aortic endothelial cells (*Corder et al., Nature 2001; 414: 863-864*).

Because of its very high potency in cell experiments sample 2 was subjected to further analysis by Sephadex LH20 chromatography and evaluation of biological activity of the fractionated material.

RESULTS:

Analysis of samples for total phenolics gave the following results.

Total Phenolics

Sample	**Nominal Value**	**Folin's CE mg/ml or mg/mg**
#1	5 mg/ml	0.95 mg/ml
#2	6.9 mg/ml	22.13 mg/ml
#3	3.9 mg/ml	0.82 mg/ml
#4	23.2 mg/ml	14.44 mg/ml
#5	Powder	0.41 mg/mg
#6	0.35 mg/ml	<0.01 mg/ml
#7	0.25 mg/ml	0.13 mg/ml
#8	0.88 mg/ml	0.41 mg/ml
#9	0.48 mg/ml	0.21 mg/ml
#10	0.25 mg/ml	0.12 mg/ml

It should be noted that sample 2 may have become more concentrated through loss of acetone on storage or during transport as it had approximately three times the anticipated amount of phenolics. Sample 5 was difficult to dissolve under the conditions used. It is only approximately 41% phenolics, which is comparable with our last report where a value of 40.1% was observed compared to the grape seed extract samples, which exceed 60%.

Results from evaluation of these extracts are shown below in Figures 1 and 2. In Figure 1 the results are expressed relative to the nominal values for each sample provided by GAT. In Figure 2 corrected values are used based on measurements of total phenolics so that concentrations of each extract are corrected for polyphenol content. Although this correction allows comparison of the activity of the polyphenol constituents as catechin equivalents, it does not provide any insights into the relative purity of the samples.

Samples 1, 2 and 5 had approximately similar potency when expressed as catechin equivalents. Sample 3 prepared with ethyl acetate appears to be both low purity and mainly polyphenols with low biological activity. Even when expressed as catechin equivalents, sample 4 was lower potency than samples 1, 2 and 5. This indicates ethyl acetate is not a good solvent for extracting biologically active phenolics from red wine pomace.

Samples 6 – 10 had no biological activity in the concentrations tested (maximum concentration 20 µg/ml based on dilutions calculated using values provided by GAT). Because a large volume of fraction 10 was provided this was concentrated and re-evaluated (Figure 3).

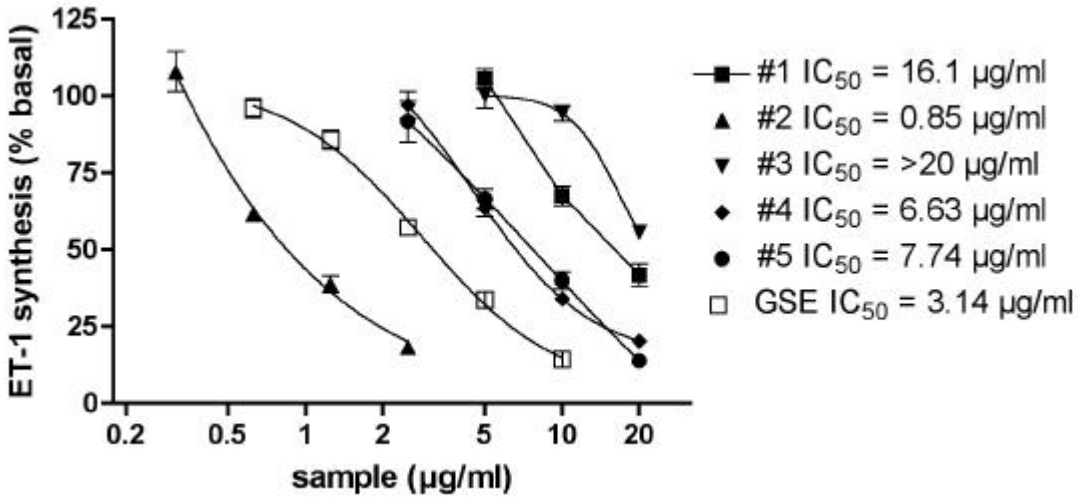

Figure 1 *– Concentration dependent inhibition of endothelin-1 synthesis by red wine pomace extract samples 1 to 5; Polyphenolic's grape seed extract included as a comparison.*

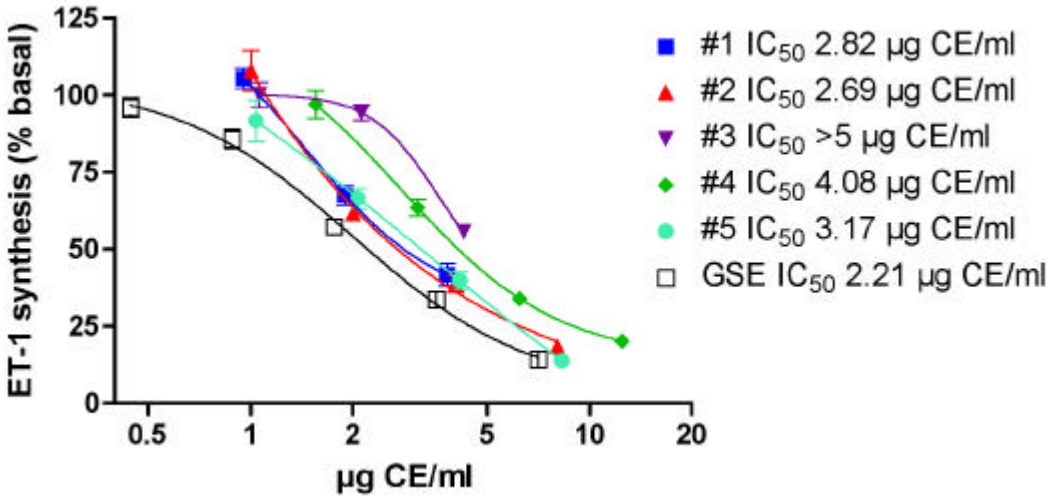

Figure 2 *– Concentration dependent inhibition of endothelin-1 synthesis by red wine pomace extract samples 1 to 5, and Polyphenolic's grape seed extract; concentrations of each sample expressed as catechin equivalents µg/ml.*

The results for sample 2 shown in Figure 1 and Figure 2 indicate that this acetone extract has the highest relative amounts of biologically active polyphenols. Therefore we subjected this sample to analysis on Sephadex LH20 to quantify the proportion of low molecular weight phenolics (methanol eluate) and oligomeric procyanidins (acetone eluate). These sub-fractions were also re-tested for biological activity.

Previous analyses of the red wine pomace extract has shown that approximately 60% of the polyphenol content is low molecular weight phenolics and 37% are oligomeric procyanidins. In comparison grape seed extracts typically contained 45 – 52% low molecular weight phenolics and 45 – 54% oligomeric procyanidins. Analysis of sample 2 showed approximately 34% low

molecular weight phenolics and 65% oligomeric procyanidins. Which is in agreement with the profile of potent biological activity of this sample.

Results for sample 10 are also included in Figure 3. This shows only low potency, but Sephadex LH20 analysis revealed sample 10 contained <30% oligomeric procyanidins.

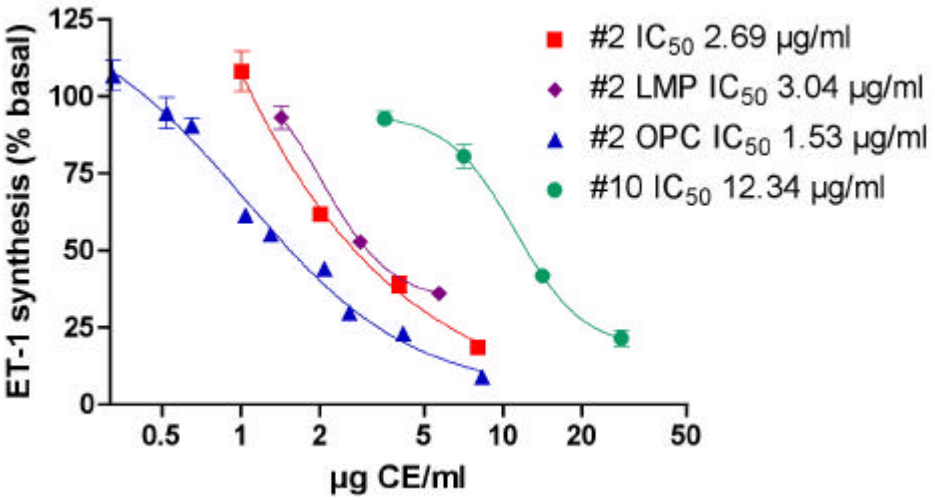

Figure 3 *– Concentration dependent inhibition of endothelin-1 synthesis by red wine pomace extract sample 2 and the fractionated low molecular weight phenolics and oligomeric procyanidins. Results from a concentrated preparation of sample 10 are also included.*

CONCLUSIONS: By weight the CO_2 precipitated polyphenol extract (#5) is approximately half the potency of commercial grape seed extract. Acetone extraction of red wine pomace may yield the highest relative amounts of oligomeric procyanidins.

EVALUATION OF THE RED WINE POMACE EXTRACT USED FOR VOLUNTEER STUDIES: EFFECT ON ENDOTHELIN-1 SYNTHESIS BY CULTURED ENDOTHELIAL CELLS

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AIMS OF THESE TESTS:

1. To assess the relative potency of the Red Wine Pomace Extract (RWPE) prepared for volunteer studies on inhibition of endothelin-1 synthesis by cultured endothelial cells.
2. To analyse this extract for total phenolics using Folin and Ciocalteu's method, and after Sephadex LH20 chromatography.
3. To determine whether chromatography on Sephadex LH20 can improve the potency of the RWPE.
4. Additional studies included testing how Dowex treatment affected the phenolic components of extracts assessed after Sephadex LH20 chromatography.

METHODS:

The RWPE was dissolved in 5 mM HCl containg 40% ethanol. This reconstituted sample was used for all subsequent analyses.

The reconstituted RWPE was analysed for total phenolics using Folin and Ciocalteu's reagent with dilutions of (+)-catechin as a reference standard. To provide information on the purity of the extract further analysis was undertaken by using Sephadex LH20 chromatography. Phenolics were assayed on the 60% methanol eluate (low molecular weight phenolics) and 70% acetone eluate (oligomeric procyanidins), and these fractions were subjected to testing on endothelial cells to determine biological activity. A sample of total phenolics extracted on Sephadex LH20 was also prepared by elution with 70% acetone after omitting the 60% methanol elution step.

Experiments to assess the effect of each extract on endothelin-1 synthesis were carried out as previously described using bovine aortic endothelial cells (*Corder et al., Nature 2001; 414: 863-864*).

RESULTS:

Phenols assay of the RWPE gave the following results:

	Catechin equivalents (µg/mg extract)
Total phenolics for RWPE	334
LH20 methanol eluate (low molecular weight phenolics)	97
LH20 acetone eluate (oligomeric procyanidins)	223
LH20 pooled eluate (total extractable phenolics)	292

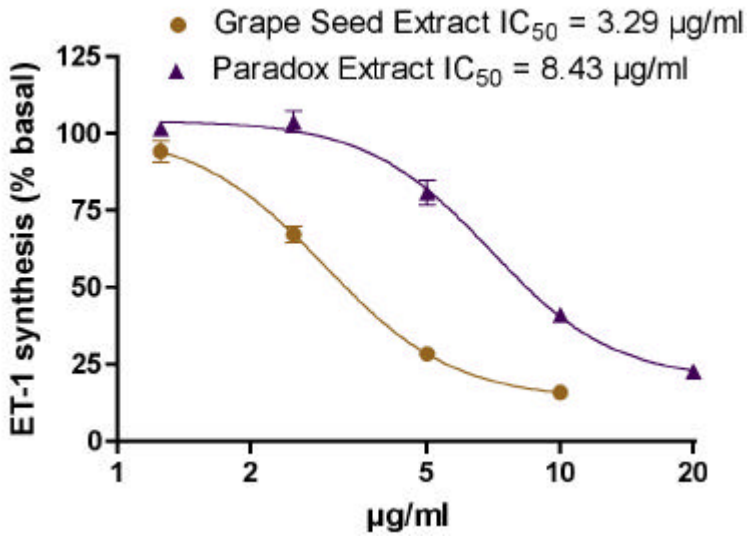

Figure 1 *– Concentration dependent inhibition of endothelin-1 synthesis by red wine pomace extract compared with grape seed extract from Polyphenolics.*

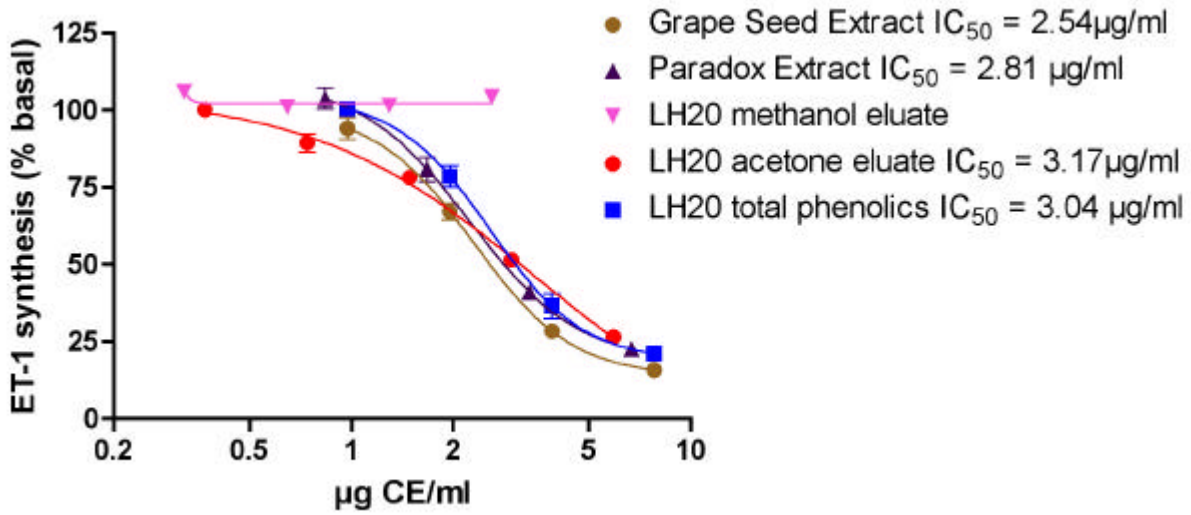

Figure 2 *– Concentration dependent inhibition of endothelin-1 synthesis by red wine pomace extract, LH20 fractionated red wine pomace extracts, and grape seed extract; concentrations of each sample are expressed as catechin equivalents µg/ml.*

The results of these studies show that this RWPE is comparable in potency with the CO_2 precipitated extract evaluated in November 2005. The phenolic components of this extract are approximately 68% oligomeric procyanidins and 29% low molecular phenolics. This is consistent with the increased potency of this extract compared to early preparations (e.g. May 2005), which showed IC_{50} values of around 15 µg/ml and procyanidin content of <40%. However, the proportion of this extract that is composed of phenolics is only 334 µg/mg, which indicates a substantial non-phenolic component. Elimination of these non-phenolic components could substantially improve the relative potency of the RWPE.

Chromatography on Sephadex LH20 showed there was no significant change in potency of the RWPE expressed as µg CE/ml even though the low molecular weight phenolics (methanol

eluate) showed no biological activity in the concentrations tested. Therefore the key to further improvements in the quality of the RWPE should focus mainly on eliminating non-phenolic components.

Therefore we evaluated how treatment of extracts with Dowex cation-exchange resin altered the phenolic components of ethanolic extracts (samples provided by GAT in November 2005). This showed that expressed as catechin equivalents Dowex-treatment reduced the level of phenolics in the crude extract (-17.6 ± 2.8%). This was mainly related to lower levels of low molecular weight phenolics (-22.3 ± 3.4%), rather than the procyanidin-content of the extract (-14.3 ± 7.1%; N.B. mean reduction only -5.1 ± 3.8% if 10% ethanol extracts are excluded)

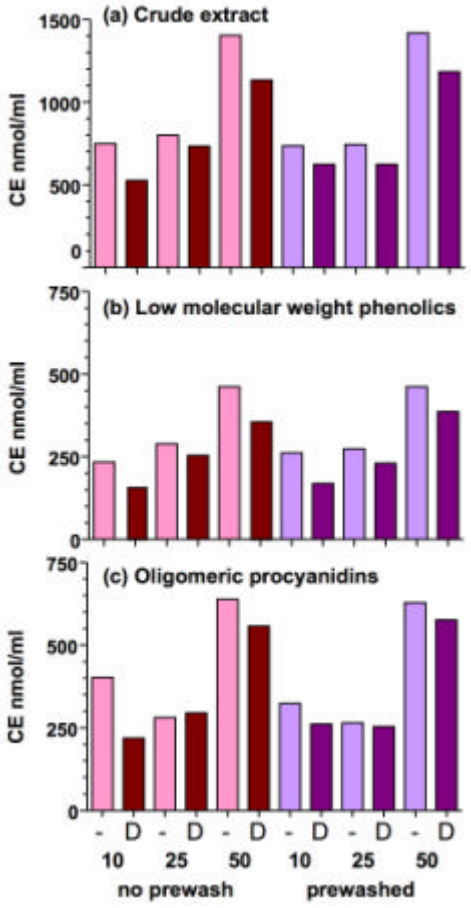

Figure 3 *– Measurement of phenols with Folin and Ciocalteu's reagent in crude extracts and after Sephadex LH20 chromatography. D = Dowex treatment; 10, 25, 50 = % ethanol used to prepare each extract. For the prewashed samples wine pomace was rinsed with 10mM citric acid prior to ethanolic extraction.*

CONCLUSIONS: By weight the CO_2 precipitated acetone extracted Red Wine Pomace Extract has ˜50% activity of commercial grape seed extract. The main difference appears to be the level of non-phenolic constituents.

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PARADOX report (8 Dec 2005)

AIMS OF THE TESTS:

To compare the effects of the ten extracts received by GAT on cell growth, both in normal or high glucose, and to compare their possible antioxidant effect.

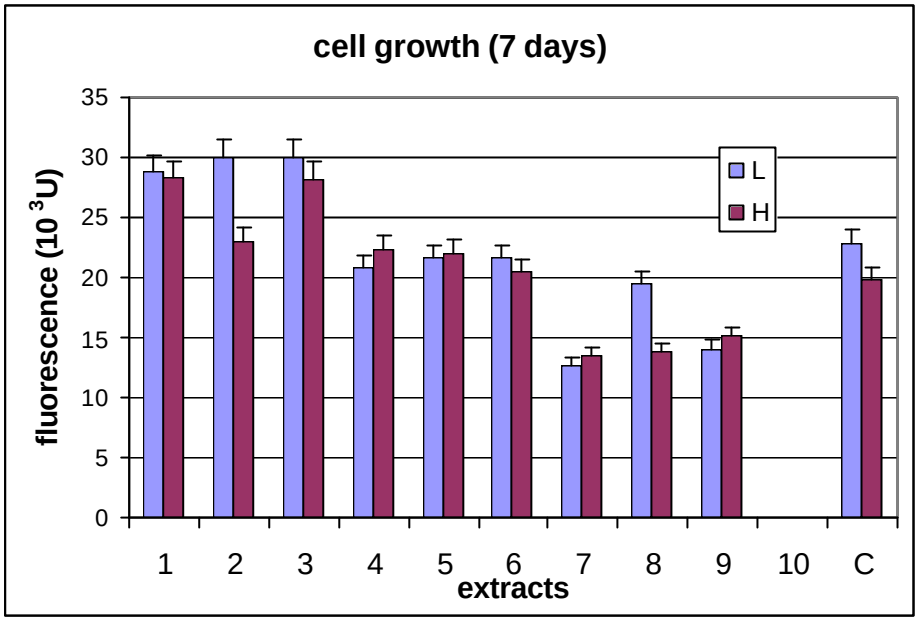
METHODS:

HUVECs were isolated and pooled from umbilical cords obtained from normal vaginal deliveries by the procedure described by Jaffe et al. The cells were cultured in gelatin-coated 60 mm Petri dishes (Sarstedt, Nümbrecht, Germany) and grown in medium 199 (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 2mM gluthamine (GIBCO), 20% heat inactivated fetal bovine serum (GIBCO), 25 µg/ml endothelial cell growth supplement, 90 µg/ml heparin (GIBCO), and 0.25 µg/ml fungizone (GIBCO). The Petri dishes were incubated at 37°C, in 5% CO_2 –95% air gas mixture. HUVECs were seeded at equal density (1.3×10^5) in gelatin coated 60mm Petri dishes and allowed to attach overnight. Then they were exposed to the experimental conditions for 7 days, receiving fresh media every 24 h. Following Paolo Grassi's short report on the samples, media were prepared adding separately 10 µg/ml of the extracts to the standard 5mM glucose medium and to the 30 mM high glucose medium.
After 7 days of treatment, experiments were assessed to determine effects of the extracts on cell growth and reactive oxigen species (ROS) production.

Cell growth was determined with Cell Titer Blue commercial kit (Promega), ROS production was determined with the fluorescent probe carboxy-H_2DCFDA, as previously reported (Wang and coll. *Free Radical Biology and Medicine*, 27, 612-616, 1999).

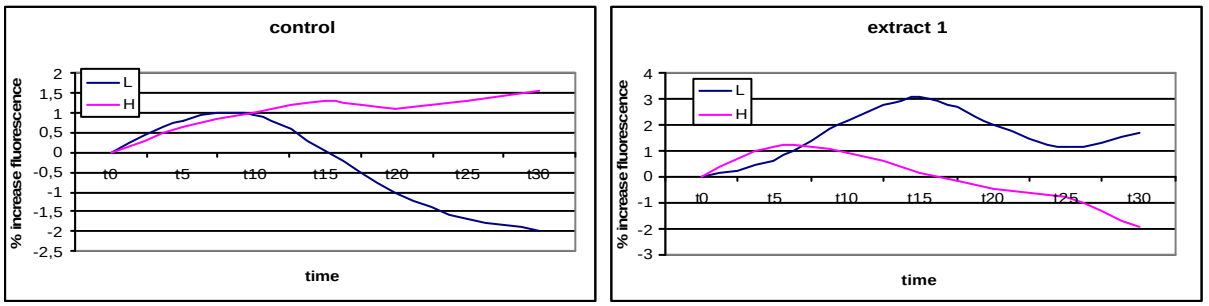
RESULTS:

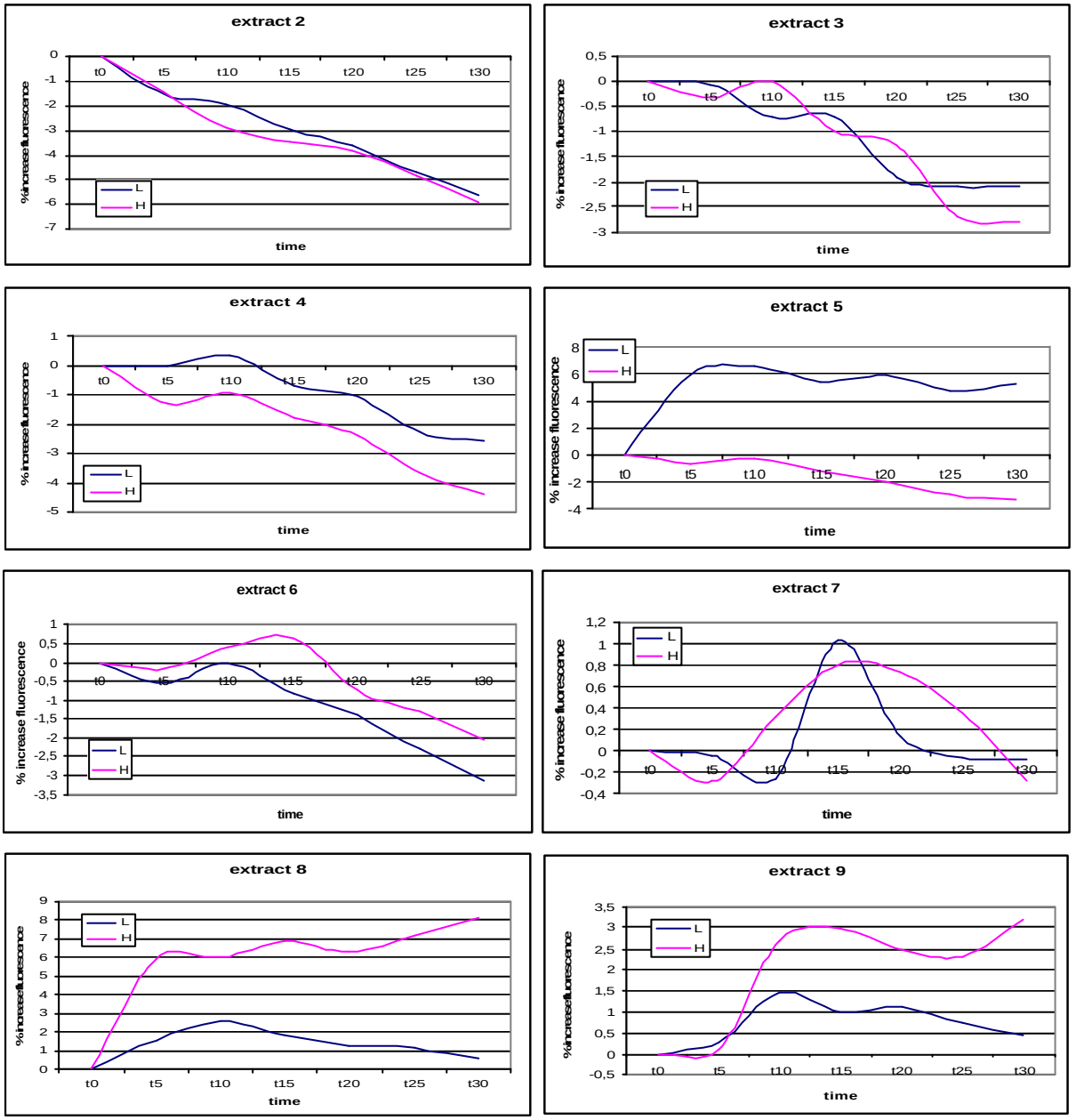
Cell Titer Blue



As previously reported in the literature, cells of the control group treated with high glucose showed a growth delay compared with the normal glucose condition. The extract treated cells could be branched in different groups with similar behaviour. Extracts 1 and 3 seem to be effective in cell protection against high glucose, however, they seem to have a stimulating effects on their growth. Extract 2 seems to have stimulating effects, but lacks the protective effect. Extracts from 4 to 6 have a protective effect, while extracts 7, 9 seem to be harmful for HUVEC. Extract 8 doesn't have any protective effect and seems to inhibit cell growth. It was not possible to terminate the experiment with extract 10 because after 3 days of cell culture the extract determined cell detachment and death.

carboxy-H_2DCFDA





As previously reported, the high glucose condition enhances ROS production inside the cell.

The extracts treated cells, like for the former Cell titer blue test, showed different behaviour.

Extracts 2, 3, 4 and partially 6 showed a high antioxidant effect, inhibiting ROS formation in the high as well as in the low glucose condition. Extracts 1 and 5 lowered ROS formation in the high glucose condition, while it was augmented in the low glucose one. Extracts 7, 8 and 9 don't seem to have any antioxidant effect.

CONCLUSIONS:

Extracts 1 seemed to stimulate cell growth, and seemed to have a protective effect on high glucose exposed cell, where there is a decrease in ROS production. Even extract 5 acts in a similar way, lacking the stimulatory effect on HUVEC growth rate. However, it is difficult to explain the action of both extracts in normal glucose, where ROS production resulted in an increase.

Extract 2 has a stimulatory effect on cell growth and seems to abolish ROS generation, but the growth gap between high glucose and normal glucose treated cells is evident after 7 days of culture. Cells were "blebbing" and even if the growth rate was not reduced, the monolayer has a clear suffering aspect, in both normal and high glucose treated cells.

Extracts 3, 4 and 6 had a good antioxidant effect, seemed to have protective action and, except for extract 4, didn't show stimulatory effect on cell growth.

Extracts 7, 8 and 9 didn't have any effects as antioxidant, moreover they seem to have a detrimental effect on HUVEC growth, as number of cell diminished during the experiment.

Extract 10 caused cell detachment and death.

Project no. : COOP-CT-2003-508649

Acronym : PARADOX

Project title : French Paradox
Red wine extract food additives

Instrument : Special Research Project for SME`s
Cooperative Research (CRAFT)

Deliverable No : D6

Deliverable Title : Process Development

Due date of deliverable : January 2006

Actual submission date : February 2006

Start date of project : 15.02.2004 Duration : 24 months

Organisation name of lead contractor for this deliverable :

GAT Formulation GmbH

Project co-founded by the European Commission within the Sixth Framework Programme (2002-2006)		
Dissemination Level		
PU	Public	
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	**X**

Project no. : COOP-CT-2003-508649

Acronym : PARADOX

Project title : French Paradox
Red wine extract food additives

Instrument : Special Research Project for SME`s
Cooperative Research (CRAFT)

Deliverable No : D7

Deliverable Title : Training, Sales Manual

Due date of deliverable : February 2006

Actual submission date : October 2005

Start date of project : 15.02.2004 Duration : 24 months

Organisation name of lead contractor for this deliverable :

GAT Formulation GmbH

Project co-founded by the European Commission within the Sixth Framework Programme (2002-2006)		
Dissemination Level		
PU	Public	
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WP7 has been completed.

Training seminars for technical sales staff of dissemination partners have been held at GAT and at dissemination partners´ locations to provide an overview of the company's objectives as well as gain a deep understanding for the technology and product benefits. Technical sales material, including but not limited to product specifications, material data safety sheets, labeling and nutritional information have been prepared. In addition, marketing material such as product catalogs, presentations, web sites, etc, have been finished and distributed amongst dissemination partners for their sales efforts. The brand name PARADOX has been created and established in all sales and marketing material

Examples of communications material are attached to this report.

[PRODUCT SPECIFICATIONS]

Product Name: PARADOX 41 *(valid for 41-0, 41-1, 41-2)*

1. Product Name and Identity

PARADOX 41 (product type 41-0, 41-1, or 41-2) by GAT Food Essentials;
POLYPHENOLS from RED WINE POMACE source; micro-encapsulated functional food ingredient.

2. Product Description

water-based micro-capsule suspension of red wine pomace extract;
red colored liquid, self-emulsifying and easily miscible in water, taste and smell masked.

3. Product Stability

stable in suspensions at pH ≥ 3.6 ; stable during heat exposure, high pressure homogenization of up to 150 bar, and pasteurization; stable against oxidation and isomerization.

4. Storage and Shelf Life

originally sealed: max. 6 months at 4°C, or 3 months at RT (20°C);
once opened use immediately; product cannot be re-used once opened.

5. Pack Size

single-use bag-in-box (10kg) sterile; [samples: single-use bag (100g), sterile].

6. Product Characteristics

Parameter	Unit	Method of Analysis	Range
pH $_{(20°C)}$	-	GAT-0702-PH	4.0 - 5.5
Particle Size $_{(50\%)}$	µm	GAT-0702-PS	< 5
Total polyphenols (eq. catechin)	g/100g	GAT-0604-AOX-HPLC	4.0 - 5.0
Total proanthocyanidins (eq. procyanidine B2)	g/100g	GAT-0904-PAC-BASM	3.5 - 4.5
Total OPC (oligomeric proanthocyanidins) (eq. catechin)	g/100g	GAT-0904-OPC-VANM	1.8 - 2.2
Palmitic acid	g/100g	GAT-0704-FXO01-GC	2.4 - 3.4
Stearic acid	g/100g	GAT-0704-FXO01-GC	3.2 - 4.2
Oleic acid ω9	g/100g	GAT-0704-FXO01-GC	6.0 - 7.0
Linoleic acid ω6	g/100g	GAT-0704-FXO01-GC	22.0 - 25.0

7. General Guidelines for Use

approximately 0.3% to 0.6% PARADOX 41 in final food product (dosage depends on, and should be adjusted to, typical consumption habits of servings per day and positioning of final food product); please refer to our Web Site for a detailed description of dosage guidelines and a calculation tool.

8. Additional Information

please refer to our Web Site at www.gat-foodessentials.com or contact us at GAT Formulation GmbH for further information and assistance.

[LABELING & NUTRITIONAL INFORMATION]

Product Name: PARADOX 41-0

1. General Information

Product Name / Code	paradox / 41-0
Description	polyphenols from red wine pomace source micro-encapsulated functional food ingredient
Pack Type / Size	bag-in-box / bag: 10kg net weight (61 gram bag); box 41.5x56cm
Origin	AUSTRIA

2. Product Description

Appearance	Water-based micro-capsule suspension
Color	Red colored liquid
Texture	Slightly viscous
Impurities	N/A

3. Storage Conditions and Shelf Life

Storage Location	Fridge
Temperature Range	1°C to 4°C
Humidity	< 70% RH
Maximum Shelf Life	Originally sealed max. 6 months at 4°C
Handling	Handle in accordance to good industrial practices; store in sealed original packaging.

4. General Statement

The product complies with EU Food Laws per November 2004. Quality Control according to GLP; processing according to HACCP protocols and EFSA and FDA general and specific rules for food production, in particular concerning the prevention of microbiological contamination and oxidation of raw and finished material. The product is packaged in a new food-grade plastic bag-in-box; compliance with 2002/72/EC. No radioactive γ-rays treatment has been used for any of the ingredients or final product.

5. GMO-Free Statement

GAT Formulation GmbH hereby confirms that the mentioned product does not contain any genetically modified organisms according to "Regulation (EU) 1829/2003 on genetically modified food and feed" and "Regulation (EU) 1830/2003 concerning the traceability and labeling of genetically modified organisms and amending Directive 2001/18/EC. **This statement is valid for all raw materials, including additives and flavor, used.**

6. GMO Questionnaire

GMO Questionnaire	Yes	No
Is the Product a GMO or does it contain GMOs?	☐	☒
Does the Product contain “Novel Foods” according to regulation (EU) 258/97?	☐	☒
Is product labeling according to Common Position (EU) 1829/2003 and 1830/2003 necessary?	☐	☒
Does the Product contain matter which contains GMO, consists of GMO, or was manufactured using GMO? Excluded are technically not avoidable GMO traces smaller than 0.9%.	☐	☒
Does the Product contain matter of GM-maize or roundup ready soy-fragments?	☐	☒
Does the Product contain matter of other cultivated genetically modified plants (i.e. colza, tomatoes, etc.)?	☐	☒
During production was there any GMO or a derivative of a GMO (i.e. enzymes) used?	☐	☒
Can genetically modified matter be proved in product?	☐	☒

7. Main Ingredients List

Ingredient	Origin	%
Red Wine Pomace Extract	EU	5
Technological additives	EU	10
Water	AUSTRIA	52
Grape Seed oil	EU	33

8. Allergen Information

Ingredients – Food Intolerance (checked if applicable)					
cow's milk protein	☐	peanuts	☐	hydrolyzed plant protein	☐
lactose	☐	peanut oil	☐	textured vegetable protein	☐
egg	☐	sesame	☐	ethanol	☐
soy protein	☐	sesame oil	☐	gelatin	☐
soy oil	☐	glutamate (E620 - E625)	☐	gallate	☐
soy lecithin	☒	sulfite (E220 - E228) > 10 ppm	☐	glutamic acid / glutamate	☐
gluten	☐	benzoic acid (E210 - E213)	☐	fructose	☐
wheat	☐	azo dyes	☐	sucrose	☐
rye	☐	tartrazine (E102)	☐	colorants (E101 - E180)	☐
beef	☐	cinnamon	☐	conservants (E202 - E219)	☐
pork	☐	coriander	☐	antioxidants (E300 - E321)	☐
chicken	☐	celery	☐	hydrocolloids (E400 - E418)	☒
fish	☐	umbelliferae	☐	aspartame (E951)	☐
shellfish and crustaceans	☐	carrot	☐		
maize	☐	lupine	☐		
cocoa	☐	mustard	☐		
yeast	☐	honey	☐		
legumes/pulse	☐	garlic	☐		
nuts	☐	caffeine	☐		
nut oil	☐	products of animal origin	☐		

9. Product Suitability

The Product is Suitable For	**Yes**
Diabetics?	☒
Vegetarians?	☒
Vegans?	☒
Celiacs?	☒
Persons with intolerance to lactose?	☒

10. Nutrition Type Statement

An organic/bio-, kosher-, or halal-statement is available under special request.

11. Nutritional Information

Nutritional Information			
Carbohydrates		**Protein**	
Total Carbohydrates	0.0 g/100g	Total Proteins	0.0 g/100g
Mono/disaccharide	0.0 g/100g	Vegetable Protein	0.0 g/100g
Polysaccharide	0.0 g/100g	Animal Protein	0.0 g/100g
Energy		**Fat**	
Kcal	336.2 Kcal/100g	Total Fat	37.4 g/100g
KJ	1406.9 KJ/100g	Saturated	6.5 g/100g
		Polyunsaturated	24.5 g/100g
Dietary Fiber		Monounsaturated	6.4 g/100g
Total Dietary Fiber	2.3 g/100g		
Minerals		**Essential Amino Acids**	
Sodium	1.6 mg/100g	Lysine	n/a
Potassium	0.1 mg/100g	Methionine	n/a
Magnesium	1.0 mg/100g	Cystine	n/a
Calcium	2.7 mg/100g	Threonin	n/a
Iron	< 0.1 mg/100g	Leucine	n/a
		Isoleucine	n/a
		Phenylaline	n/a
		Valine	n/a
		Tryptophane	n/a

12. Heavy Metals

Cadmium	< 0.05 ppm	below critical limit
Lead	< 0.02 ppm	below critical limit
Arsenic	< 0.01 ppm	below critical limit
Mercury	< 0.05 ppm	below critical limit

13. Microbiological Specifications

Microbe	Method of Analysis*)	Specifications
Aerobic Plate Count	AW06601/04/4	< 1000 cfu/g
Mould	AW06601/17/3	< 100 cfu/g
Yeast	AW06601/17/3	< 100 cfu/g
Enterobacteriaceae	AW06601/05/2	< 100 cfu/g
Lactobacillus	AW06601/18/1	< 100 cfu/g

*) based on SOP's of BIOANALYTICUM, Institut für Mikrobiologie und Hygiene, www.hygiene.co.at

14. Process Description

Production Process Description
The raw material is provided in a refined and heavy metal free form from the provider and is kept under refrigeration until processing.
The raw material is micro-encapsulated with food-grade materials (including machinery) in a water continuous medium.
The heat needed for the micro-encapsulation process is measured in such a way that it serves to kill all pathogen microbes at the same time.
The water suspension of micro-encapsulated Product is packaged under sterile conditions in food-grade bags; bags are further packed in food-grade boxes.
The Product is kept refrigerated until delivery to customer.

15. GAT Formulation GmbH

GAT Formulation GmbH hereby confirms that all information provided herein is accurate to the best of our knowledge; all data is derived from past analysis, future values may differ.

Should you require a signature on this document for your files, please contact us by sending a notification to info@gat-foodessentials.com indicating your fax number and we will return the signed document to you promptly.

[HEALTH CLAIMS]

Product Name: PARADOX 41 *(valid for 41-0, 41-1, 41-2)*

- Helps to reduce the risk of cancer
- Contributes to reduce the risk of cardiovascular heart diseases

Introduction

French Paradox is a term that is commonly used to describe the strikingly lower incidences of cardiovascular disease in France compared to other industrialized countries despite the same risk factors, such as diets high in fat and cholesterol, tobacco, as well as hypertension. This paradox has been attributed to a higher consumption of red wine and may in part be explained by the presence of polyphenols found in grapes. Food manufacturers thus have the opportunity to establish new brands or products with unique nutritional value by incorporating red wine polyphenols into processed foods.

Health Claims Associated with Polyphenols

Grape marc extract (grape seeds and peels) is a natural source of healthy and powerful antioxidants, with polyphenols (resveratrol[1], proanthocyanidins[2] and oligomers of proanthocyanidins[3]) being the most important elements. The common characteristic of these polyphenols is that they have been described as beneficial for the prevention and the treatment of various diseases[4], especially cardiovascular disease (CVD) and cancer. Current research knowledge suggests that these beneficial effects are mainly based on the antioxidant properties of the compounds of wine and grape marc extract. These compounds appear in a higher concentration in grape marc extract than in wine. Initially (related to the French Paradox), the effects of antioxidants present in grapes were only attributed as protective against CVD[5,6,7]. Only later, the anticarcinogenic properties of antioxidants were discovered. The long known and so-called “French Paradox” is just an epidemiological proof that wine is beneficial for the prevention of cardiovascular disease[8,9]. With improved scientific methods, the chemical compounds present in wine responsible for this effect have been identified afterwards.

Resveratrol and proanthocyanidin are the most important antioxidants of red wine. Resveratrol is present mainly in grape skin while proanthocyanidin is present in the seeds. The same polyphenolic antioxidants are present in grape marc extract, and as wine is a “diluted fermented grape extract”, the concentration of these antioxidants in the grape marc extract is naturally higher than in wine.

Reduced Risk of Cardiovascular Disease: Grape marc extract has been known for years to favorably influence vascular function. Sufficient polyphenols from grape marc extract appear to be absorbed to influence endothelial nitric oxide production (an oxidative stressor)[10].

Compounds belonging to the class of anthocyanidins are widely present in the plant kingdom since they are responsible for the color of grapes (they are more concentrated in red grapes) and for the colors of many flowers. The cardioprotective effect of polyphenols is attributed to antioxidants present in the anthocyanidins extracts. Proantocyanidins have been extensively reported as beneficial for vascular processes[11], even at capillary level[12] (e.g., in eye’s tissues, preventing the oxidation that causes cataracts).

Resveratrol has been discovered to support the reduction of bad cholesterol levels[13,14,15]. In a pioneering research of Prof. Corder group (Queen Mary’s School of Medicine, London), evidence was found that oligomers of proanthocyanidins from grape marc extracts (seeds) have preventive and reparative effects on epithelial cardiac cells (which are important to prevent CVD), with a mechanism that has nothing to do with the antioxidant

properties of these oligomers[16]. Research of Dr. Castanas (University of Crete)[17] also points out in the direction of multiple biological pathways (namely, not only antioxidant) for the beneficial grape marc extract.

In a report of the University of Connecticut, the authors provide evidence that red wine extract as well as resveratrol and proanthocyanidins are equally effective in reducing myocardial ischemic reperfusion injury, which suggests that these red wine polyphenolic antioxidants play a crucial role in cardio protection[18]. The results of a study from the Cardiovascular Research Center (Connecticut) indicate, that grape seed polyphenols extract functioned as an in vivo antioxidant, and its cardioprotective properties may be at least partially attributed to its ability to modulate an antideath signal[19]. All the scientific data based on basic research has been confirmed by clinical assays (extensive tests in humans), as for example the one performed by the Georgetown University Medical Center, where a clear relationship in between consumption of grape seed extract and lower levels of bad cholesterol was identified[20].

Reduce the Risk of Cancer: Generally, all biological processes leading to cancer are oxidation-mediated reactions. As a consequence of that, many types of antioxidants protect against cancer. As mentioned above, grape marc extract is an excellent source of certain types of highly active antioxidants. Resveratrol has been recently pointed out as the main responsible antioxidant related to the anticarcinogenic properties of wine[21,22,23,24,25,26,27]. Resveratrol is preferentially present in the peels of the grapes, while other antioxidants above mentioned are located in the seeds. However, grape marc extract contains both peel and seed extract. Anthocyanines[28,29] and proanthocyanidines (a type of anthocyanines) are other polyphenols with anticarcinogenic effects present in grape extracts. Oligomers of proanthocyanidins have been as well investigated agaist cancer, showing a direct relationship in between its consumption and the decrease of risk of certain cancers[30].

Other Benefits: Proanthocyanidin-rich grape extracts showed to have preventive actions on diseases such as atherosclerosis, gastric ulcer, large bowel cancer, cataracts and diabetes. In human intervention trials, grape seed extract was shown to have preventive effects on the increase in lipid peroxides in human plasma, responsible of these beneficial properties[31].

Dosage Recommendations

GAT Food Essentials recommends adding between 20–50 mg of polyphenols per serving size. The dosage of active ingredient per serving size depends on a.) how many serving sizes per day are consumed of a particular food given average consumption habits in a particular country or region, b.) the level of deficiency of the active ingredient given the dietary and nutritional habits in a particular country or region, and c.) the positioning of the final food product.

The recommended daily intake of polyphenols for preventive antioxidative purposes is about 50–100 mg. In cases of therapeutic purposes the dosage is set higher at about 150–300 mg/day.

(For any questions regarding dosage recommendations and/or how much of our functional food additive to use in order to reach the desired amount of active ingredient per serving size, please feel free to contact us through our Web Site at www.gat-foodessentials.com.)

Advantages of GAT Food Essentials paradox 41

paradox 41 by GAT Food Essentials is a liquid, natural functional food additive containing polyphenols from pomace extract that can easily be incorporated into processed foods during the production process. Due to our proprietary micro-encapsulation technology, paradox 41 is stable at pH ≥ 3.6, stable during the production process including homogenization, pasteurization, and high temperatures, as well as stable during the entire shelf life of the final food product.

Process Stability / Ease-Of-Use: GAT Food Essentials technology leads to functional food additives that can easily be incorporated into the production process. The micro-capsules show extreme process stability, remaining stable without any loss of active ingredients during food processing. Production forces, such as homogenization, high temperatures, and pasteurization, do not represent any problems to the food additive. These characteristics lead to high flexibility and low risk for the food manufacturer as neither additional production machinery nor an alteration in the regular and optimized production process is needed.

Product Stability: Due to GAT Food Essentials' micro-encapsulation technology the polyphenols are stable and protected during the entire shelf life of the final food product. The active ingredient is protected from oxygen, enzymes, metals, and other food compounds by a wall of cross-linked polymers and is stable in any environment above pH 3.6. Furthermore, the polyphenols are taste and smell masked and the outer environment is protected from the active ingredient itself. The micro-capsules' protective wall only opens once the pH level drops below 3.6, i.e. after consumption of the food product, thus allowing a targeted, rather than timed, and complete release of the active ingredient providing the essential health properties and benefits.

Natural Ingredients: GAT Food Essentials, unlike many other commercially available sources of polyphenols for incorporation into foods, does not use any genetically modified organisms and compounds (GMO-free) with all of the ingredients being derived from natural sources only. The micro-encapsulation process can be certified as organic with the ability of extending the certification to the formulations/products at our partners' and clients' request.

Summary

paradox 41 is a functional food additive containing polyphenols from natural sources. Through a unique micro-encapsulation technology the food additive shows excellent process stability and stability in the final food product, thus providing the technological prerequisites to easily incorporate important health properties into regular foods. paradox 41 is suitable for a wide variety of applications, including dairy products, fruit juices and drinks, bread and baked goods, chocolates and sweets, as well as sausages and pates and many other foods.

Literature / Sources

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[MATERIAL SAFETY DATA SHEET]

Product Name: PARADOX 41 *(valid for 41-0, 41-1, 41-2)*

1. Identification

Trade Name	paradox 41 *(valid for 41-0, 41-1, 41-2)*		
Type of Product	functional food additive		
Description	polyphenols from red wine pomace source micro-encapsulated functional food ingredient		
Origin	AUSTRIA		
Manufacturer	GAT Formulation GmbH Gewerbezone 1 2490 Ebenfurth Austria	Tel: Fax: Emergency:	+43 2624 53922 +43 2624 53922 +43 664 101 5048

2. Composition

PARADOX 41 is a water based microcapsule suspension of red wine pomace extract and inert wall forming materials.

Component	%	CAS No.	Hazardous components
Red Wine Pomace Extract	5	11029-12-2	None
Technological Additives	10	-	None
Water	52	7732-18-5	None
Grape Seed Oil	33	85594-37-2	None

3. Hazard Identification

This product is used as a food additive and consists mainly of vegetable oil and inert microcapsule forming materials. The formed emulsion is not considered to present any hazard during normal use.

4. First Aid Measures

Inhalation	At ambient handling temperatures (0-38°C), no adverse effects due to inhalation are expected.
Skin Contact	No adverse effects due to skin contact are expected; clean with water and soap.
Eye Contact	No adverse effects are expected; flush with water.
Ingestion	No adverse effects due to ingestion are expected.

5. Fire Fighting Measures

Extinguishing Media	Foam, dry chemical powder, carbon dioxide
Not Suitable	Water
Fire and Explosion Hazards	Combustible material, low hazard due to high content of water and inert ingredients; treat as an oil fire.
Special Fire Fighting Procedures	None known to GAT Formulation GmbH.

6. Accidental Release Measures

Personal Precautions	See section 8.
Environmental Precautions	Avoid discharge into sewers and waterways; absorb onto a suitable inert material and sweep up, if necessary, dispose of absorbed residues as directed in Section 13.

7. Handling and Storage

Storage Location	Fridge
Temperature Range	1 °C to 4 °C
Humidity	< 70% RH
Maximum Shelf Life	Originally sealed max. 6 months at 4°C
Handling Conditions	Handle in accordance to good industrial practices; store in sealed original containers or bag in box chilled or frozen.

8. Exposure controls and Personal Protection

Hand Protection	Suitable protective gloves
Eye Protection	Wear glasses
Skin Protection	Suitable protective clothing is recommended to avoid unnecessary contact.

9. Physical and Chemical Properties

Appearance / Odor	Red colored, viscous liquid / neutral, characteristic odor
Density, 20 °C (g/cm³)	0.950 – 1.050
pH Value, 20 °C	4.5 – 5.5
Solubility in Water	Unlimited miscible
Viscosity, 20 °C (Pa*s)	1 - 5
Boiling Point (°C)	> 100
Melting Point (°C)	N/A
Flash Point (°C)	N/A
Vapor Pressure (°C)	N/A

10. Stability and Reactivity

Thermal Decomposition	Stable under normal conditions of use.
Hazardous Reactions	None under normal conditions of use.

11. Toxicological Information

Skin Contact	Not expected to be an irritant.
Eye Contact	Not expected to be an irritant.
Inhalation	Not expected to be an irritant.
Ingestion	No hazard of low to moderate quantities.

12. Ecological Information

Biodegradation	Expected to be biodegradable
Fish Toxicity	No data
Bacterial Toxicity	No data
WGK Class	WGK 1, self-classification

13. Disposal Considerations

Collect and dispose of waste product at an authorized disposal facility, in conformance with national and local regulations.

14. Transport Information

No hazardous goods according to these transport regulations: RID/ADR/ADNR, IMO/IMDG, IATA/ICAO.

15. Regulatory Information

Not classified according to EEC directives 67/548/EEC and 88/379/EEC; refer to your national legislation implementing the EEC directive 91/155/EEC.

16. Other Information

The recommendations presented in this Material Safety Data Sheet were compiled from actual test data (when available, comparison with similar products, component information from suppliers and recognized codes of good practice). Handle in accordance to good industrial hygiene and safety practices.

Date: 01.01.2005

GAT Food Essentials

smart natural food ingredients

[Pro_duct Catalog] 2005_06

GAT Food Essentials is a division of GAT Formulation GmbH.

GAT Food Essentials

smart natural food ingredients

[Pro_duct Catalog] 2005_06

[INDEX]

[INTRODUCTION]

GAT Food Essentials is an innovative product line of functional food ingredients based on natural sources that help people achieve a balanced diet and general well being. Our strength and passion are innovative formulation concepts and technologies that allow our partners to easily incorporate our ingredients into a wide variety of applications for modern nutrition concepts, as well as to differentiate and add value to their products.

[COMPANY_ABOUT]

At GAT Formulation we are a group of natural scientists that are passionate about developing innovative formulation technologies and functional food additives for the food industry. Research and development is at the core of our business and we are proud to be recognized as a technologically leading micro-encapsulation specialist in the European Union. However, not past achievements but rather future possibilities are what drive us and we are continuing our hard work to provide you with the most innovative solutions for your products.

[COMPANY_HISTORY]

GAT Formulation was founded in 1997 by a group of highly specialized scientists to provide consulting services to the industry. Staying true to our passion for R&D we quickly realized our dream and built our own research labs to provide analytical and formulation services as well as to develop portfolio technologies. After a few years of encouraging growth and positive response from our partners we moved to new state of the art laboratory and manufacturing buildings at a new site in 2003 to continue and intensify our focus on applied research, product development, and production for the life-sciences industry.

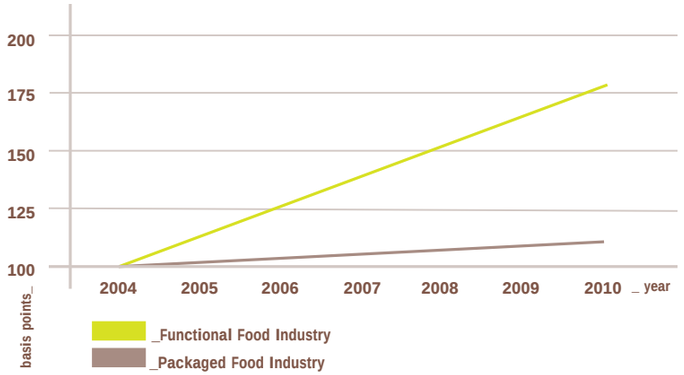
"Functional Foods are defined as any food or food component that may provide health benefits beyond nutritional value to achieve a healthy diet and general well-being."

[FUNCTIONAL FOOD INDUSTRY]

In recent years consumers have become increasingly aware of the importance of a balanced diet. In addition to paying close attention to the nutritional value of foods, consumers demand products that enable them to maintain their health or reduce the risk of certain life-style related diseases. Food companies thus have started to shift their focus towards developing products with important health properties to meet changing market trends.

With low sales growth performances of less than 1.5% per year for the food industry, functional foods start to play a key role for food producers to take advantage of high-margin growth opportunities. The functional food industry is **growing rapidly at 10% per year** with this trend expected to continue well past the year 2010. Furthermore, differentiation as well as the positioning of functional foods on the market allows an increase in **prices of up to 40% - with minimal incremental costs** - and **profit margins of more than 200%** compared to those of the conventional food industry.

[SALES GROWTH FUNCTIONAL vs. PACKAGED FOOD INDUSTRY]

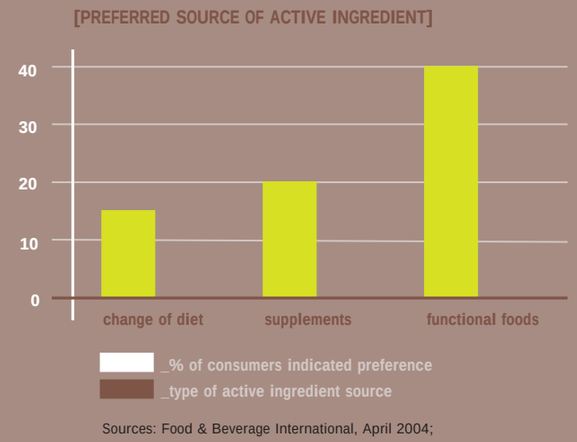


Sources: Eurostat; NBJ Functional Foods Report 2002; WSJ Online March 18th, 2004;

[CONSUMER BEHAVIOR]

Although a fairly new industry trend, functional foods already enjoy a wide acceptance and popularity among consumers. Increasing interest in quality of life, maintaining health, and reducing the risk of diseases, further fueled by a rise in available information regarding the direct link between diet and health, consumers are more and more looking towards functional foods to play an important role in daily life.

Consumer behavior studies done on the acceptance and popularity of functional foods indicate a promising future for food companies that incorporate health functions into their foods. Whereas **30% of consumers are aware of functional foods** and their important health properties, almost **50% of consumers intend to buy functional foods** once available. In addition, consumers indicated a strong preference of increasing their intake of ingredients with health properties through functional foods rather than by taking supplements in the form of pills.
As the media will continue to report on new links between active ingredients from natural sources and human health, the awareness of and demand for functional foods will certainly play a crucial role in promoting functional food products in the immediate future and in shaping consumer behavior in the long-run.



Sources: Food & Beverage International, April 2004;

[PRODUCT & TECHNOLOGY OVERVIEW]

GAT Food Essentials products are micro-encapsulated active ingredients from natural sources in a ready-to-use liquid and self-emulsifying water-based concentrate and thus can easily be added to foods without having to alternate the manufacturing process. Through our innovative micro-encapsulation technology the active ingredients are **stable during the production process** including homogenization of up to 150 bar, pasteurization, and high temperatures, as well as **stable during the entire shelf-life of the final product.** The active ingredients are **completely protected in any environment with a pH ≥ 3.6** and are only released after consumption.

[WHY_GAT FOOD ESSENTIALS]

We specialize in micro-encapsulation technology to be applied to a wide variety of active ingredients. Although we have a range of products in our portfolio, limits in the choice of active ingredients are few. All of our liquid functional food additives realized so far or to be developed in the future show the same characteristics in terms of stability, form, and handling, providing the food manufacturer extreme flexibility in deciding for an active ingredient to be incorporated into food products.

In developing our technology we aimed at making the process of implementing our functional food additives as simple as possible while ensuring absolute stability of the active ingredient during production and in the final food product. GAT Food Essentials have been successfully added to all types of dairy products, fruit juices and drinks, bread and baked goods, sausages and spreads, as well as snacks and chocolates, opening a variety of new opportunities for successful brand extension or product differentiation.

[PRODUCT LINE]

Our innovative micro-encapsulation technology defines few boundaries when it comes to new product development. As health properties of natural sources are re-discovered and scientifically substantiated on a regular basis, our portfolio of functional food additives is growing rapidly. The following pages will give a short overview of the **GAT Food Essentials** additives we have, out of personal curiosity or due to their high popularity and attention in the media, realized so far.

[PRODUCT LIST]

PRODUCT NAME	ACTIVE INGREDIENT	SOURCE
pro corde 36	omega 3 [ALA]	flax
pro corde 37	omega 3 [EPA, DPA]	fish
pro mente 38	antioxidants [EGCG]	green tea
pro corpore 39	CLA [conjugated linoleic acid]	vegetable oil
mediterraneum 40	antioxidants [hydroxytyrosol]	olives
paradox 41	polyphenols	red wine
intelligentia 42	omega 6, omega 3 [LA, ALA]	safflower/flax
aconcagua 43	caffeine	guarana
aconcagua 44	caffeine	mate
ferrum 45	iron	iron salts

pro corde
GAT Food Essentials

[PRO CORDE]

Omega 3 from plant or fish origin are polyunsaturated fatty acids that have been widely studied for their beneficial health properties for human nutrition and are known to **help lower cholesterol levels** and **reduce the risk of heart disease.** As modern diets lack an appropriate amount of omega 3 intake, the food industry can benefit from incorporating omega 3 and their health properties into processed foods, establishing unique brands and products that fulfill essential nutritional needs.

pro corde by GAT Food Essentials is a liquid, natural functional food ingredient containing omega 3 from flax or fish that can easily be incorporated into processed foods during the production process. Due to our proprietary micro-encapsulation technology, pro corde is stable at pH ≥ 3.6, stable during the production process including homogenization, pasteurization, and high temperatures, as well as stable during the entire shelf-life in the final product. pro corde is suitable for a wide variety of applications including dairy products, fruit juices and drinks, bread and baked goods, chocolates and sweets, as well as sausages and pates.

pro mente
GAT Food Essentials

[PRO MENTE]

Antioxidants from green tea have long been recognized for their valuable stimulating and protective effects on human's health. Among other health properties, antioxidants from green tea are known to **help increase mental performance** as well as help **slow the ageing process.**
Food manufacturers can thus benefit from incorporating green tea antioxidants and their health properties into processed foods, creating uniquely positioned brands and products with important nutritional value.
pro mente by GAT Food Essentials is a liquid, natural functional food ingredient containing antioxidants from green tea that can easily be incorporated into processed foods during the production process. Due to our proprietary micro-encapsulation technology, pro mente is stable at pH ≥ 3.6, stable during the production process including homogenization, pasteurization, and high temperatures, as well as stable during the entire shelf-life in the final product. pro mente is suitable for a wide variety of applications including dairy products, fruit juices and drinks, bread and baked goods, chocolates and sweets, as well as sausages and pates.

pro corpore
GAT Food Essentials

[PRO CORPORE]

CLA stands for conjugated linoleic acid, naturally incurring fatty acids known to trigger the mechanism of reducing body fat mass by decreasing the amount of fat stored after food consumption. By **increasing the rate of fat metabolism** CLA ultimately helps to **decrease the total number of fat cells** in the body. As naturally occurring CLA sources diminish due to changes in dietary habits or food processing, CLA can be added to regular foods as a functional ingredient in order to create uniquely positioned products with beneficial health properties for the consumer.

pro corpore by GAT Food Essentials is a liquid, natural functional food ingredient containing CLA from vegetable oil that can easily be incorporated into processed foods during the production process. Due to our proprietary micro-encapsulation technology, pro corpore is stable at pH ≥ 3.6, stable during the production process including homogenization, pasteurization, and high temperatures, as well as stable during the entire shelf-life in the final product. pro corpore is suitable for a wide variety of applications including dairy products, fruit juices and drinks, bread and baked goods, chocolates and sweets, as well as sausages and pates.

mediterraneum
GAT Food Essentials

[MEDITERRANEUM]

Antioxidants from olives are a principle constituent of the Mediterranean diet being known for its beneficial impact on **quality of life** and **long life-expectancy.** Natural antioxidants with hydroxy-tyrosol as the most potent phenolic compound of the olive as well as omega 9 fatty acids found in the oil have been studied for their health properties and are associated with a lower incidence of coronary heart disease and certain cancers. Adding antioxidants from olive extract and oil into processed foods can thus result in new products with important nutritional characteristics, incorporating the idea and health properties of Mediterranean diets into regular foods.
mediterraneum by GAT Food Essentials is a liquid, natural functional food ingredient containing antioxidants from olives that can easily be incorporated into processed foods during the production process. Due to our proprietary micro-encapsulation technology, mediterraneum is stable at pH ≥ 3.6, stable during the production process including homogenization, pasteurization, and high temperatures, as well as stable during the entire shelf-life in the final product. mediterraneum is suitable for a wide variety of applications including dairy products, fruit juices and drinks, bread and baked goods, chocolates and sweets, as well as sausages and pates.

[PARADOX]

French Paradox is a term that is commonly used to describe the **strikingly lower incidences of cardiovascular disease** in France compared to other industrialized countries despite the same risk factors, such as diets high in fat and cholesterol, tobacco, as well as hypertension. This paradox has been attributed to a higher consumption of red wine and may in part be explained by the presence of polyphenols found in grapes. Food manufacturers thus have the opportunity to establish new brands or products with unique nutritional value by incorporating red wine polyphenols into processed foods.

paradox by GAT Food Essentials is a liquid, natural functional food ingredient containing polyphenols from red wine that can easily be incorporated into processed foods during the production process. Due to our proprietary micro-encapsulation technology, paradox is stable at pH ≥ 3.6, stable during the production process including homogenization, pasteurization, and high temperatures, as well as stable during the entire shelf-life in the final product. paradox is suitable for a wide variety of applications including dairy products, fruit juices and drinks, bread and baked goods, chocolates and sweets, as well as sausages and pates.

intelligentia
GAT Food Essentials

[INTELLIGENTIA]

Omega 6 & Omega 3 polyunsaturated fatty acids have been widely studied for their important health properties in infant nutrition and are, when consumed in a special relationship, known to **help the development of the brain** and the **development of unborn and born babies and infants.** By incorporating this special relationship of omega 6 & omega 3 into regular foods to make up for its lack in modern day diets, new products with valuable health benefits can be specifically designed for infants, children, and pregnant women and benefit food manufacturers by creating uniquely positioned brands.

intelligentia by GAT Food Essentials is a liquid, natural functional food ingredient containing omega 6 and omega 3 from safflower and flax oil that can easily be incorporated into processed foods during the production process. Due to our proprietary micro-encapsulation technology, intelligentia is stable at pH ≥ 3.6, stable during the production process including homogenization, pasteurization, and high temperatures, as well as stable during the entire shelf-life in the final product. intelligentia is suitable for a wide variety of applications including dairy products, fruit juices and drinks, bread and baked goods, chocolates and sweets, as well as sausages and pates.

aconcagua
GAT Food Essentials

[ACONCAGUA]

Guarana & Mate are a fruit and plant typical to South America and are known to act as strong stimulants due to their high caffeine content and some compounds claimed by Latin-American natives to act as an aphrodisiac. Guarana and Mate can be used as a functional food additive, **providing their legendary energy** to endure strenuous days, opening up the opportunity to create foods or drinks specifically designed for mornings, during breaks, or to overcome low energy levels during the day.

aconcagua by GAT Food Essentials is a liquid, natural functional food ingredient containing caffeine from guarana or mate that can easily be incorporated into processed foods during the production process. Due to our proprietary micro-encapsulation technology, aconcagua is stable at pH ≥ 3.6, stable during the production process including homogenization, pasteurization, and high temperatures, as well as stable during the entire shelf-life in the final product. aconcagua is suitable for a wide variety of applications including dairy products, fruit juices and drinks, bread and baked goods, chocolates and sweets, as well as sausages and pates.

ferrum
GAT Food Essentials

[FERRUM]

Iron is an essential mineral for the oxygenation of body cells. Modern diets are characterized by a pronounced lack of iron leading to a deficit in red blood cells that are crucial for **providing oxygen to our organs.** Incorporating iron into foods thus opens up the potential for new products and brands with important health benefits to the consumer. However, as iron is highly reactive with other food ingredients, it is important to choose an iron source with high bioavailability in a format that ensures stabilization of the iron itself, as well as protection of other ingredients from iron as an active ingredient.

ferrum by GAT Food Essentials is a liquid, natural functional food ingredient containing iron from highly bio-available iron salts that can easily be incorporated into processed foods during the production process. Due to our proprietary micro-encapsulation technology, ferrum is stable at pH ≥ 3.6, stable during the production process including homogenization, pasteurization, and high temperatures, as well as stable during the entire shelf-life in the final product. ferrum is suitable for a wide variety of applications including dairy products, fruit juices and drinks, bread and baked goods, chocolates and sweets, as well as sausages and pates.

[MICRO-ENCAPSULATION]

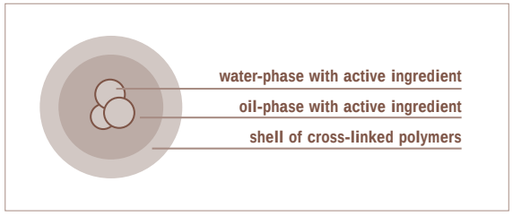
GAT Food Essentials products are micro-encapsulated active ingredients from natural sources in a liquid and self-emulsifying water-based concentrate. More accurately, the process applied for stabilization of bioactive ingredients is a **multiple micro-encapsulation** resulting in a water in oil in water system (w/o/w) protected in a shell of cross-linked polymers.

The formulation is characterized as a continuous water phase wherein micro-capsules are suspended. The micro-capsules contain oil droplets which may contain **oil soluble bioactive ingredients;** in the center of each oil droplet a water-phase is enclosed which in itself may contain **water soluble bioactive ingredients.** A polymerized material of natural sources covers and protects the droplets, thus stabilizing the active ingredients.

The innovative approach of GAT Food Essentials micro-encapsulation technology results in **liquid functional food additives** that are water-suspensions of tiny micro-capsules and are extremely flexible and elastic and remain stable in any environment with a pH level of 3.6 or higher. Once a final food product containing our food additives is consumed and exposed to low pH in the digestive tract, the micro-capsule wall opens and thus, fully releases the bioactive ingredients.

GAT FOOD ESSENTIALS TECHNOLOGY [MULTIPLE MICRO-ENCAPSULATION]

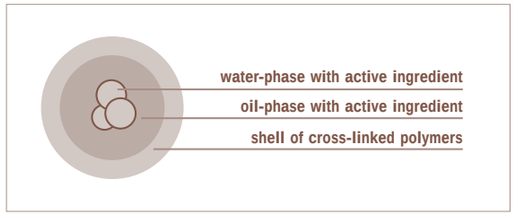
	GAT Food Essentials Technology Multiple Micro-Encapsulation
functional food additive characteristic	liquid, self-emulsifying in water or oil
incorporation of active ingredient	saturated solution of active ingredients in the water-phase and in the oil-phase
protection of active ingredient	multiple micro-encapsulation system protected in a shell of cross-linked biopolymers
approximate size of capsules	2 - 5 µm
stability of micro-capsules	- stable in suspensions at pH ≥ 3.6 - stable during heat exposure - stable during high pressure homogenization - stable against oxidation - stable against isomerization
application in food products	liquid or solid foods with pH ≥ 3.6
release of active ingredient	targeted/complete release at pH < 3.0



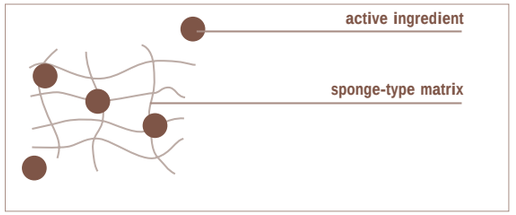
[MICRO-ENCAPSULATION]

COMPARISON: GAT FOOD ESSENTIALS TECHNOLOGY [MULTIPLE MICRO-ENCAPSULATION] & OTHER TECHNOLOGY [MATRIX-ENCAPSULATION]

	GAT Food Essentials Technology Multiple Micro-Encapsulation	Other Technology Matrix-Encapsulation
functional food additive characteristic	liquid, self-emulsifying in water or oil	powder
incorporation of active ingredient	saturated solution of active ingredients in the water-phase and in the oil-phase	entrapement of active ingredient in a matrix
protection of active ingredient	multiple micro-encapsulation system protected in a shell of cross-linked biopolymers	sponge-type matrix
approximate size of capsules	2 - 5 µm	30 - 50 µm
stability of micro-capsules	- stable in suspensions at pH ≥ 3.6 - stable during heat exposure - stable during high pressure homogenization - stable against oxidation - stable against isomerization	- stable in suspensions at pH 5.5 - unstable during heat exposure - unstable during high pressure homogenization - unstable against oxidation - unstable against isomerization
application in food products	liquid or solid foods with pH ≥ 3.6	liquid or solid foods with pH ≥ 5.5; limited process stability
release of active ingredient	targeted/complete release at pH < 3.0	gradual and incomplete release by diffusion and dissolution at pH < 5.5



[MICRO-ENCAPSULATION]



[MATRIX-ENCAPSULATION]

[CONTACT_INFORMATION]

We hope that we were able to provide you with an interesting overview of our company, technology, and products. Should you share our passion for functional food additives and think that we can help you realize your ideas, please contact us at **www.gat-foodessentials.com.**

GAT Food Essentials_smart natural food ingredients

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Project no. : COOP-CT-2003-508649

Acronym : PARADOX

Project title : French Paradox
Red wine extract food additives

Instrument : Special Research Project for SME`s
Cooperative Research (CRAFT)

Deliverable No : D8

Deliverable Title : Dissemination

Due date of deliverable : February 2006

Actual submission date : 2005 - 2006

Start date of project : 15.02.2004 Duration : 24 months

Organisation name of lead contractor for this deliverable :

Campi y Jove

Project co-founded by the European Commission within the Sixth Framework Programme (2002-2006)		
Dissemination Level		
PU	Public	**X**
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	

WP8 has been completed.

Publication of product brand name was completed by establishing the brand name in all GAT and dissemination partners' communications material. Presentations of the micro-encapsulation technology and the product PARADOX were given at various regional and international food manufacturing companies throughout Europe. Presentations and meetings were held either directly by GAT or in connection with dissemination partners. First presentations were directed towards technical staff of food manufacturing companies with marketing and purchasing staff following at a later point during the product development cycle.

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KUK Seminar - Ringe Kuhlmann & GAT Food Essentials

- Begrüßung und Vorstellung
  - Vorstellung GAT Formulation GmbH
  - Funktionelle Lebensmittelindustrie
- Multiple Mikro-Verkapselung
  - Ziele der Technologieentwicklung
  - Technologiedarstellung
  - Funktionelle Lebensmittelzusätze im Detail
- Funktionelle Lebensmittelindustrie
  - Anwendungspraktiken
  - Anwendungsgebiete
  - Produktbeispiele
- Diskussion, Fragen, Abschluss

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  - *Produktbeispiele*
- *Diskussion, Fragen, Abschluss*

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GAT FORMULATION GMBH

- GAT Formulation GmbH
  - Technologieunternehmen im Life Sciences Bereich
  - Mikro-Verkapselungs Spezialist
  - R&D Fokus
- GAT Food Essentials
  - Division von GAT Formulation GmbH
  - “smart natural food ingredients”

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GAT FORMULATION GMBH

- Geschichte / Entwicklung
  - 1999: Gründung von GAT Formulation GmbH als Beratungsunternehmen
  - 2003: Ausdehnung der Aktivitäten auf Analysen und Spezialformulierungen; Entwicklung der multiplen Mikro-Verkapselung als Technologieplattform
  - 2004: Adaption der Technologie auf funktionellen Lebensmittelbereich
  - 2004: Gründung von GAT Food Essentials
- Standort
  - Ebenfurth, Niederösterreich (südlich von Wien)
  - Forschung und Entwicklung, Produktion
  - 35 Mitarbeiter (Stand August 2005)

FUNKTIONELLE LEBENSMITTELINDUSTRIE

- Konventionelle Nahrungsmittelindustrie
  - Stagnierendes Umsatzwachstum mit weniger als 1.5% pro Jahr
  - Bisherige Wachstumstrends erreichen Sättigung und sind limitiert (Komfort-, Bio-, Light-Produkte; Brand Extensions)
  - Preisdruck auf Grund der Stärke des Handels und Eigenmarken
  - Minimale Margen
- Funktionelle Nahrungsmittelindustrie
  - Starkes Wachstum mit mehr als 10% pro Jahr bis 2010
  - Neue Wachstumsfaktoren beflügeln Industrie
  - Differenzierung und gesteigerter Kundennutzen erlauben höhere Preise mit minimalen Zusatzkosten
  - Margen bis zu 200 % höher als in der gesamten konventionellen Nahrungsmittelindustrie

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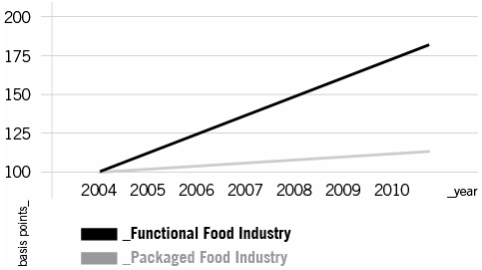
FUNKTIONELLE LEBENSMITTELINDUSTRIE

- Umsatzwachstum Funktionelle und Konventionelle Lebensmittelindustrie



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FUNKTIONELLE LEBENSMITTELINDUSTRIE

- Funktionelle Nahrungsmittelindustrie: Wachstumsfaktoren
  - Zunehmendes Gesundheitsbewusstsein der Konsumenten
  - Laufend neue wissenschaftliche Erkenntnisse bzgl. Ernährung und Wirkung von natürlichen Stoffen
  - Essgewohnheiten im Alltagsleben / Alternde Bevölkerung
  - Institutionelle- und Regierungskampagnen gegen Fehlernährung (steigende Kosten im Gesundheitswesen wegen falschen Ernährungsgewohnheiten)

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FUNKTIONELLE LEBENSMITTELINDUSTRIE

- Funktionelle Nahrungsmittelindustrie: Herausforderungen
  - Positionierung: Produkte sollen Gesundheit aufrechterhalten und nicht Krankheiten heilen
  - Geschmack: Nur Funktion alleine reicht nicht; funktionelle Nahrungsmittel müssen schmecken
  - Verschwommene Grenzen zwischen funktionellen Nahrungsmitteln und Pharmaindustrie
  - Auslobung von gesundheitsfördernden Faktoren / Legislatur

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FUNKTIONELLE LEBENSMITTELINDUSTRIE (Konsumentenverhalten)

- Bekanntheit von Funktionellen Nahrungsmitteln
  - 30 % der Konsumenten haben schon davon gehört
  - 50 % der Konsumenten würden funktionelle Nahrungsmittel kaufen, wenn diese erhältlich sind
- Vorteile Funktioneller Nahrungsmittel
  - Image zur Aufrechterhaltung der Gesundheit anstatt Krankheit zu kurieren
  - Konsumenten sind über gesundheitlichen Nutzen durch Medienpräsenz gut informiert
  - Komfort des Konsums funktioneller Nahrungsmittel vs. Pillen
- Konsumpräferenzen bei Aktiven Inhaltsstoffen
  - 40 % der Konsumenten würden Konsum durch funktionelle Nahrungsmittel erhöhen
  - 20 % würden Konsum durch Ergänzungsmittel (Pillen) erhöhen
  - 15 % würden Konsum durch einen Wechsel der Ernährungsgewohnheiten erhöhen

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FUNKTIONELLE LEBENSMITTELINDUSTRIE (Definitionen)

- Funktionelle Nahrungsmittel
  - Jegliche Nahrungsmittel oder Nahrungsmittelkomponente, die eventuell zusätzlichen gesundheitlichen Nutzen liefert, der über den Nutzen des normalen Nährwerts hinausgeht, um eine gesunde Ernährung und generelles Wohlbefinden zu erzielen.
- Novel Food - Neue Lebensmittel
  - Erzeugnisse, die bislang noch nicht in nennenswertem Umfang für den menschlichen Verzehr verwendet wurden und von pflanzlicher Quelle, tierischer Quelle oder Mikroorganismus kommen und bei deren Herstellung ein nicht übliches Verfahren angewandt wurde und dieses Verfahren eine bedeutende Änderung ihrer Zusammensetzung oder Struktur bewirkt hat, was sich auf ihren Nährwert, ihren Stoffwechsel oder auf die Menge unerwünschter Stoffe im Lebensmittel auswirkt.

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- *Diskussion, Fragen, Abschluss*

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ZIELE DER TECHNOLOGIEENTWICKLUNG (Grundprinzipien)

- Fokus auf Natürliche Inhaltsstoffe
  - Keine gentechnisch manipulierte Bestandteile
  - Aktive Inhaltsstoffe von natürlichen Quellen
  - Mikroverkapselungsprozess ist bio-zertifizierbar
  - Bio-Zertifizierung kann bei Bedarf auf Produkte ausgeweitet werden

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ZIELE DER TECHNOLOGIEENTWICKLUNG

- 1. Ziel: Einfache Anwendung
  - Ready-to-Use Produkte: einfache Beifügung zum Lebensmittel
  - Keine Änderung der etablierten und optimierten Produktionsprozesse notwendig
  - Schnelle Reaktionszeit auf Markttrends durch leicht austauschbare Zusätze
- 2. Ziel: Prozessstabilität
  - Stabilität der Mikro-Kapseln während der gesamten Produktion
  - Prozessstabilität bei hohen Temperaturen, hohem Druck, Pasteurisierung, usw.
  - Dosierung des aktiven Wirkstoffes bleibt erhalten während des Produktion
- 3. Ziel: Produktstabilität
  - Stabilität des aktiven Wirkstoffes im Endprodukt (keine Oxidation, kein Abbau)
  - Schutz des aktiven Wirkstoffes vor der Umgebung
  - Schutz der Umgebung vom aktiven Wirkstoff

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TECHNOLOGIEDARSTELLUNG

- Warum Mikro-Verkapselung
  - Technologie um empfindliche aktive Wirkstoffe Lebensmitteln beifügen zu können
  - Lösung zur Problematik von Oxidation, Abbau, Geschmack und Geruch
  - Erhalt der beigefügten Dosis des aktiven Wirkstoffes (Zugabe Produktion bis Ende Haltbarkeit)
  - Verlässliche, sichere, und gesunde Produkte für den Konsumenten
- Mikro-Verkapselungs Technologie
  - Stabilisierung aktiver Wirkstoffe durch “multiple Mikro-Verkapselung” (Wasser/Öl/Wasser Emulsion)
  - Verkapselungsmaterial umgibt und schützt aktive Wirkstoffe
  - Extreme Flexibilität und Elastizität der Kapseln
  - Zielgenaue und komplette Freisetzung der Wirkstoffe

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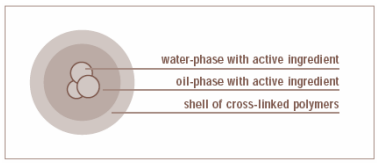
TECHNOLOGIEDARTELLUNG

- Vergleich: GAT Food Essentials Mikroverkapselung & Andere Technologie



[MICRO-ENCAPSULATION]

Kapselgröße	2 - 5 µm (sensorischer Vorteil)
Freisetzung des Inhaltsstoffes	gezielte, komplette Freisetzung pH < 3.0
Breitere Anwendungsmöglichkeiten	flüssige und feste Nahrungsmittel mit pH > 3.6
Stabilität der Mikro-Kapseln	- hitzestabil - stabil bei Hochdruckhomogenisierung - stabil gegen Oxidierung

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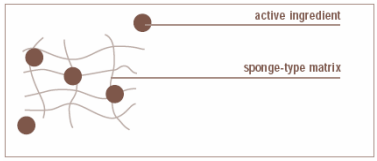
TECHNOLOGIEDARTELLUNG

- Vergleich: GAT Food Essentials Mikroverkapselung & Andere Technologie



[MATRIX-ENCAPSULATION]

Kapselgröße	30 - 50 µm
Freisetzung des Inhaltsstoffes	schrittweise und unvollständige Freisetzung bei pH < 5.5
Breitere Anwendungsmöglichkeiten	flüssige und feste Nahrungsmittel mit pH > 5.5
Stabilität der Mikro-Kapseln	- instabil bei Hitze - nicht stabil bei Hochdruckhomogenisierung - nicht stabil gegen Oxidierung

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TECHNOLOGIEDARSTELLUNG

- Vorteile der Mikro-Verkapselung
  - Flexibilität und Einfachheit der Anwendung
  - Reduzierung von “Time to Market”
  - Technologieplattform
  - Vielfalt der aktiven Wirkstoffe
  - Vielfalt der Anwendungsmöglichkeiten

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FUNKTIONELLE LEBENSMITTELZUSÄTZE IM DETAIL

PRODUKT	AKTIVER WIRKSTOFF	QUELLE
pro corde 36	omega 3 [ALA]	flax oil
pro corde 37	omega 3 [EPA, DHA]	fish oil
pro mente 38	antioxidants [EGCG]	green tea extract
pro corpore 39	CLA, conjugated linoleic acid	CLA
mediterraneum 40	antioxidants [hydroxytyrosol]	olive extract and oil
paradox 41	polyphenols	pomace extract
intelligentia 42	omega 6, omega 3 [LA, ALA]	safflower/flax oil
aconcagua 43	caffeine	guarana extract
aconcagua 44	caffeine	mate extract
ferrum 45	iron	iron gluconate
ferrum 46	iron	iron lactate

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  - Produktbeispiele
- *Diskussion, Fragen, Abschluss*

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ANWENDUNSPRAKTIKEN

- Mix and Taste
  - Gruppenbildung (ca. 8 Personen)
  - Arbeiten mit GAT Food Essentials
  - Verkostung
  - Feed Back
- Viel Spass!

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ANWENDUNGSGEBIETE & PRODUKTBEISPIELE

- Milchprodukte
  - UHT + Frischmilch
  - Joghurt und Trinkjoghurt
- Getränke
  - Fruchtsäfte
  - Energy Drinks
- Backwaren
  - Brot
  - Müsliriegel
  - Cerealien
- Fleischwaren
  - Fleisch
  - Fleischaufstriche

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DISKUSSION, FRAGEN, ABSCHLUSS

Vielen Dank für Ihr Interesse an unserm Unternehmen, unseren Produkten und unserer Technologie!

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GAT Food Essentials

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PRESENTATION OVERVIEW

- Introduction
- Functional vs. Packaged Food Industry
- Consumer Behavior
- Functional Food Definition
- Product & Technology Overview
- Micro-Encapsulation
- Product Line

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INTRODUCTION

- GAT Formulation GmbH
  - specialist in micro-encapsulation technology
  - focus on R&D and technology
- History
  - 1999: founded to provide consulting services to the industry
  - 2003: provided analytical and formulation services and developed portfolio technologies
  - 2004: adaptation of technology for functional food industry; market entry with GAT Food Essentials
- Other
  - located south of Vienna, Austria
  - currently 35 employees, 80% in scientific department

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FUNCTIONAL vs. PACKAGED FOOD INDUSTRY

- Packaged Food Industry
  - stagnant growth rates at less than 1.5% per year
  - former growth drives reach maturity and face limits (convenience-, organic-, brand-extension-trend)
  - price pressure due to retailer bargaining power and private labels
  - minimal margins
- Functional Food Industry
  - strong growth rates at more than 10% per year well into 2010
  - new growth drivers leverage growth
  - differentiation and added value to consumers allow for higher prices at minimal incremental costs
  - margins of up to 200% higher than in overall packaged food industry

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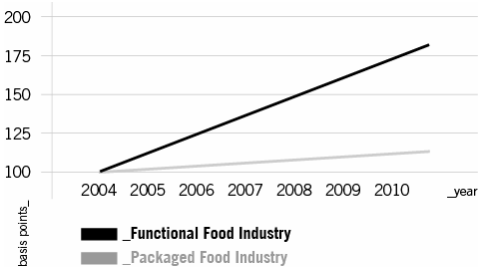
FUNCTIONAL vs. PACKAGED FOOD INDUSTRY

- Sales Growth Functional vs. Packaged Food Industry



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FUNCTIONAL vs. PACKAGED FOOD INDUSTRY

- Functional Food Industry: Driving Forces
  - increasing health awareness of consumers
  - ageing population
  - institutional and governmental campaigns (rising health care costs due to wrong nutrition habits)
- Functional Food Industry: Challenges
  - positioning: products must be positioned to help maintain health rather than treat sickness
  - taste: function alone will be out-functioned
  - blurring of lines between the functional food industry and pharmaceutical industry

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CONSUMER BEHAVIOR

- Functional Food Awareness
  - 30% of consumers have heard of functional foods
  - 50% of consumers would buy functional foods if available
- Functional Food Advantages
  - image of functional foods consumption to maintain health rather than treat sickness
  - consumers are well informed of health benefits due to presence in media
  - convenience of functional foods vs. pills
- Preference of Active Ingredient Intake
  - 40% of consumers would increase intake through functional foods
  - 20% would increase intake through supplements (pills)
  - 15% would increase intake through a change of diet

FUNCTIONAL FOOD DEFINITION

- Functional Foods
  - any food or food component that may provide health benefits beyond nutritional value to achieve a healthy diet and general well-being
- Novel Food
  - a substance that has not been previously been applied to foods or a food that is derived from a plant, animal, or microorganism that has been genetically modified

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PRODUCT & TECHNOLOGY OVERVIEW

- Focus on Natural Ingredients
  - no genetically modified compounds
  - active ingredients are from natural sources
  - micro-encapsulation process certified organic

PRODUCT & TECHNOLOGY OVERVIEW

- Focus on Ease-Of-Use
  - products can be easily added/incorporated into food processing (ready-to-use liquid)
  - established/optimized production processes do not have to be changed
  - early adaptation of market trends (additives are easily interchangeable)
- Focus on Process Stability
  - active ingredients are stable throughout production stress
  - process stability at high temperatures, pressure, pasteurization, etc.
  - active ingredient dosage stable from beginning of production process to end of shelf life
- Focus on Product Stability
  - stability of active ingredients in final food product (no oxidation, degradation)
  - protection of active ingredients from outer environment
  - protection of outer environment from active ingredients

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MICRO-ENCAPSULATION

- Why Micro-Encapsulation
  - technology to incorporate sensitive active ingredients into foods
  - solution to problematic of oxidation, degradation, taste and smell
  - consistent dosage of active ingredient
  - reliable and safe end products for consumers
- Micro-Encapsulation Technology
  - stabilization of active ingredients through multiple micro-encapsulation (w/o/w emulsion)
  - polymerized material covering and protecting active ingredients
  - extreme flexibility and elasticity of micro-capsules
  - targeted and complete release of active ingredients

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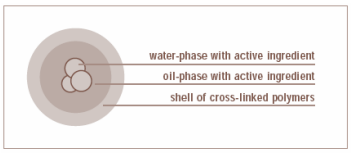
MICRO-ENCAPSULATION

- GAT Food Essentials Technology: Multiple Micro-Encapsulation

functional food additive characteristics:	liquid, self-emulsifying in water or oil
incorporation of active ingredient:	saturated solution of active ingredients in the water-phase and in the oil-phase
protection of active ingredient:	multiple micro-encapsulation system protected in shell of cross-linked biopolymers
approximate size of micro-capsules:	2 - 5 µm
application in food:	liquid or solid foods with pH ≥ 3.6
release of active ingredient:	targeted/complete release at pH < 3.0



[MICRO-ENCAPSULATION]

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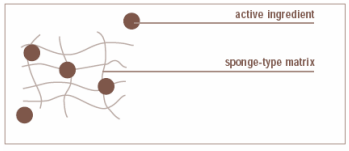
MICRO-ENCAPSULATION

- Other Technology: Matrix Encapsulation

functional food additive characteristics:	powder
incorporation of active ingredient:	entrapment of active ingredient in a matrix
protection of active ingredient:	sponge type matrix
approximate size of micro-capsules:	30 - 50 µm
application in food:	liquid or solid foods with pH ≥ 5.5 limited process stability
release of active ingredient:	gradual/incomplete release by diffusion and dissolution at pH < 5.5



[MATRIX-ENCAPSULATION]

MICRO-ENCAPSULATION

- Micro-Encapsulation Benefits
  - flexibility and ease-of-use
  - short time to market
  - technology platform
  - wide range of active ingredients
  - wide range of applications

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PRODUCT LINE

PRODUCT	ACTIVE INGREDIENT	SOURCE
pro corde 36	omega 3 [ALA]	flax oil
pro corde 37	omega 3 [EPA, DHA]	fish oil
pro mente 38	antioxidants [EGCG]	green tea extract
pro corpore 39	CLA, conjugated linoleic acid	CLA
mediterraneum 40	antioxidants [hydroxytyrosol]	olive extract and oil
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aconcagua 44	caffeine	mate extract
ferrum 45	iron	iron gluconate
ferrum 46	iron	iron lactate

CONTACT

Thank you for your interest in our company, technology, and products!

Should you have any questions, please visit our Web Site at www.gat-foodessentials.com for further information or contact us via e-mail at info@gat-foodessentials.com or phone at +43 2624 53922.

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Sources:
Industry Data: Eurostat; NBJ Functional Food Report; WSJ Online, March 18th, 2004
Consumer Behavior Data: Food & Beverage International, April 2004

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(43) Fecha de publicación de la solicitud: **01.07.2005**

(43) Fecha de publicación del folleto de la solicitud: **01.07.2005**

(71) Solicitante/s: **GAT FORMULATION GmbH**
Gewerbezone, 1
2490 Ebenfurth, AT

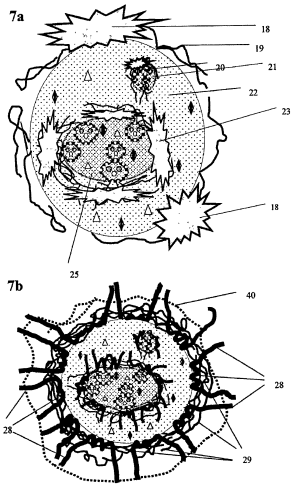
(72) Inventor/es: **Gimeno Sierra, Miguel;**
Gimeno Sierra, Bárbara;
Moser, Martha y
Casaña Giner, Víctor

(74) Agente: **Soler Lerma, Santiago**

(54) Título: **Proceso de multi-microencapsulación continuo para la mejora de la estabilidad y almacenamiento de ingredientes biológicamente activos.**

(57) Resumen:
Proceso continuo de multi-microencapsulación para la mejora de la estabilidad de ingredientes biológicamente activos, microcápsulas y sus usos.
La invención se refiere a microcápsulas y proceso continuo de microencapsulación que comprende una fase acuosa continua con dispersión de microcápsulas conteniendo gotas de aceite, y en el interior de cada gota de fase aceite -opcionalmente conteniendo materiales solubles en aceite-, existe dispersión de agua, o extracto acuoso o material dispensable en agua o material soluble en agua. Las gotas de aceite son encapsuladas con un material polimerizable de origen natural. Tales microcápsulas sirven para procesos de secado por spray, ser usadas como un polvo seco, liofilizadas, polvo autoemulsionable, gel, crema y cualquier forma líquida. Los compuestos activos incluidos en las microcápsulas son beneficiosos para la salud u otros fines biológicos. Tales formulaciones permiten su incorporación en alimentos, especialmente para producir nutracéuticos y cosméticos. Las preparaciones estabilizan compuestos añadidos a alimentos, medios de cultivo de microbios y nutracéuticos, en especial, los fácilmente degradables u oxidables.

Fig. 7



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DESCRIPCIÓN

Proceso continuo de multi-microencapsulación para la mejora de la estabilidad de ingredientes biológicamente activos, microcápsulas y sus usos.

Notas

Una expresión que contenga "A, B y/o C" quiere decir que permite las combinaciones A, A + B, B, C, A + C, B + C, A + B + C, esto es, sus permutaciones.

Abreviaturas

La siguiente lista de abreviaturas consiste en términos comúnmente empleados en el campo de la invención.

W = agua

O = aceite

W/O = emulsión agua en aceite

O/W = emulsión aceite en agua

(W/O)/W = emulsión agua en aceite en agua

Cuando se hace referencia a la fase agua, se sobreentiende que la fase agua puede no consistir solamente en agua, sino que, puede contener compuestos solubles o dispersables en agua, así como otros compuestos químicos, especialmente alcoholes de cadena corta (menos de 9 carbonos).

i.a. = ingrediente(s) activo(s), en la presente invención supone(n) ingrediente(s) biológicamente activo(s), excepto cuando es evidente que se refiere a ingredientes no usados para funciones biológicas. El uso del singular o plural se deduce del texto. En caso de duda, se refiere siempre al plural (i.a. = ingredientes activos).

UV = luz ultravioleta (incluye todas sus longitudes de onda, esto es, UVA, UVB, etc.)

Ácido graso es un ácido orgánico de cadena larga (más de 6 carbonos). En ocasiones se abrevia el término como "FA" o, para el plural, "FAs".

Ácido graso saturado es un ácido graso sin ninguna insaturación (sin dobles enlaces)

Ácido graso insaturado es un ácido graso con al menos una insaturación. En ocasiones es abreviado como UFA en singular o "UFAs", en plural.

Ácido graso monoinsaturado (1 insaturación)

Ácido graso poliinsaturado (2 o más insaturaciones)

Ácido graso altamente insaturado (4 o más insaturaciones)

Omega-3 = ácido graso insaturado omega-3, esto es, que posee al menos una insaturación en el carbono 3, numerando de modo creciente la cadena carbonada desde el extremo opuesto del grupo carbonilo.

Omega-6 = ácido graso insaturado omega-6, definido igual que omega-3, excepto que la primera insaturación (al menos una), numerando de modo creciente la cadena carbonada desde el extremo opuesto del grupo carbonilo, esté en el carbono 6 y no en el 3.

Omega-9 = ácido graso insaturado omega-9, definido igual que omega-3, excepto que la primera insaturación (al menos una), numerando de modo creciente la cadena carbonada desde el extremo opuesto del grupo carbonilo, esté en el carbono 6 y no en el 3.

Como abreviaturas se han empleado:

Omega-3/-6 omega-3 y omega-6

Omega-3/-6/-9 omega-3 y omega-6 y omega-9

Las abreviaturas omega-3 y omega-6 y omega-9 se refieren tanto en singular como en plural.

El "ácido linoleico", en el contexto de esta patente, incluye tanto el ácido linoleico (que posee varios números CAS en virtud de las posibilidades estéricas de los dobles enlaces, esto es, cis o trans o ambos mezclados), definido por los números CAS [60-33-3], [506-21-8], [2197-37-7], [2420-42-0], [2420-55-5], así como cualquier variación isomérica, respecto a la posición de los dobles enlaces, del ácido linoleico, y más en particular a los llamados (erróneamente en opinión de los inventores) "ácidos linoleicos conjugados", en los que el término "conjugado" se refiere a la presencia en la molécula de un fragmento (-CH=CH-CH=CH-).

La presente invención se refiere a microcápsulas y proceso continuo de microencapsulación agua en aceite en agua mediante una polimerización *in-situ* e interfacial de la emulsión. La formulación comprende una fase acuosa continua teniendo una dispersión de microcápsulas que contienen gotas de aceite, y en donde en el interior de cada gota de fase aceite -opcionalmente conteniendo materiales solubles en aceite-, existe una dispersión de agua, o extracto acuoso o material dispersable en agua o material soluble en agua. Las gotas de aceite son encapsuladas con un material polimerizable de origen natural. Tales microcápsulas son adecuadas para procesos de secado por atomización, para ser usadas como un polvo seco, liofilizadas, polvo autoemulsionable, gel, crema y cualquier forma líquida. Los compuestos activos incluidos en las microcápsulas son beneficiosos para la salud u otros fines biológicos. Tales formulaciones demuestran ser adecuadas para su incorporación en cualquier clase de alimentos, especialmente para la producción de nutracéuticos, así como productos cosméticos (como cremas rejuvenecedoras, antiarrugas, geles, consumibles de baño y ducha y en sprays.) Las preparaciones son adecuadas para estabilizar compuestos añadidos a los alimentos, medios de cultivo de microbios y nutracéuticos, en especial, aquellos que son fácilmente degradables u oxidables.

El campo de la presente invención corresponde con métodos de formulación y uso de materiales biológicamente activos, en especial en alimentos, y más concretamente en alimentos funcionales o nutracéuticos; comprende método de microencapsulación, microcápsulas producidas y aplicación (uso) de las mismas conteniendo ciertos compuestos, algunos de ellos descritos aquí por primera vez.

Estado de la técnica: antecedentes de la invención

Microencapsulación

La técnica de microencapsulación es conocida y empleada en campos muy distintos (farmacia, agroquímica, colorantes, etc.) Existen descritas formas diferentes de microencapsular compuestos, de forma que son liberados de forma controlada. Para una correcta y detallada definición del término microcápsula y una detallada revisión del estado de la técnica, consúltese Fong, W. "Technologies of microencapsulation" en el libro Controlled Release Systems: Fabrication Technology, 1988 Vol I Editor Dean Hsieh, CRD Press, Florida. En dicha cita, se menciona que muchas veces se confunde el término microcápsula con otras maneras de formulación, como emulsiones, microesferas, liposomas, etc. Las verdaderas microcápsulas se basan en una separación física de fases por medio de una pared (polímero) que ocluye dentro -el núcleo- al material microencapsulado; no se deben confundir con formulaciones que contienen materiales dispersos en polímeros o mezclados en matrices de polímeros. Tampoco hay que confundir las microcápsulas con simples emulsiones. Esta advertencia es necesaria para no confundir el amplio estado de la técnica referido a dispersiones de a.i.. en matrices hechas de polímeros, así como referido a emulsiones W/O y/o (W/O)/W conteniendo a.i. en. Una diferencia fundamental de nuestra invención con respecto a prácticamente todas las patentes referentes a microcápsulas es que nosotros creamos una emulsión (W/O) que es encerrada por la pared de una microcápsula, y las microcápsulas se encuentran dispersas o emulsionadas en W, y, además, las microcápsulas pueden contener microcápsulas menores en su núcleo, creándose, por tanto, multimicrocápsulas Por otro lado nuestras microcápsulas y proceso de microencapsulación, se caracterizan porque la pared está hecha de una mezcla de hidrocoloides que se polimerizan y entrecruzan, y se fija su estructura definitivamente por medio de un incremento en la temperatura; el proceso transcurre sin esperas de tiempo entre etapas de proceso y bajo agitación continua. Ninguna patente o artículo científico presenta un método de microencapsulación similar al descrito aquí. La patente más cercana a nuestro proceso de microencapsulación es la descrita en US 6,234,464.

US 6,234,464 describe un método de microencapsulación de FAs, en particular omega-3, omega-6 o derivados. Las diferencias con respecto a la presente invención estriban en que: (i) en US 6,234,464 el material microencapsulado en núcleo de la microcápsula es una emulsión O/W; en nuestra invención el núcleo contiene una emulsión W/O y, además, microcápsulas menores; (ii) en US 6,234,464 cada gota de agua está protegida con una pared; en nuestra invención existen múltiples gotas de agua dentro de las gotas de aceite, y no todas las gotas de agua están protegidas con una pared; (iii) en US 6,234,464 la pared está limitada a estar formada por dos hidrocoloides, además separados en dos capas diferentes definidas como "interior" y "exterior"; en nuestro proceso es posible, y conveniente, combinar más de dos hidrocoloides para formar la pared y, además, no existe una estructura definida de la pared en dos (o cualquier número) de capas, sino que nuestras microcápsulas poseen una capa mixta en donde los hidrocoloides (no necesariamente limitados a dos) están entremezclados; (iv) durante el proceso descrito en el Ejemplo 1 de US 6,234,464 existe un paso para fijar (curar) la primera capa de hidrocoloide, por medio de variación del pH, y así depositar la segunda capa encima; mientras que en nuestro proceso, no ejercemos ningún paso intermedio para curar ninguno de los hidrocoloides, sino que se curan al final del proceso todos los hidrocoloides empleados sin variación de pH intencionada (no-adición de compuestos que varíen el pH); (v) en US 6,234,464 cada partícula de ácido graso -entendemos que se refiere a gotas de ácido graso- está recubierta de dos capas de hidrocoloide; nuestras microcápsulas no necesitan que los ácidos grasos -en el caso de que se elijan tales como a.i..- estén recubiertos con dos capas, sino que, mucho más ventajosamente para la calidad del producto, es conveniente que los ácidos grasos estén en contacto con otros compuestos, incluso provenientes de la fase acuosa, que actúen como estabilizantes y prevengan su

oxidación; (vi) el curado de las microcápsulas en US 6,234,464 se realiza mediante enfriado; mientras que nosotros lo hacemos por aumento de temperatura, resultando en nuestro caso, una pared más firme; (vii) para eliminar el agua de las paredes en US 6,234,464 se emplea etanol como reemplazante del agua y secado para obtener un polvo de microcápsulas, mientras que nosotros podemos conseguir el polvo de microcápsulas sin la intervención de etanol.

Aunque las diferencias mencionadas son abundantes, se han descrito sólo las que se refieren a pasos del proceso; las microcápsulas producidas mediante US 6,234,464 y las descritas en la presente invención también tienen diferentes propiedades: térmicas, de protección de a.i.., de emisión controlada de los a.i.., del contenido de las microcápsulas (US 6,234,464 se limita a FAs), etc.

Uso de FAs en alimentos

Multitud de patentes reivindican el uso de FAs. La práctica totalidad de ellas han sido precedidas por artículos científicos en los que se demuestra los beneficios para la salud de los FAs, entre ellos, como antiinflamatorios, reductores de riesgo de cáncer y enfermedades coronarias y aterosclerosis en general, aplicación en fibrosis quística, correctores de esterilidad masculina, etc. Diversas corporaciones han obtenido un amplio número de patentes que hacen referencia a los FA omega-3 y omega-6, como es el caso de BASF, Milupa, Puleva, Clanidin, Omegatech, Unilever, Calgene, Abbott Lab, Wisconsin Alumni Research Foundation, Hoffmann-LaRoche, Martek Corp., etc.

Un estudio detallado de las diversas patentes revela que las diferencias entre ellas se basan en:

1.- Diferentes fuentes de omega-3 y omega-6, naturales o de organismos genéticamente modificados o microorganismos.

2.- Aplicaciones de los mismos compuestos omega-3 y omega-6 en diferentes enfermedades

3.- Diferencias en las dosis y proporciones de omega-3 / omega-6

4.- Métodos de obtención de omega-3 y omega-6

5.- Formulaciones de omega-3 y omega-6

Respecto a los 5 puntos arriba señalados, los inventores han encontrado referencias bibliográficas, en la inmensa mayoría de los casos, de muy diversos artículos en los cuales se describen fuentes, aplicaciones, dosis, métodos de obtención de omega-3/-6/-9 desde fechas muy anteriores a patentes que hacen referencia a dichas fuentes bibliográficas que sirven de ciencia básica para la aplicación industrial. La novedad de las patentes concedidas estriba en particularizaciones sujetas a un estudio más detallado que lo perteneciente al estado de la técnica.

WO200074669 muestra la aplicación de los omega-3/-6 para problemas de esterilidad masculina. WO2003056939-A1 muestra el uso de ácidos grasos poliinsaturados en alimentos para prevenir enfermedades cardiovasculares. WO2003017945-A2 muestra suplementos nutricionales para mujeres y niños (preconcepción, embarazo, lactancia y posparto).WO2002925540-A muestra la obtención de omega-3/-6 a partir de microbios. WO200291853-A muestra la aplicación de los omega-3/-6 a la alimentación de pájaros. WO200210322-A muestra la preparación de una mezcla de aceite purificado conteniendo omega-3/-6. WO200200028-A describe un agente protector para los omega-3/-6 destinados a animales que producen leche. WO200149282-A muestra una composición terapéutica para el tratamiento de la fibrosis quística basada en omega-3/-6. WO200146115-A muestra el uso de FA de 18 carbonos contra enfermedades coronarias. WO200110424-A muestra la aplicación terapéutica de omega-3/-6 combinados con vitamina E. WO9836745-A muestra métodos y composiciones para reducir enterocolitis basándose en el uso de FA altamente insaturados, fosfolípidos y colina. WO9735488-A muestra el uso de FAs para fórmulas infantiles. WO9212711-A muestra otra formula infantil más usando omega-3/-6 de microbios combinados con fuentes naturales conocidas (antes de la presentación de la patente) de omega-3/-6. WO9213086 muestra métodos de producción de ácido araquidónico y sus usos, mediante microbios. WO0054575 muestra una formulación infantil más en la que se incluyen omega-3/-6 y también fosfolípidos (los cuales están presentes de modo natural en prácticamente todas las formulaciones infantiles desde hace décadas). Otra variante de formulación infantil conteniendo omega-3/-6 se encuentra descrita en la patente US 5,550,156.

No obstante, en ninguna de ellas se describe un método de microencapsulación parecido al descrito en la presente patente, ni un estudio detallado de la estabilidad frente a la oxidación y deterioro químico de los omega-3/-6/-9 referente a los productos tóxicos que se pueden derivar de una mala formulación de estos ácidos grasos, como lo son determinados aldehídos, cetonas y otros productos de descomposición. La presente invención demuestra la estabilidad de los ácidos grasos insaturados microencapsulados según el proceso aquí descrito, en el tiempo, y en los procesos industriales de elaboración de alimentos.

Desarrollo de la inteligencia

Es sobradamente conocido para los expertos en la materia que ciertos ácidos grasos insaturados son beneficiosos para la salud, en especial los monoinsaturados (oleico preferentemente) y poliinsaturados. Dentro de estos grupos se diferencian los omega-3, los omega-6 y los omega-9. A la publicación por parte de científicos y estudios epidemio-

lógicos han seguido multitud de patentes que, basándose en estos estudios, reivindican el uso de estos compuestos naturales consumidos de modo natural por la humanidad desde sus inicios. Los inventores de la presente invención no conocen patente alguna que reivindique el uso combinado de FAs con esfingolípidos, ni con cerebrósidos. Los métodos de aplicación de estos compuestos en alimentos son muy variados, incluyendo microencapsulación, pero en ningún método se describe una microencapsulación de FAs como la presente invención (que precisamente se caracteriza por permitir incorporar, a toda clase de alimentos, FAs microencapsulados sin que se produzca degradación apreciable de los mismos. Está descrita la combinación de FAs con antioxidantes (p. ej., EP 0404058, US 5,855,944), pero en ningún caso se aplican microcápsulas parecidas a las aquí descritas, ni tampoco se reivindican ciertos antioxidantes complementarios aquí descritos y carecen de los estudios muy rigurosos de la calidad de los UFAs una vez procesados los alimentos (esto es, que permanezcan sin degradación tras proceso industrial), o, simplemente, su estabilidad con el tiempo.

Desarrollo de la inteligencia mediante omega-3/-6 combinados con esfingolípidos, en especial cerebrósidos

Un aspecto importante de la invención es la aplicación de nuestra formulación en productos infantiles, pues la leche de vaca carece de ciertos UFAs que sí están presentes en la leche materna. Sobre este tema de complementación de productos para embarazadas, lactantes y niños hay también muchas patentes, no obstante, ninguna emplea una microencapsulación con características parecidas a la descrita aquí para la óptima conservación de los UFAs hasta el consumo final.

En los últimos tiempos la sociedad en general se encuentra en un debate abierto sobre las posibilidades de incrementar la inteligencia, o al menos el potencial para desarrollar mayor inteligencia, mediante técnicas de ADN recombinante o selección de genes parentales. Los autores de la presente invención, basándose en diversos artículos que relacionan el desarrollo del cortex cerebral (donde reside la inteligencia) con una correcta administración y equilibrada dieta conteniendo omega-3/-6/-9, así corno relacionando el papel que juegan determinados esfingolípidos en transmisiones neuronales, y siendo los inventores conocedores de rutas metabólicas humanas, han encontrado una solución a una nueva demanda latente de la sociedad: desarrollar al máximo la potencialidad del ser humano, y en especial la inteligencia, como máxima distintiva del género humano, mediante la incorporación de ciertos compuestos naturales a la dieta. Así pues, aquí describimos el uso conjugado de omega-3/-6/-9 y esfingolípidos, preferiblemente cerebrósidos, para incrementar el potencial de desarrollo de inteligencia. No existe ninguna patente de invención que reivindique el uso de cerebrósidos en combinación con una equilibrada mezcla de omega-3/-6/-9 para el desarrollo de la inteligencia. Si que existen en el estado de la técnica algunos artículos que relacionan el consumo de omega-3/-6/-9 con mayor potencial desarrollo de inteligencia [ver C. Maurage, P. Guesnet, M. Pinault, *et al*. "Effect of two types of fish oil supplementation on plasma and erythrocyte phospholipids in formula-fed term infants." Biol Neonate 1998; 74: 416-29. y Crawford-MA Bloom-M Broadhurst-CL Schmidt-WF Cunnane-SC Galli-C Gehbremeskel-K Linseisen-F Lloydsmith-J Parkington-J; "Evidence for the Unique Function of Docosahexaenoic Acid During the Evolution of the Modern Hominid Brain"; Lipids 1999, Vol 34, Iss S, pp S39-S47] pero éstos ni ningún otro artículo, apuntan al importante papel metabólico que juegan en conjunto los ácidos omega-3/-6/-9 junto con los esfingolípidos y los cerebrósidos en particular en procesos de desarrollo del cerebro.

Uso de antioxidantes, protectores y/o bloqueadores de UV y bloqueadores de radicales libres.

Es de sobra conocido que el origen de muchísimas enfermedades, desde muy diversos tipos de cáncer hasta cataratas, es debido a reacciones de oxidación, de degradación de cadenas de ADN, todo esto debido por procesos de oxidación, inducidos por oxidantes, luz UV y/o radicales libres. También son muchas las patentes que reivindican el uso de extractos naturales, compuestos antioxidantes, etc. (EP 1344516, EP 1064910) para prevenir un gran abanico de enfermedades. La presente invención, a diferencia de todas las demás, muestra la particularidad de que los compuestos antioxidantes preservan su capacidad antioxidante gracias a la estructura y configuración de las microcápsulas o sus formulaciones, y permiten que estos antioxidantes sean añadidos a alimentos con todas las propiedades intactas microencapsulados de acuerdo con el proceso que se describe aquí, permitiendo procesos industriales sin merma de calidad.

Descripción detallada de la invención

§1 El proceso de multiencapsulación propuesto es por multi-microencapsulación continua, mediante polimerización interfacial e *in-situ*, de materiales biológicamente activos caracterizado porque.

(a) en un primer paso se adiciona una fase agua que contiene un iniciador de polimerización y, opcionalmente, al menos un material biológicamente activo, a una fase aceite, que contiene opcionalmente al menos un material biológicamente activo; adicionalmente existe al menos un emulgente en al menos una de las dos fases mencionadas, y existe un material biológicamente activo en al menos una de las dos fases.

(b) en un segundo paso se añade una solución o dispersión acuosa conteniendo al menos un hidrocoloide, que provoca una inversión de fases, y al mismo tiempo el hidrocoloide comienza a depositarse y a polimerizarse en las paredes de las nuevas gotas consistentes en una emulsión agua en aceite, ocurriendo también un entrecruzamiento de los polímeros.

(c) en un tercer paso, se añade una solución o dispersión acuosa que contiene al menos un coloide protector, el cual comienza a depositarse en la superficie de las gotas de agua en aceite, polimerizarse y entrecruzarse consigo mismo y con el hidrocoloide.

(d) después se añade una solución o dispersión acuosa de emulgente primario que permite una notable reducción del tamaño de las gotas de agua en aceite.

(e) en el proceso de reducción de tamaño de gotas, las parcialmente formadas microcápsulas se separan y se juntan, ocurriendo eventualmente un encerramiento de gotas dentro de otras mayores (multi-microencapsulación).

(f) cuando ha pasado suficiente tiempo para que las gotas de agua en aceite se recubran de, al menos, un hidrocoloide y, al menos, un coloide protector, se incrementa la temperatura para fortalecer la pared de las mencionadas gotas, que este instante ya son microcápsulas o multi-microcápsulas en suspensión acuosa.

(g) opcionalmente se seca la formulación para obtener polvo, y se reformula mediante técnicas pertenecientes al estado de la técnica para obtener (o ser mezcladas las microcápsulas en) polvos mojables, gel, cremas cosméticas o medicinales, productos de baño, medios de cultivo de microorganismos.

(h) todo el proceso -opcionalmente excepto el paso g)- se produce bajo agitación continua.

§2 En una descripción más detallada del proceso podemos referirnos a las Figuras adjuntas:

(a) dos soluciones diferentes (Fig.1) 1a (aceite) y 1b (agua) se mezclan mediante la adición de 1b a 1a, estas soluciones conteniendo ingredientes activos y opcionalmente cationes libres o secuestrados para ser liberados posteriormente.

(b) gracias a un emulgente alimentario que puede estar en la solución 1a o en la 1b, se forma una emulsión de gotas de agua (10) en la fase aceite (9). Este paso se concluye con la formación de la emulsión 1c, en donde en la fase aceite (9) están solubilizadas o dispersados los, preferiblemente, ingredientes activos liposolubles; también se forma una emulsión de agua en aceite, con las gotas de agua (10) conteniendo, preferiblemente, los ingredientes activos hidrosolubles.

(c) después se añade la solución 2b de al menos un hidrocoloide -capaz de ser polimerizado y entrecruzado, y opcionalmente conteniendo al menos un ingrediente activo, a la emulsión existente en 1c.

(d) seguidamente ocurre una inversión de fases, y tenemos gotas dispersas (11) que son una emulsión de agua (12) en aceite, dispersas en el medio continua (24), es decir, agua.

(e) después, (Fig. 5) añadimos una solución o dispersión 5a, conteniendo al menos un hidrocoloide (15), que actúa como coloide protector. La solución o dispersión 6a que contiene el emulgente primario se añade a la emulsión 2a.

(f) cuando las reacciones de polimerización y entrecruzamiento se consideran finalizadas y se alcanza un grado de reducción del tamaño de partícula en el rango de 1 μm a 30 μm, la temperatura que permanecía entre 30ºC y 70ºC se aumenta a 60ºC - 100ºC.

(g) finalmente se añade un modificador de viscosidad alimentario Opcionalmente, la formulación puede ser secada mediante atomización (spray-dry), o cualquier forma perteneciente al estado de la técnica, y ser recogidas y formar polvos secos, polvos autoemulsionables, geles, cremas o cualquier forma líquida que las contenga (incluyendo una dispersión en aceite), así como también pueden ser liofilizadas.

Puesto que una de las formas de realización preferidas es el uso para su adición a alimentos es una forma de realización preferida, las microcápsulas obtenidas mediante este proceso han sido probadas exitosamente desde el punto de vista de resistencia a degradación térmica, por presión, cambios en rangos de pH específicos, etc.

§3 El (los) hidrocoloide(s) como el (los) coloide(s) protector(es) se pueden añadir conjuntamente en forma de solución o dispersión acuosa inicial.

§4 El emulgente primario y el coloide protector pueden ser elegido entre el grupo de los hidrocoloides, así como el modificador de viscosidad, puesto que los hidrocoloides tienen todo este tipo de propiedades diferentes.

§5 El grupo de compuestos más apropiado para el éxito de una formulación acorde con el proceso descrito se corresponde con el grupo: quitosanas, almidón, dextrinas, ciclodextrinas, celulosas, lignina, pectinas, agar, alginatos, carragenatos, gelatinas, goma guar, goma arábiga, gelatina, tragacantos, lignosulfonatos, goma de Caraya, goma de *Ceratonia siliqua*, saponina, goma xantana, gomas de semillas, galactomananas, arabanogalactanas, beta-glucanos, inulina, psyllium, goma acacia; en todas sus formas isoméricas y estereoquímicas, en todas sus variantes con respecto a la cantidad y proporción de monómeros u oligómeros constituyentes del hidrocoloide, en todas sus variantes de presentación, como sales de cationes metálicos o sales de compuestos nitrogenados o fosforados o sulfurados, así como de cualquiera de los productos de derivatización de los mencionados hidrocoloides.

§6 El balance hidrofílico lipofílico, conocido como HLB, es una estimación del poder emulgente de un compuesto con capacidad emulgente, variando según la conveniencia de formar una emulsión W/O o una emulsión O/W. El HLB del emulgente primario de la presente invención debe estar comprendido entre 9 y 16, preferiblemente entre 12 y 14.

§7 La emulsión mostrada en 1c (10) debe tener un tamaño de partícula, determinado mediante un equipo medidor del tipo Master Sizer, de entre 50-500 μm, preferiblemente entre 70-200 μm.

§8 Al final del proceso, las microcápsulas formadas (7b) tienen un tamaño de partícula de en el rango 0.1-100 μm, preferiblemente en el rango 1-30 μm, mas preferiblemente en el rango 1-5 μm.

§9 Este tamaño puede variar con el tiempo por procesos de agregación, que en cierto modo son deseables, mientras la estructura de la formulación no se vea muy afectada.

§10-11 Uno de los factores que influyen en el éxito de formar una emulsión o dispersión estriba en la energía proporcionada a la solución o dispersión en donde se va a formar la emulsión. Esta energía se puede proporcionar en forma de tensión de corte, por medio de agitadores, preferiblemente con agitadores de dientes, de ancla, o ambos combinados. La velocidad de giro en revoluciones por minuto debe permanecer entre 3000 y 25000 rpm, aunque estos valores deben ser considerados dependiendo de las dimensiones de los reactores o recipientes en donde se realice la emulsión / dispersión. Cuando las microcápsulas están formadas es conveniente no proporcionar demasiada energía cinética / térmica para no provocar su indeseada ruptura.

§12 Un tipo particular de coloides son los hidrogeles, así pues los hidrocoloides pueden ser substituidos por hidrogeles, opcionalmente aquellos basados en albúmina, alginatos, policarboxilatos, poli-L-lactido, almidón, y derivados.

§13 Dependiendo de la velocidad de liberación del material microencapsulado, se pueden emplear diversas mezclas de hidrocoloides o hidrogeles, así podemos variar el grado de polimerización, la dureza de las paredes, su grosor, sus propiedades eléctricas, su permeabilidad a determinados compuestos, para obtener la microcápsula con la resistencia a los procesos alimentarios y al medio en el que se encontrará (p. ej. en un yogur) hasta su consumo final.

§14 Esta variabilidad de los componentes de la pared de la microcápsula también es aplicable a los modificadores de viscosidad y emulgentes usados, tanto el usado inicialmente para formar 1c (preferiblemente un polisorbato) como para emulgente primario (preferiblemente un compuesto basado en lecitina de soja).

§15 Las microcápsulas pueden obtenerse en estado seco, o también redispersarlas en otras matrices líquidas o incluso sólidas o solidificables. El medio exterior puede contener compuestos que ayuden a mantener la pared de la microcápsula, como reguladores de la fuerza fónica de la solución en la que se encuentran las microcápsulas, de la presión osmótica, etc. También puede haber, por ejemplo, cationes metálicos en el interior de las microcápsulas que, una vez completamente formadas, ayuden a que la pared no se destruya, como sería el caso de iones calcio dentro de una microcápsula formada con pectinas en su pared.

§16 Los ingredientes activos pueden ser añadidos en cualquier paso del proceso, incluso en la fase de procesado del alimento (mezclado con microcápsulas) pero obviamente, lo más importante es que los materiales activos y beneficiosos para la salud se encuentren en el interior de las microcápsulas, sea porque provienen de las soluciones 1a, de 1b, de 2b, 5a o añadidos en cualquier instante del proceso.

§17 Puesto que una de las realizaciones constituye el añadir a cualquier tipo de alimento compuestos beneficiosos para la salud, en partículas antioxidantes y UFAs, es importante prevenir oxidaciones durante la formación de las microcápsulas. Bajo las siguientes condiciones de proceso se reduce grandemente la oxidación: en vacío, presión reducida, en presencia de un gas inerte, preferentemente nitrógeno o helio, protegido de luz de cualquier longitud de onda, en condiciones estériles.

§18 Cuando nos referimos a fase agua, en todo el presente documento, debe entenderse que bajo ese término se incluyen soluciones o dispersiones: (i) basadas en extractos acuosos, (ii) con un contenido en alcoholes no superior al 40%, siendo el resto agua (iii) de compuestos solubles o dispersables en agua.

§19 También se entiende que la fase aceite puede ser cualquier fase hidrófoba, como ceras o incluso mezclas complejas como miel.

§20 Por las propiedades térmicas del agua, alcoholes o aceites, así como coeficientes de transmisión de calor de una fase a otra, se puede mejorar la resistencia de los compuestos microencapsulados frente procesos alimentarios (incluso cocinado en el hogar del consumidor) mediante un balance apropiado de la proporción de fase agua y aceite. Esto es, la acumulación de la energía térmica puede emplearse para proteger los a.i. de su deterioro.

§21 Es obvio que en ocasiones será necesario añadir un estabilizante microbiológico (bactericidas, fungicidas, bacteriostáticos, fungistáticos, etc.) a la formulación puesto que eventualmente va a ser utilizada en alimentos. También corresponde con el uso alimentario que los antimicrobianos sean autorizados en alimentación.

§22 Una realización de la invención consiste en una formulación seca de las microcápsulas, recubiertas con el estabilizante microbiológico.

§23 Para ciertas aplicaciones, sobre todo cosméticas, una vez secas las microcápsulas, se deben reincorporar en otros medios como geles, aceites, soluciones alcohólicas para perfumes, etc. En una realización de la invención, las microcápsulas contienen aromas para ser empleados en perfumería o para perfumar geles y cremas de baño.

§24 Las microcápsulas pueden ser aplicadas a todo tipo de alimentos, de modo no limitante los siguientes ejemplos: cereales y derivados (opcionalmente muesli, cereales para leche), bollería y pastelería, azúcares y derivados (opcionalmente chocolates, dulces, turrones, mazapanes), dulces dietéticos (con bajo nivel de calorías), en alimentos de régimen y para diabéticos, aceites y derivados, lácteos y derivados, huevos, verduras y hortalizas, legumbres, frutas, tubérculos y derivados, tallos comestibles, snacks, aperitivos, raíces comestibles (opcionalmente regaliz), bayas y productos silvestres, conservas de frutas, frutos secos, carnes, embutidos, pescados, mariscos y crustáceos y sus conservas, bebidas alcohólicas y no alcohólicas, bebidas carbonatadas o no carbonatadas, zumos, jarabes, néctares, especias, condimentos, comidas precocinadas, alimentos pre-procesados (masa de pan congelada), pizzas, miel.

§25 Aunque la principal y más útil realización de la invención se refiere a alimentación (de humanos y otros animales, incluso peces y también microorganismos), las microcápsulas pueden ser empleadas para otros fines, en particular para encapsular semioquímicos, atrayentes, repelentes, insecticidas, esterilizantes, herbicidas, fungicidas, bactericidas, viricidas (o materiales que previenen las infecciones víricas), vectores de genes (para terapia génica o para objetivos de técnicas de ADN recombinante), aromas, pungentes indicadores de presencia de compuestos químicos inodoros, astringentes para evitar la ingestión de productos tóxicos (preferiblemente etanol, alcohol isopropílico, agua oxigenada, limpiamuebles y otros productos similares de uso en el hogar).

§26 En ocasiones, la invención se realizará para evitar aromas, con la consiguiente adaptación de los materiales de la pared y otros factores, con el objeto de evitar al máximo la liberación de los materiales encapsulados. Esto es especialmente útil para productos enriquecidos con omega-3/-6/-9 provenientes de aceites de pescado, de tal forma que los olores no deseables sean reducidos al mínimo.

§27 En un ejemplo presentado más adelante, veremos que el solicitante ha empleado técnicas estadísticas avanzadas no usuales para reducir el número de pruebas necesarias para determinar los parámetros más adecuados para encapsular ciertos compuestos, o para obtener la velocidad de liberación deseada, etc. Para seleccionar las variables independientes: tipo de compuestos de la pared, tamaño de partícula, emulgente(s), velocidad de rotación del agitador, tipo de agitador, modificador de viscosidad, tipo de compuesto a microencapsular -dependientes de una variable independiente que representa la calidad de la formulación o de las microcápsulas- se ha empleado el análisis de varianza o múltiple análisis de varianza con diseño de fracciones factoriales, preferiblemente factorial en 2, 4, 8, 16, 32, y 64 bloques, media fracción saturada, diseño Box-Behnken, compuesto central, Plackett-Burman. La presente invención es el resultado de cinco años de experimentación con más de 50,000 formulaciones distintas ensayadas, sin embargo, sin el empleo de estas técnicas estadísticas, el número de ensayos ascendería a, por lo menos, un número mayor en 10 órdenes de magnitud.

§28 Definiendo un aspecto de la invención podemos referirnos a las microcápsulas producidas mediante un proceso continuo de multi-microencapsulación caracterizadas porque (a) contienen ingredientes activos beneficiosos para la salud humana; (b) la pared de las microcápsulas esta compuesta por una mezcla de al menos dos hidrocoloides, tal mezcla polimerizada y entrecruzada, tales hidocoloides son comestibles; (c) el grado de polimerización, entrecruzamiento y naturaleza de los hidrocoloides influye en la liberación controlada de los compuestos activos y la protección contra el oxígeno y/o luz y/o temperatura; (d) las microcápsulas contienen en su interior una emulsión de agua en aceite, existiendo opcionalmente ingredientes activos en la fase aceite, opcionalmente en la fase agua u opcionalmente en ambas fases y además, pueden contener microcápsulas menores (multi-encapsulación posible hasta, al menos, 5 grados de multi-encapsulación); (e) la media del tamaño de las microcápsulas se encuentra en el rango 0,1 μm - 100 μm, preferiblemente en el rango 1 μm - 10 μm (f) son producidas mediante un proceso continuo de multi-microencapsulación por polimerización interfacial *in situ*.

§29 Las microcápsulas producidas según el proceso aquí descrito pueden liberar su contenido por motivo de al menos un factor elegido del grupo de: pH, temperatura, presión, fuerza fónica, osmosis, volatilización, presencia de compuestos que disuelven la pared de la microcápsula.

§30 Las microcápsulas formadas, en una realización correspondiente a consumo humano, deben resistir los procesos alimentarios usuales, en particular a operaciones, pertenecientes al estado de la técnica, concernientes a protección contra microorganismos, nocivos y/o no deseados tanto en la formulación recién terminada o posibles microorganismos colonizadores de la formulación o alimento al que se destina, siendo éstas operaciones eventualmente: esterilización, estabilización de microorganismos, pasteurización, UHT, ozonización, rayos UV, adición de productos antimicrobianos químicos (tanto de síntesis como naturales), irradiaciones esterilizantes.

§31 Los estabilizadores microbiológicos pueden añadirse también en el proceso industrial, por tanto, en una realización, en el interior de las microcápsulas (opcionalmente en la fase aceite, o en la fase agua, o en ambas) y/o en la fase que contiene las microcápsulas, se encuentra un material estabilizador desde el punto de vista de calidad microbiológica.

§32 En una realización, la formulación se acompaña con un certificado de calidad en donde se analiza la inexistencia de metales pesados, productos nocivos de degradación de los materiales biológicamente activos, productos agroquímicos usados en la producción de los materiales biológicamente activos y demás compuestos que son nocivos para la salud.

§33-36 En una realización de la invención, las microcápsulas se emplean para proporcionar nutrientes, anabolitos, compuestos que ayuden a identificar microbios causantes de enfermedades (como anabolitos selectivos o productos fluorescentes o marcados radioactivos), y estos compuestos pueden opcionalmente ser liberados por cambios de pH en el medio de cultivo (p. ej., agar patata-dextrosa), por producción de enzimas (del mismo cultivo microbiano, p.ej.) o otros metabolitos (como alcohol o enzimas liberados).

§37 Las microcápsulas se pueden añadir a edulcorantes naturales o artificiales, sal, pimienta, especias y condimentos en general, de tal forma que la adición de los citados condimentos a los alimentos hace que se incremente el valor nutritivo, o beneficio para la salud, de los alimentos.

§38 Para una mayor protección de la pared de la microcápsula misma, o los compuestos activos contenidos en ella, es conveniente un compuesto que prevenga la acción oxidativa y/o destructiva de los rayos ultravioleta.

§39 Una realización preferida es aquella en la que se microencapsulan materiales que son hartamente conocidos por los científicos y por el público -con un cierto nivel de cultura- como muy apropiados para mantener la salud o prevenir enfermedades, o incluso curar enfermedades. No obstante el número de patentes que reivindican el uso de ciertos compuestos (antioxidantes y ácidos grasos omega-3, omega-6 y w-9 sobre todo), hay que tener presente que el un porcentaje abrumador, estas patentes han sido solicitadas mucho después de que se describieran los efectos beneficiosos de dichos compuestos. Es pues, el objetivo de nuestra invención, aplicar compuestos conocidos como sanos en forma microencapsulada, puesto que nuestro método de microencapsulación consigue mantener hastta el consumo final por parte del hombre / mujer o de cualquier otro animal, todas las propiedades beneficiosas de los compuestos activos (evitar su degradación). La práctica totalidad de productos los cuales se describen en esta patente, han sido descritos como beneficiosos desde hace más de 20 años, o incluso usados por la humanidad conscientemente o inconscientemente por su bondad desde hace milenios, e incluso desde los orígenes del género humano. En este sentido, los inventores escogen el grupo de compuestos, (mezclados totalmente o parcialmente o usados individualmente), para ser microencapsulado siguiente: té verde, té negro, cacao, vino tinto o uvas tintas u orujos de unas tintas, sidra o manzana o zumo de manzana, germen o salvado de cereales, carlotas o zanahorias, chili, ajo, rábano (en especial, rábano picante).

§40 Del mismo modo que se ha explicado en la reivindicación anterior, la presente invención muestra un método novedoso de formulación de muchos tipos diferentes de compuestos, constituyendo una completa novedad respecto el estado de la técnica el que los compuestos son microencapsulados con materiales comestibles de tal forma que protegen los ingredientes activos de degradación en los procesos de la industria alimentaria y/o en la cocina, de una forma superior a lo que existe descrito, gracias a la estructura de multi-microcápsula que dota de una multitud de capas protectoras a un porcentaje de los productos microencapsulados, gracias a la emulsión agua en aceite dentro de las microcápsulas que permite que se microencapsulen tanto productos hidrófilos como hidrófobos, y que las mezclas de estos compuestos permite que algunos compuestos protejan de la oxidación a otros compuestos, así como los detalles y pasos del proceso de producción que resultan en microcápsulas que pueden ser confeccionadas a medida de los compuestos a microencapsular, en términos de protección óptima y velocidad de liberación adecuada. Tras el elevado número de experimentos realizados por el solicitante, y considerando que compuestos químicamente similares se comportan de manera similar en el proceso y la microcápsula (p.ej., el limoneno y el pineno, siendo ambos monoterpenos, no deben presentar gran diferencia a la hora de encapsular ni a la hora de ser liberados por las microcápsulas, incluso el copaeno -que es un sesquiterpeno- tampoco diferirá mucho de los monoterpenos, ni tampoco el óxido de limoneno, con un grupo funcional adicional, presentará grandes diferencias a la hora de su microencapsulación según el proceso aquí descrito, puesto que los grupos funcionales no repercuten en la formación de las emulsiones ni en la constitución de la pared de las microcápsulas de modo drástico. En los casos que ciertos compuestos sí que modifiquen grandemente las necesidades de emulgentes especiales, los inventores han previsto estos casos, y para ello se emplean diferentes emulgentes, polímeros, etc., limitados a los ya mencionados -pero capaces de superar cualquier dificultad en el proceso de los compuestos que seguidamente se mencionan-. Así pues, preferiblemente pero de modo no limitante, son objeto de microencapsulación:

(a) flavonoides en general y sus derivados: antocianidinas, pro-antocianidinas, oligomero-procianidina, isoflavonas, chalconas, catequina, epicatequina, epicatequina galato, epigalocatequina, epigalocatequina gallato, eriocitrina, narirutina, rutina, naringina, miricitrina, hesperidina, miricetina, eriodictiol, fisetina, quercetina, naringenina, luteolina, hesperitina, kaempferol, isorhamnetina, apigenina, rhamnetina, galangina, quercitrina, quercetina, diosmetina, taxifolina, galandina, biochanina A, genisteina, eriodictiol, chrysina, hidroxitirosol, oleuropeina, glabridina, licochalcona, daidzeina, matairesinol, secoisolariciresinol, enterodiol, enterolactona, equol, desmetilangolensina, luteoferol, luteolinidina, apiferol, apigenidina, leucocianidina, pelargonidina.

(b) ácidos fenólicos en general y sus derivados (preferiblemente esteres, éteres, glicósidos, rutinósidos y aminas): gálico, sinápico, síringico, caféico, clorogénico, ferúlico, (o-, m- or p-) cumárico, guaiacol, (o-, m- or p-) cresol, 4-etilfenol, 4-vinilguaicol, p-hidroxibenzoico, procatecuico, vainíllico, hidroxicinámico, taninos en general, elagiotaninos, galotaninos.

(c) amidas estructuralmente combinadas comprendiendo ácidos hidroxicinámicos y ácidos antranílicos (avenantramidas), avenasterol, ácidos hidroxicinámicos estructuralmente combinados con ácidos grasos de cadena larga saturados o insaturados, ácidos hidroxicinámicos estructuralmente combinados con alcoholes, indoleaminas, melatonina, inulina; glutatión.

(d) terpenoides en general y sus derivados, monoterpenos, diterpenos, sesquiterpenos, triterpenos, tetraterpenos, incluyendo los carotenoides, alfa-caroteno, fitotoeno, ciclo-artenol, beta-caroteno, ionona, zeaxantina, capsantina, astaxantina, cantaxantina, violaxantina, mutatoxantina, luteoxantina, auroxantina, neoxantina, apo-carotinal, xantofilas.

(e) antioxidantes usados comúnmente en la industria alimentaria (y sus derivados) del tipo de butilhidroxianisol, 2,6-di-ter-butilhidroxitolueno, ter-butilhidroquinona, 2,6-di-ter-butilhidroquinona, 2,6-diterbuty1-4-hidroximetilfenol, 2,4,5-trihidroxibutirofenona, tocoferoles y sus derivados, [alfa-, beta-, gamma- y delta-] tocoferol; tocotrienoles y sus derivados, [alfa-, beta-, gamma- y delta-] tocotrienoles; tococromanoles

(f) ácido alfa-lipoico; coenzima Q-10; escualeno; fitoestrógenos; clorofila; vitaminas; aminoácidos, preferiblemente L-arginina, y sus correspondientes polímeros orgánicos como lo son el glutatión los oligopeptidos, preferiblemente carnitina y carnosina, peptidos, enzimas; inhibidores enzimáticos, preferiblemente inibidores de las fenolasas, oxigenasas, lipooxigenasas, peroxidasas y lipasas;

(g) así como minerales, oligoelementos, en especial aquellos que participan en procesos redox *in vivo* como el selenio, zinc y magnesio.

§41 Las fuentes naturales de los compuestos arriba indicados, o también de otros compuestos que todavía no se conocen, o de otros compuestos conocidos pero no indicados en el párrafo anterior, se pueden elegir entre el grupo de vegetales que son ya aceptados por multitud de naciones como aditivos alimentarios, considerando aditivos a algo que se añade al alimento, sea o no parte fundamental o predominante del alimento. También los inventores consideran que ciertas plantas productoras de narcóticos son fuentes de compuestos que pueden ser (o ya son usados) en medicina. Por último, en esta lista se incluyen plantas que son conocidas por sus cualidades terapéuticas y empleadas en herboricultura y para-farmacia. Esta lista es un ejemplo no limitantes de fuentes naturales de compuestos activos a microencapsular, tanto por aislamiento de compuestos, como por soluciones acuosas o alcohólicas de las mencionadas fuentes como por dispersiones de hojas, tallos, raíces, flores frutos, etc. machacados hasta cierto grado de tamaño de partícula, así como de preparaciones liofilizadas de los mismos o pre-procesadas en cualquier modo. La lista referida es: *Medicago sativa, Pimenal officinalis, Hibiscus abelmoschus, Angelica archangelica, Galipea officinalis, Pimpinella anisum, Ferula foetida, Ferula asafetida, Melissa officinalis, Myroxylon pereirae, Ocimum basilicum, Pimenta acris, Citrus aurantium bergamia, Prunus amygdalus, Citrus aurantium, Citrus aurantium amara, Piper nigrum, Prunus spinosa, Aniba rosaeodora, Camelia oleifera, Camelia sinensis, Carum carvi, Elettaria cardamomum, Ceratonia siliqua, Daucus carota, Dacus carota sativa, Cascarilla, Apium graveolens, Anthemis nobilis, Matricaria chamomilla, Anthemis nobilis, Anthriscus cerefolium, Cichorium intybus, Cinnamomum spp., Cinnamomum zeylanicum, Cymbopogon nardus, Salvia sclarea, Trifolium pratense, Theobroma cacao, Coffea arabica, Coriandrium sativum, Cuminum cyminum, Taraxacum officinale, Sambucus nigra, Elderweiss, Helichrysum italicum, Foeniculum vulgare, Trigonella foenumgraecum, Arabidopsis spp., Zingiber officinale, Citrus grandis, Psidium guajava, Humulus lupus, Marrubium vulgare, Monarda punctata, Hyssopus officinals, Jasminum officinale, Jasminum grandiflorum, Juniperus spp. Juniperus comunis, Eucaliptus officinalis, Cola acuminata, Laurus nobilis, Lavandula spp. Lavandula hybrida, Taxus baccata, Citrus medica limonum, Myristica fragans, Marjorana hortensis, Thymus spp., Thymus officinalis, Thymus mastichina, Ilex paraguarensis, Chamomilla recutita, Saccharum officinarum, Myristica fragans, Allium cepa, Citrus aurantium dulcis, Carum petroselinum, Mentha pulegium, Mentha piperita, Pimenta officinalis, Chimaphila umbellate, Punica granatum, Pelargonium spp., Pelargonium graveolens, Rosmarinus officinalis, Crocus sativus, Salvia app., Salvia officinalis, Mentha spicata, Mentha viridis, Satureia hortensis, Satureja hortensis, Origanum majorana, Tamarindus indica, Citrus reticulata, Artemisia dracunculus, Thea sinensis, Thymus vulgaris, Polianthes tuberosa, Curcuma longa, Prunus serotina, Thymus serpillum, Satureja Montana, Cananga odorata, Curcuma zedoaria, Plantago major, Adansonia digitata, Ananas comosus, Artocarpus altilis, Carica papaya, Lycopersicon esculentum, Cephalophus spp., Vaccinium myrtillus, Thymus aragonensis, Thymus spp., Citrus aurantiifolia, Citrus paradisi, Cucumis melo, Cucurbita spp., Vitis spp., Vitis vinifera, Mangifera indica, Lamiaceae* (*Coleus, Hedeoma, Hyptis, Leonurus, Leucas, Lycopus, Marrubium, Mentha, Monarda, Perilla, Prunella, Salvia, Stachys, Teucrium, Thymus*), *Cannabis spp., Digitalis lanata, Adonis vernalis, Aesculus hippocastanum, Frazinus rhychophylla, Agrimonia supatoria, Rauvolfia sepentina, Andrographis paniculata, Areca catechu, Atropa belladonna, Berberis vulgaris, Ardisia japonica, Betula alba, Ananas comosus, Camellia sinensis, Cinnamomum camphora, Camptotheca acuminata, Potentilla fragarioides, Erythroxylum coca, Papaver somniferum, Colchicum autumnale, Claviceps purpurea, Digitalis purpurea, Digitalis lanata, Glaucium flavum, Papaver somniferum, Gossypium spp., Hyoscyamus niger, Camptotheca acuminata, Piper methysticum, Lobelia inflata, Crotalaria sessiliflora, Nicotiana tabacum, Physostigma venenosum, Ephedra sinica, Cinchona ledgeriana, Rhododendron molle, Datura spp., Taxus brevifolia, Strychnos nux-vomica, Stevia rebaudiana, Theobroma cacao, Valeriana officinalis, Pausinystalia yohimbe, Ephedra spp. Crataegus oxyacantha, Hamamelis virginiana, Hydrastis Canadensis, Hypericum perforatum, Potentilla erectra, Ledum palustre, Salvia officinalis, Chamomilla recutita, Arctostaphylos uva, Eucommia ulmoides, Mytilus galloprovincialis, Diplazium esculentum, Manihot utillissima, Sauropous androgynus, Terminalia arjuna, Iberis amara, Crataegus spp., Arbutus unedo, Cynara scolymus, Amaranthus caudatus, Alchornea laxiflora, Alpinia officinarum, Xanthophyllomyces dendrorhous, Crataegus monogyna, Taxus yunnanensis, Bacopa monniera, Cistus albidus, Ocimum basilicum, Rosmarinus officinalis, Thymus vulgaris, Bixa orellana, Centella asiatica, Urtica dioica, Agrocybe aegerita, Crataegus laevigata, Satureja horten-*

sis, Crocus sativus, Coccinia indica, Brugia malayi, Rubus spp., Silybum marianum, Cannabis spp., Cannabis sativa, Hypericum perforatum, Rhus coriaria, Olea europaea, Cyclopia intermedia, Ginkgo biloba, Lentinus lepideus, Pseudomonas putida, Sargassum micracanthum, Pinus radiata, Pinus sp., Phaseoulus mungo, Cicer arietinum, Vigna sinensis, Phaseolus aureus, Dolichos lablab, Cajanus cajan, Vicia faba, Dolichos biflorus, Phaseolus lunatus, Phaseolus aconitifolius, Pisum sativum, Psophocarpus tetragonolobus, Arachis hypoagea, Brassica spp., Brassica campestris, Brassica napus, Valeriana officinalis, Echinacea purpurea, Echinacea pallida, Echinacea angustifolia, Glcyrrhiza glabra, Seronea repens, Vaccinium macrocarpon, Tancetum parthenuum, Tancetum parthenuum, Vaccinium macrocarpon, cereales, frutales de hueso, bayas silvestres, legumbres, té verde, té negro y microorganismos productores de ácidos grasos de cadena larga insaturados.

§42-45 Otro asunto que preocupa enormemente a una parte considerable de la población en países desarrollados es el consumo de organismos probióticos, entendiendo como tales, a organismos que por productos de su metabolismo o por su presencia en el organismo, protegen contra ciertas infecciones (en especial *Candidasis*), reducen niveles de colesterol y glicéridos, y ayudan en procesos de digestión y enfermedades del aparato digestivo, principalmente. Estos organismos probióticos son generalmente introducidos en yogures y productos lácteos, pero mediante la presente invención, hemos constatado que es posible microencapsular bacterias, hongos y levaduras vivas, permaneciendo vivas tras la microencapsulación y tras procesos usuales en la industria alimentaria, como homogeneización pasteurización e incluso ciertos tipos de horneado, así como cocción casera. Esto implica una novedad y la posibilidad de añadir estos organismos probióticos a un inmenso abanico de tipos de productos alimentarios. Los organismos probióticos son elegidos preferiblemente (pero no de modo limitante) entre los grupos:

(a) bacterias probióticas, opcionalmente bacterias ácido-lácticas y más preferiblemente elegidos entre el grupo de: *Lactobacillus casei., L. acidophillus, L. rhamnosus, L. paracasei, L. gasseri, L. fermentum, L. plantarum, L. salivarius, L. crispatus, L. bulgaricus, L. fermentum, L .reuteri, Bifidobacterium infantis, B. bifidum, Streptococcus termophilus, S. bovis, Enterococcus durans, E. faecalis, E. Gallinarum, Escherichia coli, Propionibacterium freudenreicheii*, o bacterias u hongos o levaduras modificadas genéticamente en las que se han insertado genes propios beneficiosos de las bacterias probióticas.

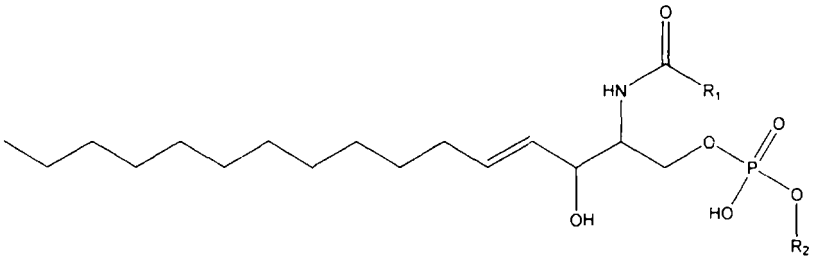
(b) levaduras probióticas, preferiblemente elegidas entre el grupo de: *Saccharomyces cerevisiae, Kluyveromyces marxianus, Rhodotorula rubra, Sporobolomyces puniceus, Aureobasidium pullulans, Leucosporidium scotti*.

(c) hongos probióticos, preferiblemente aquellos hongos presentes en, o coincidentes con, o provenientes de, quesos.

§46 El interés por el consumo de ácidos grasos insaturados omega-3, omega-6 y w-9 es un tema que durante décadas ha sido investigado por Universidades y se han realizado estudios epidemiológicos por agencias gubernamentales y Hospitales. Tras estos estudios, un número considerable de patentes ha sido presentado reivindicando el uso de estos compuestos, en muchos casos sin ningún dato adicional más que los ya conocidos públicamente. La intención del solicitante no es reivindicar el uso de estos compuestos ni cualquier combinación de ellos en un ratio determinado (aspecto en el que se basan muchas patentes para atribuirse novedad), sino el uso de estos compuestos en las microcápsulas descritas, en virtud de la protección contra la oxidación de estos compuestos UFAs mucho más efectiva que lo conocido en el estado de la técnica, tal y como mostraremos más adelante con los ejemplos. Así mismo, los inventores consideran que el uso combinado de UFAs con esfingolípidos, y más especialmente con cerebrósidos, es recomendable para un desarrollo adecuado o mejorado, del cortex cerebral y otros lugares (p. ej., retina) en donde hay un desarrollo neuronal preferente. Más aún es importante en fetos, niños o personas con problemas neuronales. La combinación del uso de cerebrósidos con UFAs no pertenece al estado de la técnica, ni tampoco su administración conjunta por medio de compuestos mixtos covalentemente unidos como los descritos más adelante, (A) y (B), sintetizados de acuerdo con Dondoni, A. *et al*., (1990) , J.Org.Chem. 55 (5): 1439-1446, y

Schmidt, R.R., Zimmermann, P., (1986), Tetrahedron 27 (4): 481-484, así como técnicas pertenecientes al conocimiento común de químicos orgánicos, relacionadas con esterificaciones. Los inventores han sintetizado el compuesto B tal que, R_3: CH_2CH_3, R_4: $CO\text{-}(CH_2)_2\text{-}(CH_2\text{-}CH{=}CH)_4\text{-}CH_2\text{-}CH_3$, con un rendimiento basado en el contenido inicial de ácido araquidónico del 35%. Debido a la pequeña cantidad sintetizada, sólo fue posible obtener datos por LC-MS data (Agilent 1100 Series LC/MSD Trap), confirmando que el compuesto tenía una fragmentación típica de esfingolípidos y también del ácido araquidónico (picos M/Z: 79, 67, 91, 55, 108, 318 [M+]). El análisis del lado esfingolípido realizado tras esterificación y benzoilación. No observamos absorción UV a 205 nm, indicando que no obtuvimos transisomerización (remarcamos que es conveniente que los dobles enlaces de los ingredientes activos permanezcan en posición cis). Así pues, en la presente invención mostramos un proceso de microencapsulación de materiales biológicamente activos de acuerdo con las reivindicaciones anteriores caracterizado porque al menos uno de los compuestos activos se elige entre el grupo de compuestos que corresponden a las siguientes estructuras moleculares (A) y (B), en todas sus variantes estereoisoméricas, y/o isoméricas:

Compuesto(s) A

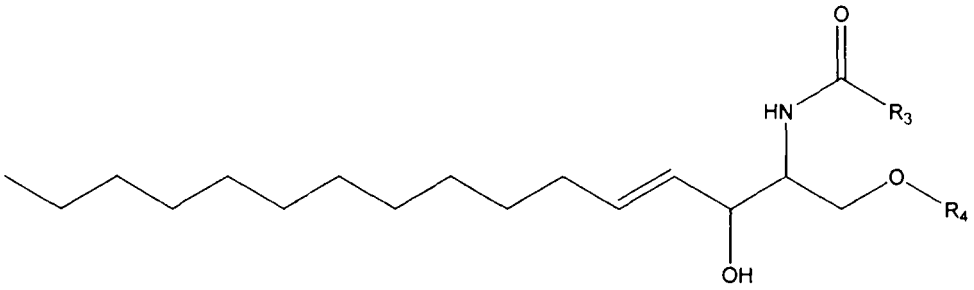


en donde,

R_1 es un éster de un ácido graso omega-3 o de un ácido graso omega-6

R_2 es un éster de un ácido graso omega-3 o de un ácido graso omega-6

Compuesto(s) B



en donde,

R_3 es un éster de un ácido graso omega-3 o de un ácido graso omega-6 o de un ácido graso w-9

R_4 es un éster de un ácido graso omega-3 o de un ácido graso omega-6 o de un ácido graso w-9 o de un oligosacárido unido covalentemente

§47 Una de las realizaciones consiste en un proceso de microencapsulación caracterizado porque al menos uno de los materiales biológicamente activos presentes en la formulación consiste preferiblemente en, al menos, un ácido graso de cadena larga (al menos 6 carbonos) insaturado, en cualquier configuración isomérica y/o estereoquímica, así como derivados de él (los) mismo(s) -preferiblemente ésteres, éteres, glicéridos, fosfolípidos, esfingolípidos y más con mayor preferencia, diglicéridos, triglicéridos, fosfolípidos, compuestos (A) y/o (B)- estereadiónico, eicosapentaenoico, docosahexaenoico, docosapentaenoico, linoleico, linolénico, gamma-linoleico, alfa-linoleico, dihomogamma-linolénico, araquidónico y/o oleico.

§48 Los ácidos grasos se eligen preferiblemente del grupo de ácidos: oleico, estereadiónico, eicosapentaenoico, docosahexaenoico, docosapentaenoico, linoleico, linoleicos conjugados, linolénico, gamma-linolénico, alfa-linolénico, dihomogamma-linolénico, araquidónico.

§49 Estos ácidos grasos pueden estar conjugados con otros compuestos que los liberen posteriormente en condiciones del estómago o aparato digestivo o procesos enzimáticos en el hígado, así pues, de acuerdo con esta invención, los ácidos grasos pueden estar conjugados, manteniendo o no manteniendo intactas todas o parte de las insaturaciones, y/o unidos covalentemente con glicéridos (esterificados) -más con mayor preferencia, formando ésteres monogilicéridos, diglicéridos y ésteres triglicéridos-; fosofolípidos; esfingolípidos; mielina; aminas; amidas; éteres; azúcares, oligosacáridos, polisacáridos; heterociclos nitrogenados, fosforados, oxigenados, sulfurados o anillos aromáticos sustituidos.

§50 Multitud de virtudes medicinales están asociadas con el uso de ácidos grasos, pero el objeto de la invención es conseguir que el efecto medicinal realmente sea posible gracias a que estos UFAs que son muy lábiles, en especial el ácido araquidónico, por su elevado número de insaturaciones (4), lleguen al consumidor en perfectas condiciones. Los inventores se han dado cuenta de que la adición simple de UFAs a alimentos, sin protección alguna, resulta en la formación de productos no deseables (aldehídos, cetonas, peróxidos, etc.) en el alimento. La novedad de esta invención estriba en que estos ácidos grasos se protegen mediante la singularidad de las microcápsulas descritas, por la provisión de antioxidantes en la cercanía física de los UFAs encapsulados, y la procedencia natural, en su caso, de

los antioxidantes, así como las propiedades de liberación controlada del contenido de las microcápsulas, que en una realización muy preferida, permanecen estables a pH > 3, y por lo tanto, en la mayoría de pH presentes en alimentos y son destruidas solamente en el estómago, cuando el pH es menos a 3.

§51-54 Los ácidos grasos insaturados de cadena larga (de más de 6 carbonos) provienen de fuentes naturales, los omega-6 y w-9 son comunes en plantas, pero los omega-3 son más difíciles de encontrar en el reino vegetal, y abundan en pescados. Además de las fuentes usuales de omega-6 y w-9, fuentes que también proporciones omega-3 son:

(a) origen vegetal: con mayor preferencia de las familias: *Boraginaceae*, en especial (*Borago spp*. y en especial *Borago officinalis*); *Linaceae* (*Linum usitatissimum, Linum arvense, Linum sativum*); *Onograceae (Oenothera biennis); Grossulariaceae* (*Ribes nigrum*), *Zea Mais, Gossypium hirsutum, Carthamus tinctorius, Glycine max*.

(b) algas, con mayor preferencia de las familias: *Graciliariceae* (*Gracilaria spp*); *Gigartinaceae* (*Iridaea spp*.); *Kallymeniaceae* (*Callopyllis variegata*); *Durvillaceae* (*Durvillaea antartica*); *Solieriaceae* (*Euchema cottoni*); *Gelidiaceae (*Gelidium spp*);* Lossoniaceae *(*Lesonia nigrescens*);* Gigantinaceae *(Gigartina spp*.); *Lessoniaceae* (*Macrocystis spp*.); *Bangiaceae* (*Porphyra spp*.) y *Crypthecodinium spp*.

(c) origen animal, generalmente de aceites de pescado, con mayor preferencia de las familias -entre paréntesis, géneros y/o especies especialmente preferidas)-: *Engaulidae (Lycengraulis olidus); Clupeidae (Sardina pilchardus); Scomberesocidae (Scomberesox saurus scombroides); Berycidae (Beryx splendens); Engraulidae (Engraulis ringens); Ophichthyidae (Ophichthus spp.); Serranidae (Hemilutjanus macrophthalmus); Scombridae (Thunnus spp.*, en especial, *Thunnus albacares, Thunnus alalunga, Thunnus obesus); Sciaenidae (Cynoscion analis); Carcharhinidae (Prionace glauca); Normanichthyidae (Normanichthys crockeri); Percichthyidae (Polyprion oxygeneios); Nototheniidae (Dissostichus eleginoides); Apogonidae (Epigonus crassicaudus); Branchiostegidae (Prolatilus jugularis); Scombridae (Thunnus spp., Thunnus albacares, Thunnus alalunga, Thunnus obesus, Sarda spp., Sarda chiliensis, Scomber japonicus peruanus), Sciaenidae (Cynoscion analis), Carcharhinidae, Normanichthyidae (Normanichthys crockeri); Percichthyidae (Polyprion oxygeneios); Nototheniidae* (Bacalao de profundidad); *Apogonidae (Epigonus crassicaudus); Branchiostegidae (Prolatilus jugularis); Cheilodactylidae (Cheilodactylus gayi); Gadidae (Salilota australis); Pomadasyidae; Scorpaenidae; Serranidae; Cyprinidae; Monacanthidae; Centrolophidae; Ophidiidae; Scorpaenidae; Coryphaenidae; Channichthydae; Sciaenidae; Aplodactylidae; Carangidae (Trachurus symetricus murphyi); Bothidae (Paralichthys microps); Mugilidae; Clupeidae; Priacathidae; Merlucciidae (Merluccius gayi, Merluccius australis); Macruronidae (Macruronus magellanicus); Gadidae (Micromesistius australis); Girellidae; Trachichthyidae; Carangidae; Kyphosidae; Callorhynchidae; Labridae ; Macrouridae; Atherinidae; Gobiesocidae; Alopiidae; Galaxiidae; Rajidae; Bramidae; Carangidae; Nototheniidae; Scianidae; Mugiloididae; Salmonidae (Salmo spp., Salmo salar, Oncorhynchus spp., Oncorhynchus kisutch, Oncorhynchus mykiss, Oncorhynchus tshawytscha); Clupeidae (Sardinops spp., Sardinops sagax, Clupea bentincki); Pomadasyidae; Gempylidae; Lamnidae (Isurus spp., Isurus oxyrinchus); Triakidae; Clinidae; Scophthalmidae; Labridae*.

◦ De especial preferencia son las especies *Atlantic mackerel, Engraulis encrasicholus, Pomatomus saltatrix, Sarda sarda, Sardina pilchardus, Brevoortia tyrannus, Brevoortia patronus, Chloroscombrus chrysurus, Auxis thazard, Scomber scombrus, Scomber japonicus, Alosa aestivalis, Clupea harengus, Etrumeus teres, Argentina silus, Ictalurus punctatus*.

(d) de origen microbiano, con mayor preferencia: *Saccharomices cerevisiae, Escherichia coli, Schizochytrium spp., Thraustochytrium aureum, Thraustochytrium roseum, Thraustochytrium striatum, Mortiriella spp., Phytium spp., Aspergillus spp. Aspergillus nidulans, Aspergillus sydowi, Fusarium spp., Fusarium equiseti, Fusarium oxysporum*

§55 Una realización de la invención es una formulación microencapsulada destinada a incrementar el desarrollo neuronal, en especial del cerebro y más especialmente en fetos, recién nacidos, lactantes y niños caracterizada porque al menos existe un compuesto caracterizado por las formulas B y/o A.

§56-58 Otra realización es una formulación microencapsulada destinada a incrementar la inteligencia potencial en fetos y bebés lactantes de leche materna -mediante el consumo de la leche materna con un vehículo alimentario para la madre apropiado en el que se añade la formulación microencapsulada-, en formulaciones de leche para lactantes y en niños, caracterizada porque contiene ácidos grasos omega-3 y omega-6 en una proporción entre 0.5 y 10.0, preferiblemente entre 1.4 y 5.7 y además contiene cerebrósidos en un porcentaje entre el 0,005% y 1% o/y opcionalmente compuestos A y/o B. La novedad de esta formulación es la incorporación de cerebrósidos y opcionalmente compuestos A o B, así como el método de proporcionar microcápsulas sin productos nocivos resultantes de degradación de FA. También existe un modo de realización consistente en una formulación microencapsulada para su empleo en fórmulas infantiles, caracterizada porque opcionalmente se prescinde de cualquier ácido graso omega-6 e, y opcionalmente se añade ácido gamma linolénico en una proporción del 1.25%. Otra realización de la invención sería una formulación microencapsulada destinada a incrementar el desarrollo del cortex cerebral y la inteligencia, caracterizada porque contiene ácidos grasos omega-3 y omega-6 en una proporción entre 0.5 y 10.0, preferiblemente entre 1.4 y 5.7 y además contiene cerebrósidos en un porcentaje entre el 0,005% y 1% y opcionalmente compuestos A y/o B.

§59, 83 Los inventores han formulado una bebida refrescante conteniendo una formulación de microcápsulas, producida de acuerdo con la reivindicación 1, caracterizada porque dicha bebida contiene microcápsulas, y estas a su vez contienen en su fase aceite ácidos omega-6 y/o omega-3, opcionalmente con antioxidantes añadidos en la fase discon-

tinúa acuosa de la microcápsula o en la fase continua hidrofóbica de la microcápsula o en ambas, y la bebida contiene aromas o extractos de: uva, piña y al menos algún cítrico, preferiblemente entre el grupo de tangerina, naranja, mandarina, limón, lima, y los ácidos grasos omega-3 y/o omega-6 permanecen estables en la bebida, una vez finalizado todo el proceso industrial (incluyendo procesos usuales de estabilización microbiológica como la pasteurización), al menos durante un mes (pérdida de omega-3 menor al 7%). Tras más de un centenar de pruebas para enmascarar el aroma extraño proporcionado por las fuentes de omega-3, al final, los inventores han obtenido una bebida la cual, mediante un panel de expertos en catas, se logro enmascarar totalmente el mal olor de pescado o el sabor metálico del aceite de lino (n=12, d.f.= 7, P<0,05). Otra realización de la invención relacionada con ésta es Zumo conteniendo microcápsulas producidas de acuerdo con cualquier combinación adecuada de las reivindicaciones anteriores caracterizado porque (a) las microcápsulas contienen ácidos omega-3 provenientes de una formulación comercial basada en aceite comestible de lino; (b) la fase aceite contiene el aceite de lino y un emulgente basado en compuestos de soja (c) la fase agua contiene una mezcla de diferentes subclases de hidrocoloides del tipo de los alginatos y/o goma arábiga y/o kappa-carragenato y/o goma guar, además de un emulgente primario alimentario de HLB entre 10 y 14, y un modificador de viscosidad alimentario (d) y el pH de la formulación de microcápsulas esta en el rango de 3-6, el tamaño en el percentil 50 de las microcápsulas recién producidas está en el rango 1-10 μm. (e) el componente mayoritario del zumo es zumo de naranja.

§63 Las microcápsulas producidas, en virtud de su biocompatibilidad al emplear hidrocoloides adecuados, y sobre todo, en virtud de la novedad de conseguir multi-encapsulación con un tamaño de partícula final, medido con el instrumento debidamente calibrado MasterSizer®, es de una mediana de alrededor de 0,5-1,5 μm, en una realización preferente no limitante, pueden ser apropiadas para xenotransplantes con materiales (las propias microcápsulas) biocompatibles, autotransplantes, transplantes de islotes de Langherns, y otros transplantes con características similares.

§60-71 Variando el tipo de hidrocoloide o hidrogel empleado, los inventores han sido capaces de formular microcápsulas que se destruyen a pH bajo (como el presente en el estómago humano) o son resistentes a pH bajo y se liberan en función del tiempo y especialmente por el tiempo de paso por el estómago, en el intestino. También otros métodos enzimáticos (p. ej., amilasas, tripsina) se pueden romper paredes de microcápsulas producidas como se ha descrito anteriormente, con la diferencia de que las paredes poseen restos digeribles (almidón, por ejemplo). También la presión puede ser utilizada (por ejemplo, microcápsulas cuya pared consta de una mezcla de goma arábiga, gomas de algarrobo y ciclodextrinas, conteniendo mentol en su interior, que se libera por combinación de humectación o presión por masticado, produciendo una liberación muy rápida del aroma deseado. Puesto que en ningún caso la invención está limitada a la alimentación humana, las microcápsulas pueden ser diseñadas para las condiciones particulares de cada animal al que vayan a ser administradas (p. ej., pH del estómago de los perros está en torno a 1,luego una realización de la invención sería una microcápsula con una pared con un alto grado de polimerización (resistiendo así el incremento de esfuerzo de la microcápsula al ser masticada) que, en términos de liberación en el estómago, se compensa por una mayor efecto del descenso del pH). Como se verá más adelante, el grado de desarrollo de la invención permite que las microcápsulas permanezcan estables en el yogur (ácido) pero se libere el contenido en el estómago humano (más ácido aún).

§72 Las microcápsulas pueden ser empleadas en productos llamados ecológicos o biológicos, siempre que los a.i., los materiales de la pared provengan de fuentes naturales, así como los demás aditivos tecnológicos empleados proceden de fuentes naturales (p. ej., pared basada en almidón, agar y carragenatos, emulsionantes provenientes de huevos de gallina, etc.)

§73 Como contrapunto a la realización anterior, se pueden emplear, para la obtención de ingredientes activos, organismos genéticamente modificados, variedades vegetales híbridas u obtenidas mediante selección humana, así como cultivos microbiológicos seleccionados mediante cualquier técnica. Esta realización es posible pero no preferible porque los consumidores en general tienden a evitar los organismos modificados genéticamente.

§74 Las microcápsulas se pueden aplicar a alimentos destinados a consumo animal, en especial en ganadería, (opcionalmente avicultura), piscicultura, cría de animales domésticos y mascotas, en especial para aumentar el contenido de omega-3/-6 en los huevos o carne.

§75-76 Obviamente, las microcápsulas pueden formar parte de formulaciones medicinales, de farmacia o para-farmacia, sean en combinación con principios activos no presentes en las microcápsulas o siendo los ingredientes activos presentes en las microcápsulas o formulación de las microcápsulas los únicos ingredientes activos de la preparación medicinal, incluyendo bajo el término preparación medicinal también materiales de contraste en radiología, semillas para radioterapia oncológica, termoterapia o terapia por irradiación de luz de cualquier longitud de onda.

§77-78 Puesto que muchos de los a.i. beneficiosos para la salud son lábiles, especialmente a la oxidación, una realización de la invención es mantener las microcapsulas, realizadas mediante los procesos aquí descritos o mediante cualquier proceso, así como cualquier formulación de omega-3/-6/-9 y/o antioxidantes (puros, como extractos, como dispersión de sólidos, etc.), separadas del alimento o bebida hasta unos momentos antes del consumo final, opcionalmente con un receptáculo que al ser apretado libera la formulación, preferentemente seca, al alimento o a la bebida.

§79 Microcápsulas producidas de acuerdo con las reivindicaciones precedentes, caracterizadas porque los materiales de la pared de las microcápsulas se disuelven o degradan o liberan los materiales activos cuando se encuentran

en la boca del consumidor (sea humano u otro animal), siendo capaz de apreciar las cualidades organolépticas de al menos un material microencapsulado.

Ejemplos

Los ejemplos siguientes se dan de forma solamente ilustrativa, y remarcan los aspectos esenciales de la presente invención. Existen multitud de variaciones de los ejemplos presentados, que, naturalmente, caen dentro del espíritu de la presente invención.

Se adjunta una Tabla de resultados y 19 figuras.

El solicitante ha desarrollado métodos de los que es propietario, para analizar formulaciones de microcápsulas producidas de acuerdo la siguiente invención, mediante cromatografía en gel, RMN, HPLC-MS, GC-MS, FT-IR y métodos enzimáticos para determinar los polímeros empleados en las microcápsulas, así como procedimientos para análisis microscópico de la estructura de las paredes. Estos procedimientos están disponibles por parte del solicitante para el cumplimiento de leyes de organizaciones Sanitarias Gubernamentales o Internacionales para la comercialización de productos conteniendo las microcápsulas descritas en la presente invención.

Ejemplo 1

Este es ejemplo muy explicativo del alcance de la invención, aunque se muestra de modo no relevante. Se expone la realización de un modo de invención, el proceso con aplicación industrial, las microcápsulas formadas y su aplicación.

Se trata de formular un zumo de naranja que contiene un suplemento nutritivo con antioxidantes provenientes de romero, carotenoides provenientes de zumo de carlota y ácidos grasos omega-3 principalmente, aunque hay otros FA también en la formulación provenientes del aceite de lino.

La formulación de microcápsulas se realiza para una masa total de 1 kg. La concentración final de aceites de lino y de *Borago officinalis* en el zumo de naranja es del 1.5% (los cálculos se deben realizan en base a esta exigencia en este ejemplo).

1.1.- *Ingredientes*

Fase aceite	[%]
Aceite de lino+Aceite de borago [1:1 p/p]	25.00
Metarin®	1.00
Fase agua	
Agua bidestilada*	20.00
Extracto de romero**	2.80
Zumo de zanahoria	7.30
Orlistat® (inhibidor de lipasas)	1.00

1.2.- *Aditivos tecnológicos para emulsificación y encapsulación*

	[%]
Solución de alginatos***	12.35
Solución de goma Xantana****	12.35
Goma guar (4% en agua)	15.40
Lamegin®	2.50
Keltrol®	0.30

* en ella están presentes además 0.5% $CaCl_2$, 0.2% ácido cítrico, 0.08% nipagil®.

** extracto de hojas de romero en solución - al 15% (p/p) - en etanol/agua 10/90 (v/v)

*** Alginatos al 25% (v/v) en agua.

**** Goma Xantana al 5% (v/v) en agua

1.2 *Proceso*

La fase aceite se forma mezclando los componentes y se homogeneiza en baño de ultrasonidos durante 10 min. a 20ºC.

La fase agua se realiza también mezclando los componentes y homogeneizándolos en un baño de ultrasonidos durante 30 min. a 40ºC.

La emulsión W/O se forma mediante, primero situar la fase aceite en un reactor de 2 litros, calentado (o enfriado) por circulación termostatada de agua en las paredes exteriores a 40°C, y añadiendo a la fase aceite, tras un periodo de estabilización en el cual la fase aceite ha alcanzado 40°C, la fase agua a 20°C. (En ocasiones el choque térmico puede ocasionar inversión de fases, pero no ocurre en este determinado ejemplo; en tales casos es preferible mezclar las dos fases a la misma temperatura). La adición de la fase aceite a la fase agua, se realiza mediante agitación que provoca esfuerzo de corte en el líquido, por medio de un agitador a 7320 rpm, durante 25 min. Las gotas de aceite así formadas mostraron tener un tamaño de partícula (MasterSizer®) con mediana de 150 μm.

Después adiciona la solución con los hidrocoloides goma xantana y alginatos (punto 1.2) y la el agitador se ralentiza hasta 350 rpm, a una temperatura de 50ºC. Los inventores, en otros experimentos son capaces de emplear diversas configuraciones químicas de alginatos, que no afectan esencialmente al proceso, pero resultan en ligeras diferencias en las propiedades finales de la solución de microcápsulas (a igual condición del resto de factores del proceso).

De esta forma se forma la emulsión múltiple (W/O)/W. Al contrario de lo descrito en la bibliografía (véase Food Emulsions; S.E. Friberg , K. Larsson y J. Sjöloml., Marcel Decker Ink, New York, p.63 y pp. 353-421), no se forma una "doble emulsión" (una fase agua que contiene gotas de aceite con una única gota de agua), sino que, gracias a la existencia inmediata de hidrocoloides en la superficie de las gotas de agua que por afinidad polar quedan adheridos a ellas, se vence la tendencia termodinámica a que la múltiple emulsión se convierta en una doble emulsión (esto es, diversas gotas de agua dentro de cada gota de aceite, sin que se produzca coalescencia: unión de todas las pequeñas gotas de agua en una sola terminando el proceso con una emulsión doble, y no una emulsión múltiple). Este apartado es una gran diferencia con el estado de la técnica anterior, puesto que se consigue, ya durante el proceso, y por supuesto más adelante por un periodo quasi-infinito, la estabilización de la emulsión múltiple -ya que a medida que avanza la polimerización y entrecruzamiento de los hidrocoloides, las paredes de las gotas de agua se estabilizan [frente a la coalescencia] progresivamente-. En la detallada revisión realizada en el reciente libro arriba mencionado (en prensa en el 2003, publicación en el 2004), de unos autores de lo más reputados en el área de emulsiones, encapsulaciones y microencapsulaciones, se explica que la emulsión múltiple (como la que ya que comienza a formarse en este paso de nuestro proceso), deriva en una emulsión doble si no existen parámetros que eviten esta degeneración de la emulsión múltiple. Nuestro proceso no se encuadra de ningún modo posible, con los descritos para emulsiones (W/O)/W. Ni siquiera los ejemplos de emulsiones múltiples de microcápsulas que existen en la literatura científica, han alcanzado una aplicación industrial, según los autores referenciados. Obviamente la microencapsulación ayuda a la existencia de una emulsión múltiple (o como sinónimo, "multi-microencapsulación", pues aquí tratamos de microcápsulas), pero los ejemplos (sobre todo en el campo de proteínas para use en medicamentos), distan enormemente de la sencillez de este proceso de este ejemplo, más aun del tipo, coste, materiales y proceso de multi-microencapsulación.

Macrofotografías realizadas en la Universidad de Viena, mediante criofractura y por scanning, muestran que realmente existe una multi-microencapsulación en nuestro proceso, no solo la existencia de diversas gotas de agua en la gota de aceite, sino también gotas de agua encerradas en gotas de aceite y a su vez encerradas en gotas de agua (hasta cinco grados de multi-microencapsulación).

El tamaño de partícula se va reduciendo progresivamente aproximadamente a 90 μm.

Seguidamente se añade la goma arábiga, a 8500 rpm a 40ºC.

El tamaño de partícula, que se va reduciendo progresivamente durante todo el proceso, se ve altamente reducido con el siguiente paso, esto es, la adición de Lamegin®, y Keltrol®. La velocidad de giro se aumenta a 10000 rpm a 40ºC durante 20 minutos.

Para un perfecto acabado de la formulación deseada, se van tomando muestras cada minuto y se mide el tamaño de partícula. Los inventores, a diferencia muy notable del estado de la técnica, han conseguido en una formulación muy similar a la del este ejemplo, que el tamaño de partícula de las microcápsulas en este paso sea de 0.4 μm, algo que, a nuestro buen saber, no ha sido jamás descrito. Es remarcable que el rango habitual para un microcápsulas de múltiple emulsión esta entre 40-50 μm [Adv. Drug Delivery Rev., 28:85-86 (1997); J. Control Release, 38:219-228 (1996); J. Microencaps., 14:225-241 (1997)].

En el paso siguiente se añade Lamegin®, continuando el decremento de tamaño de partícula que en general se sitúa entre 5-30 μm.

A continuación se procede al curado de las microcápsulas a 4000 rpm, que en general tiene una duración de 30 min. a 75°C. En este tiempo, aún se consigue una reducción mayor de tamaño de partícula, llegando a un rango de entre 1-5 μm (variando la velocidad de giro se puede disminuir aún más el tamaño de partícula, pero cabe el riesgo de romper las microcápsulas si la combinación de parámetros temperatura-agitación-tiempo no es correcta).

En esta misma encapsulación, como se muestra en la Fig. 8, hemos obtenido (antes de añadir el modificador de viscosidad) un tamaño de partícula de media de 0,89 μm, algo realmente novedoso en el campo de la microencapsulación con emulsiones múltiples.

Finalmente, se añade el modificador de viscosidad Keltrol®, para estabilizar la suspensión de microcápsulas, a una velocidad de 4000 rpm, manteniendo esta velocidad durante 5 min.

En este momento las microcápsulas ya están completamente formadas, y la solución a aplicar en el zumo, preparada. Únicamente resta enfriar gradualmente la temperatura. Es remarcable decir que con el paso del tiempo, y dependiendo de concentraciones de aditivos, y otros muchos parámetros (los inventores consiguen evitar una agregación excesiva, en procesos muy similares a este, con variaciones en el pH, siempre dependientes de las propiedades iónicas de las materiales de la pared de la microcápsula y de la fuerza iónica del medio acuoso), el tamaño medido de partícula se incremente, debido a formación de pequeños aglomerados.

En el presente ejemplo, el tamaño de partícula (que se refiere al tamaño de las microcápsulas y/o agregados de microcápsulas) final, tras 24 horas de finalizado el proceso, es de 8 μm.

En una variante de este mismo ejemplo, con una mayor agitación en el paso de adición de Lamegin®, y un ajuste final de pH a 6.1, se consigue una formulación de microcápsulas que 24 horas tras su preparación es de 3 μm. No obstante, como se ha señalado más arriba, se ha llegado a tamaños de partícula de 0.4 μm, y es posible detener el proceso antes de la adición de Keltrol y alcanzar microcápsulas de 0.29 μm. Obviamente, no es de extrañar que una reducción a 0.01 μm pueda ser alcanzada, puesto que las fuerzas de movimiento Browntoniano pueden verse compensadas con la adición de factores de control físico-químico apropiados.

Ejemplos de 2 a 11

En la Tabla 1, se presenta una serie de procesos de microencapsulación, con un procedimiento similar al arriba indicado; es autoexplicativa, y muestra tanto diferentes componentes como los resultados obtenidos. Los datos proporcionados sobre tamaño de partícula corresponden al percentil 50 (mediana) y al percentil 90.

En la última fila podemos ver la calidad de la formulación resultante. Se observa claramente que pequeños cambios en la composición/procedimiento pueden suponer una mala microencapsulación y/o formulación.

Ejemplo 12

En la presente realización de la invención, mostramos la liberación de riboflavina de microcápsulas producidas según se describen en la presente invención, de acuerdo con variación del pH del medio.

Las presentes microcápsulas son estables a pH ácido usual en yogures, pero se rompen al pH más ácido del estómago.

Hemos comprobado la velocidad de liberación de la riboflavina microencapsulada (de acuerdo con la presente invención) que se encuentra presente en un yogur probiótico.

El yogur fue preparado (20 kg) hecho en nuestro laboratorio usando un cultivo inicial proveniente de un yogur obtenido en una quesería tradicional, cultivo de *Lactobacillus casei*.

La composición del yogur es la siguiente, en lo que respecta a ingredientes activos adicionales:

- Riboflavina: 100 μg/kg yogurt (menos del 0.1% del total de ingredientes activos)

- *Lactobacillus casei* 2% (solución en agua de un cultivo con 500 colonias por cm^2)

- *Avena sativa* extracto 98% [30% residuo seco]

La formulación ha sido preparada siguiendo un procedimiento de encapsulación muy similar al descrito en el Ejemplo 1, con una mezcla de ciclodextrinas y amilex® como hidrocoloide entrecruzado y goma de algarrobo y goma guar como hidrocoloides protectores.

En el ensayo se ha probado la liberación en medio ácido símil al estómago, liberación en símil yogur y liberación en ambos casos de material no encapsulado.

Se han realizado dos tipos de ensayo, en primer lugar la liberación en condiciones del estómago mediante una cámara y después en una solución en la que el pH es el pH aproximado típico de muchos yogures (pH = 3.7). El blanco consistió en una solución de agua de igual volumen en ambos medios. Se realizó una comparación con material microencapsulado de acuerdo con la invención US 6,234,464, entendiendo que los parámetros no descritos exactamente en la mencionada invención (pues no se describe un método detallado de como realizar la invención de forma completa) fueron optimizados respecto a material no encapsulado y restos de pared no reaccionada.

Es remarcable señalar que los efectos de la matriz alimentaria en el yogur no son relevantes es este tipo de experiencias. En ensayos realizados posteriormente, el comportamiento en yogures fue similar al del test B) (en solución

con pH = 3.7 estable, solución isotónica). No obstante, debido al incremento de artefactos en el análisis, preferimos mostrar los resultados de este ensayo, puesto que la precisión y exactitud con la que se mide la cantidad de riboflavina es mayor, a igual número de muestras.

Para el análisis de las muestras se empleo cromatografía HPLC (columna RP18) acoplada a espectrómetro de masas (AGILENT 1100®) -abreviadamente, HPLC-MS y una muestra estándar de riboflavina (pureza>95%) [Sigma-Aldrich®]. Antes del análisis se procedió a una extracción de la riboflavina de los distintos medios (liquido-liquido y flash-cromatografía).

Se observó que la liberación de la vitamina B_2 de la formulación incorporada en el yogur, de acuerdo con la presente invención, ocurre en el estómago humano, pero no en el yogur.

A-*Resultados en medio acídico del estómago*

La media de cantidad liberada de riboflavina en coediciones similares a las del estómago, de nuestra formulación microencapsulada tras 30 min. es de 41.5 μg/kg, esto es una conversión de la muestra de entre 40 - 50%. Tras 60 min. son liberados 55.7 μg/kg [esto es, una conversión de cerca de entre 50 – 60%].

En el medio estómago, la liberación de la riboflavina de acuerdo con US 6,234,464, es mayor. Tras 30 min. es de 96,8% μg/kg, esto es una conversión de la muestra de entre 95 - 100%. Tras 60 min. son liberados 97.7 μg/kg [esto es, una conversión de cerca de entre 95 – 98%].

El blanco no mostró ninguna liberación de riboflavina, obviamente.

B-*Resultados en medio acídico del yogur*

La formulación microencapsulada de nuestra invención no mostró ninguna liberación en el yogur de riboflavina detectable por el GC-MS.

La formulación de la formulación de acuerdo con US 6,234,464 mostró una liberación en el yogur de riboflavina de en el plazo de 60 min. del 90% - 95% (92.5 μg/kg).

Los blancos no mostraron liberación alguna de riboflavina en el yogur (obviamente de esperar pues no existe riboflavina).

Se deduce de esta experiencia que la formulación de acuerdo con US 6,234,464 se produce en ambos medios, tanto como en el yogur como en el estómago (siendo la liberación en el estómago superior a la liberación de nuestra fórmula microencapsulada). En cambio la formulación de acuerdo con nuestra invención mantiene la riboflavina completamente protegida de procesos de degradación-oxidación en el yogur, al estar encerrada dentro de las cápsulas, y, no obstante, permite la liberación de la riboflavina en el estómago. Precisamente este es un objeto de la invención: mantener los ingredientes activos completamente protegidos hasta su consumo, evitando cualquier deterioro de los mismos durante su permanencia en el alimento al que son añadidos, o incluso, evitar los sabores extraños asociados con ciertos a.i. que imparten al medio alimentario si no están completamente encapsulados establemente.

Ejemplo 13

Uno de las características de esta invención es la capacidad de mantener estables los ingredientes activos por un tiempo mayor con respecto el estado de la técnica.

No sólo es importante que los ingredientes activos permanezcan estables durante el procesado de los alimentos, sino que también sean estables en el tiempo.

El proceso de encapsulación es básicamente el presentado en el ejemplo 1, con la excepción de que los materiales de la pared secundaria se forman con goma de Caraya (a diferencia de goma arábiga), el emulgente es Softenol® 3767 (1%) y el modificador de viscosidad es Glycosperse® (1%), siendo la fuente de ácidos grasos insaturados aceite de pescado (*Clupea harengus*).

Los resultados de este experimento se muestran en la siguiente tabla, donde podemos apreciar la estabilidad de los ácidos grasos, durante 60 días a 45ºC, es excepcional.

	Palmítico % en aceite	Esteárico el% en aceite	Oleico el% en aceite	Linoleico el% en aceite	alfa-linolénico el% en aceite	w-3 el% en aceite
d=0	1,1	1,4	2,9	2,8	2,7	7,8
d=30; 4 °C	1,1	1,4	2,7	2,6	2,5	7,8
d=30; 25 °C	1,1	1,4	2,6	2,6	2,6	7,7
d=30; 45°C	1,1	1,3	2,6	2,5	2,5	7,7
d=60; 45°C	1,1	1,3	2,4	2,5	2,4	7,5

Ejemplo 14

El mayor problema con las formulaciones de microcápsulas y emulsiones es que hay muchos parámetros que influyen el producto final, y variaciones en los mismos pueden conducir a resultados muy diferentes. Puesto que el solicitante ha desarrollado la aplicación del método de multi-microencapsulación para muy diferentes ingredientes activos, microcápsulas y matrices en donde se encuentran estas formulaciones en los alimentos (es muy distinto una formulación para leche que para un zumo ácido -de limón, por ejemplo-).

El número de experimentos requerido para una validación estadística de los parámetros que influyen determinada formulación (dentro de un rango aceptable de variabilidad) es extremadamente elevado. El solicitante ha aplicado de modo sistemático avanzadas técnicas estadísticas para reducir el número de experimentos a la hora de desarrollar una nueva formulación.

En el ejemplo siguiente el solicitante ha empleado un diseño experimental doblado Plackett-Burman con 3 puntos centrales y un número de grados de libertad aceptable (19). En este caso, el número total de pruebas a realizar es 27, en lugar de las 64 que se emplearía en un diseño usual de experimentos.

Se ha estudiado la influencia final en la formulación los siguientes parámetros:

- Fase aceite (aceite de semillas de uva [50%] + aceite de salmón [50%]) a 2 niveles: 10% y 30% en producto final

- Extracto natural (orujos de uva [50%] + té verde descafeinado [50%]) a 2 niveles: 10% y 20%

- Solución de alginatos [3% en agua] a 2 niveles: 5% y 10%

- Solución de carragenatos [3% en agua] a 2 niveles: 5%-10%

- Extracto de Yucca glauca a 2 niveles: 3%-5%

- Homogeneización de la suspensión de microcápsulas a 2 niveles: presente o no presente

- Spray-dry a 2 niveles, presente o no presente

La variable independiente en este caso es un valor que refleja la adecuación de la formulación final para fines industriales, en particular para su adición en galletas.

Experimentalmente los inventores han determinado los coeficientes (peso en el índice de aceptabilidad de producto) que cuantifican las diversas propiedades medidas:

Aceptabilidad

$$= \frac{(0.20 * TamañoDeParticula + 0.30 * Densidad + 0.15 * Polimerosnoreaccionados + + 0.15 * gradodeencapsulacion + 0.20 * ingredienteslibresnoencapsulados)}{1} * 100$$

De este modo, obtenemos -mediante el uso del programa informático Statgraphics®- un diseño aleatorio como sigue, siendo -1 el valor más bajo y 1 el más alto (la última curva corresponde al índice de aceptabilidad resultante de cada test).

test	Aceite	Plants	Algin..	Xant.	Yucca	Homg.	Atom.	Aceptabilidad
1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0
2	1,0	-1,0	-1,0	-1,0	1,0	-1,0	-1,0	10
3	1,0	-1,0	1,0	1,0	-1,0	1,0	-1,0	95
4	1,0	1,0	-1,0	1,0	1,0	-1,0	1,0	60
5	1,0	1,0	-1,0	-1,0	-1,0	1,0	-1,0	84
6	-1,0	-1,0	1,0	-1,0	1,0	1,0	1,0	32
7	1,0	-1,0	1,0	-1,0	-1,0	-1,0	1,0	20
8	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0
9	-1,0	1,0	1,0	1,0	-1,0	-1,0	-1,0	60
10	-1,0	-1,0	-1,0	1,0	-1,0	-1,0	1,0	30
11	-1,0	-1,0	1,0	1,0	1,0	-1,0	1,0	28
12	1,0	1,0	-1,0	1,0	-1,0	-1,0	-1,0	45
13	1,0	-1,0	1,0	1,0	1,0	-1,0	-1,0	31
14	-1,0	1,0	1,0	1,0	-1,0	1,0	1,0	69
15	-1,0	-1,0	-1,0	1,0	1,0	1,0	-1,0	85
16	1,0	-1,0	-1,0	-1,0	1,0	1,0	1,0	93
17	-1,0	1,0	-1,0	-1,0	1,0	-1,0	1,0	15
18	-1,0	-1,0	-1,0	-1,0	-1,0	-1,0	-1,0	7
19	1,0	-1,0	-1,0	1,0	-1,0	1,0	1,0	54
20	-1,0	1,0	-1,0	1,0	1,0	1,0	-1,0	61
21	-1,0	-1,0	1,0	-1,0	-1,0	1,0	-1,0	12
22	1,0	1,0	1,0	1,0	1,0	1,0	1,0	69
23	1,0	1,0	1,0	-1,0	1,0	1,0	-1,0	81
24	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0
25	-1,0	1,0	1,0	-1,0	1,0	-1,0	-1,0	20
26	1,0	1,0	1,0	-1,0	-1,0	-1,0	1,0	17
27	-1,0	1,0	-1,0	-1,0	-1,0	1,0	1,0	72

Los resultados del ANOVA arriba se encuentran en la Tabla 2, y se muestra que todos los parámetros estudiados tuvieron influencia en la aceptabilidad del producto final. Esto se indica mediante el valor P (<0.05 en todos los casos). Por lo tanto, en el desarrollo de una formulación de bebida energética que mejore la salud, no podemos despreciar ninguno de estos parámetros. Es remarcable la extrema influencia que tiene la homogeneización en este tipo de formulación.

Ejemplo 15

Hemos ensayado la estabilidad de una formulación (de acuerdo con el ejemplo 9, mejorando los resultados previos con la adición de un emulgente secundario -span 65, al 5%-) de esporas de *Bacillus subtilis*. Posteriormente hemos ensayado que las esporas eran viables (sembrándolas en placas Petri en el medio de cultivo comercial denominado potato dextrose-agar y verificando el desarrollo de colonias pertenecientes a la especie *B. subtilis*).

Los resultados de la estabilidad de las microcápsulas, basados en la estabilidad del tamaño de partícula de la dispersión, a diferentes tiempos de envejecimiento de la formulación (dispersión de microcápsulas).

Los resultados de estabilidad de las microcápsulas, basados en la estabilidad conforme el tamaño de partícula, se muestran en la Fig. 9. Se observa una distribución satisfactoria de este parámetro, obedeciendo las diferentes curvas a diferentes tiempos y temperaturas, como sigue:

A = (tiempo 0, T= 25ºC)

B = tras 60 días a 3ºC

C = tras 60 días a 25ºC

D = tras 90 días a 25ºC

La forma de las curvas es homogénea, significando que durante el proceso no se ha ocurrido rotura de las microcápsulas. El porcentaje total de bacterias no encapsuladas es del 5%, medido en la solución resultante de realizar una separación física por floculación de las microcápsulas y filtrado.

Ejemplo 16

En el análisis de formulaciones de multi-microcápsulas, hemos obtenido los siguientes diagramas de viscosidad frente esfuerzo de corte. Este tipo de diagramas caracterizan a diversas realizaciones de la presente invención, es decir, con ellos podemos saber si se corresponde el producto final con la formulación pretendida.

El pico mostrado en las figuras de 10 a 12 es característico de nuestra formulación. Indica que la estructura interna de la suspensión de microcápsulas disminuye progresivamente de acuerdo con la fuerza aplicada (esfuerzo o tensión de corte), pero tras un periodo de tiempo (esto es, mayor tiempo y mayor fuerza) las fuerzas cohesivas, que mantienen estable la suspensión de microcápsulas, se rompen (exactamente hasta el pico mostrado). No ocurre una rotura de las microcápsulas, sino de la estructura de la suspensión en la que se encuentran (si no existiera cierta estructura, la solución no seria estable en diversos aspectos, p. ej., floculación, precipitación, coacervación, etc.).

En cambio, en la figura 13, no se observa ningún pico, sino un decremento de la viscosidad proporcional a la tensión de corte. En este caso, las fuerzas de cohesión, electroestáticas, son muy débiles, y el producto es de baja viscosidad (cercana a la del agua). Este tipo de comportamiento es aceptable en ciertas formulaciones, pero es más preferible el comportamiento mostrado en las figuras de 10 a 12. Si la curva es casi una recta (figura 13, curva inferior) se trata de una formulación con un comportamiento de fluido newtoniano, que no es recomendable (aunque aceptable en ciertos casos) para el objeto de la presente invención.

Ejemplo 17

En el presente ejemplo mostramos otra realización de la invención en la que se encapsulan minerales, en concreto selenio y citrato de zinc. En la microfotografía (Fig. 14) se ve claramente (encerrado por el óvalo) un cristal de citrato de zinc, presente en la fase acuosa continua. Se observa también el efecto de multi-microencapsulación (gracias a una formulación determinada de las microcápsulas, con una muy alto tamaño de poro que permite su apreciación mediante microscopio óptico).

Ejemplo 18

En el ejemplo (Fig. 15 y Fig. 16) presente mostramos dos tipos de microcápsulas, cuando todavía aún no están formadas y permiten fácilmente ver el grado de multi-microencapsulación con microscopio óptico. Se observan microcápsulas de doble emulsión y también verdaderas microcápsulas con emulsión múltiple.

Ejemplo 19

En el siguiente ejemplo mostramos una comparación de productos existentes en el mercado conteniendo ácidos grasos poliinsaturados de probadas virtudes medicinales, con las formulaciones realizadas de acuerdo con la presente invención. Se trata de un ensayo de estabilidad de los omega-3/-6 respecto a la pasteurización, homogeneización y al tiempo.

Por un lado, hemos empleado una formulación microencapsulada, de acuerdo con la presente invención y el ejemplo 1, de una mezcla de omega-3 (ácido Docosahexenoico, AHD) y omega-6 (ácido araquidónico, ARA) proporcionados por Degussa, y a partir del producto Ovothin®, obteniendo un producto con una relación ARA:DHA de 1:2. Es decir, los ingredientes activos ARA y DHA han sido multi-microencapsulados mediante el proceso descrito en la presente invención.

Por otro lado, hemos empleado los productos comerciales ARASCO® y DHASCO®, de tal forma que obtenemos una producto no microencapsulado consistente en ARA:DHA en una relación 1:2.

Los análisis de ácidos grasos se realizan en GC-MS, previa derivatización de los mismos, con un protocolo estándar validado analíticamente para análisis de omega-3 y omega-6 en leche, de acuerdo con la literatura científica pública. Al mismo tiempo, pero en muestras separadas del mismo producto y fase de ensayo, se analizan productos de oxidación.

El protocolo del ensayo es el siguiente, para ambos productos, en las mismas cantidades respecto al contenido en ARA y DHA:

1.- La formulación se mezcla con una matriz (leche) -análisis inicial-

2.- La muestra se pasteuriza durante 10 min. a 95°C

3.- Se enfría la leche a 60ºC

4.- La muestra se homogeneiza a 200-300 bar durante 10 min. -análisis tras pasteurización y homogeneización-

5.- La muestra se almacena herméticamente a 5°C y durante 14 días y también durante un mes. -análisis respecto al tiempo-

Los resultados (ensayos realizados por quintuplicado, se representa diagrama de barras correspondiente a la media) se muestran en la Fig. 17.

Se observa que la pasteurización y homogeneización provoca una degradación de ácidos grasos sustancial en la mezcla no encapsulada ARASCO-DHASCO, con una pérdida del 33% de la cantidad inicial de ARA y DHA. Sin embargo, la formulación microencapsulada acorde con la presente invención preserva el 100% de los ácidos grasos en la pasteurización y homogeneización. El almacenamiento durante 2 semanas a 5°C no afecta a la estabilidad de las formulaciones en ninguno de los dos casos, y el almacenamiento durante 25 días a 25° varia la cantidad de ARA y DHA en las muestras de ARASCO y DHASCO, así como en las muestras correspondientes a nuestro proceso de encapsulación. Evidentemente, a efectos prácticos no tiene sentido el almacenamiento de la leche durante dos semanas a 25°, pero deseamos encontrar un punto en el cual nuestra formulación mostrara (de manera similar a la de ARASCO y DHASCO) una degradación de los ARA y DHA.

Se concluye, según nuestro ensayo, que la leche a la cual se le añade ARASCO y DHASCO sin encapsular, sufre, durante los procesos tecnológicos universales de tratamiento de la leche, una pérdida del 33% de DHA y ARA. Es fácil extrapolar que estas perdidas se producirán también de una forma cualitativamente similar en otros alimentos que sufran procesos tecnológicos similares.

Uno de los problemas que resuelve la presente invención es la prevención de la formación de compuestos tóxicos que se forman ante la degradación de DHA y ARA, y ácidos grasos en general. Es obvio que el 33% de pérdidas en ARASCO y DHASCO acaba formando parte del grupo de sustancias que están sobradamente descritas en la bibliografía como productos de descomposición química de ácidos grasos, que son tóxicos. De alguna forma, un compuesto pretendidamente beneficioso se puede transformar en no beneficioso para la salud por formación de compuestos del tipo mostrado en la Fig. 18.

Con más detalle, presentamos cromatogramas de ambas muestras antes y después de la pasteurización y homogeneización. En la figura 19 vemos que antes de mencionados procesos, el perfil cromatográfico es similar (se han monitorizado exclusivamente picos que son en realidad producto del mero hecho de que las matrices iniciales de los ARA y DHA son diferentes debido principalmente a las fuentes de ácidos grasos). Se observa la presencia de propionaldehido y capronaldehido en el cromatograma (Fig. 19) relativo al estado de la leche tras la pasteurización y homogeneización cuando se emplean ARASCO y DHASCO como fuente de ácidos grasos ARA y DHA.

REIVINDICACIONES

1. Proceso de multi-microencapsulación continua, mediante polimerización interfacial e *in situ*, de materiales biológicamente activos **caracterizado** porque

(a) en un primer paso se adiciona una fase agua que contiene un iniciador de polimerización y, opcionalmente al menos un material biológicamente activo, a una fase aceite, que contiene opcionalmente al menos un material biológicamente activo; adicionalmente existe al menos un emulgente en al menos una de las dos fases mencionadas, y existe un material biológicamente activo en al menos una de las dos fases

(b) en un segundo paso se añade una solución o dispersión acuosa conteniendo al menos un hidrocoloide, que provoca una inversión de fases, y al mismo tiempo el hidrocoloide comienza a depositarse y a polimerizarse en las paredes de las nuevas gotas consistentes en una emulsión agua en aceite, ocurriendo también un entrecruzamiento de los polímeros de hidrocoloide, opcionalmente en presencia de cationes

(c) en un tercer paso, se añade una solución o dispersión acuosa que contiene al menos un coloide protector, el cual comienza a depositarse en la superficie de las gotas de agua en aceite, y a polimerizarse y entrecruzarse consigo mismo y con el hidrocoloide

(d) después se añade una solución o dispersión acuosa de emulgente primario que permite una notable reducción del tamaño de las gotas de agua en aceite

(e) en el proceso de reducción de tamaño de gotas, las parcialmente formadas microcápsulas se deaglomeran y reaglomeran, ocurriendo eventualmente un encerramiento de gotas dentro de otras mayores (multi-microencapsulación)

(f) cuando ha pasado suficiente tiempo para que las gotas de agua en aceite se recubran de, al menos, un hidrocoloide y, al menos, un coloide protector, se incrementa la temperatura para fortalecer la pared de las mencionadas gotas; en este instante ya son microcápsulas o multi-microcápsulas en suspensión acuosa

(g) opcionalmente se seca la formulación para obtener polvo, y opcionalmente se reformula mediante técnicas pertenecientes al estado de la técnica para obtener (o ser mezcladas las microcápsulas en) polvos mojables, gel, cremas cosméticas o medicinales, productos de baño, medios de cultivo de microorganismos; opcionalmente se añaden aditivos (opcionalmente antiaglomerantes) para formulaciones secas de las microcápsulas.

(h) todo el proceso -opcionalmente excepto el paso g)- se produce bajo agitación continua.

2. Proceso de preparación de una suspensión de microcápsulas **caracterizado** porque:

(a) dos soluciones diferentes (Fig.1) 1a (aceite) y 1b (agua) se mezclan mediante la adición de 1b a 1a, estas soluciones conteniendo ingredientes activos y opcionalmente cationes libres o secuestrados para ser liberados posteriormente

(b) gracias a un emulgente alimentario que puede estar en la solución 1a o en la 1b, se forma una emulsión de gotas de agua (10) en la fase aceite (9). Este paso se concluye con la formación de la emulsión 1c, en donde en la fase aceite (9) están solubilizados o dispersados los, preferiblemente, ingredientes activos liposolubles; también se forma una emulsión de agua en aceite, con las gotas de agua (10) conteniendo, preferiblemente, los ingredientes activos hidrosolubles; la solubilidad en la fase aceite o agua de los ingredientes activos pudiendo ser modificada por derivatización del (los) ingrediente(s) activo(s)

(c) después se añade la solución 2b de al menos un hidrocoloide -capaz de ser polimerizado y entrecruzado-, y opcionalmente conteniendo al menos un ingrediente activo, a la emulsión existente en 1c.

(d) seguidamente ocurre una inversión de fases, y tenemos gotas dispersas (11) que son una emulsión de agua (12) en aceite, dispersas en el medio continuo (24), es decir, agua.

(e) después, (Fig. 5) añadimos una solución o dispersión 5a, conteniendo al menos un hidrocoloide (15), que actúa como coloide protector. La solución o dispersión 6a que contiene el emulgente primario se añade a la emulsión 2a.

(f) cuando las reacciones de polimerización y entrecruzamiento se consideran terminadas y se alcanza un grado de reducción del tamaño de partícula en el rango de 0.1 μm a 30 μm, la temperatura que permanecía entre 30ºC y 70ºC se aumenta a 60ºC - 150ºC.

(g) finalmente se añade un modificador de viscosidad

(h) opcionalmente, la formulación puede ser secada mediante atomización (spray-dry), o cualquier forma perteneciente al estado de la técnica, y ser recogidas y formar polvos secos, polvos autoemulsionables, geles, cremas o

cualquier forma líquida que las contenga (incluyendo una dispersión en aceite), así como también pueden ser liofilizadas.

3. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con las reivindicaciones precedentes, **caracterizado** porque tanto el (los) hidrocoloide(s) como el (los) coloide(s) protector(es) se añaden conjuntamente en forma de solución o dispersión acuosa, evitando así, en el proceso descrito en la primera reivindicación, el paso (d), al estar incluido el coloide protector en la solución descrita en la reivindicación 1 punto (c) o reivindicación 2 punto (e).

4. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con la reivindicación 1, **caracterizado** porque el coloide protector o los coloides protectores son del grupo químico de los hidrocoloides.

5. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con las reivindicaciones 1 y 2, **caracterizado** porque los hidrocoloides y los coloides protectores son preferentemente escogidos del grupo de: quitosanas, almidón, dextrinas, ciclodextrinas, celulosas, lignina, pectinas, agar, alginatos, carragenatos, gelatinas, goma guar, goma arábiga, gelatina, tragacantos, lignosulfonatos, goma de Caraya, goma de Ceratonia siliqua, saponina, goma xantana, gomas de semillas, galactomananas, arabanogalactanas, beta-glucanos, inulina, *psyllium*, goma acacia; en todas sus formas isoméricas y estereoquímicas, en todas sus variantes con respecto a la cantidad y proporción de monómeros u oligómeros constituyentes del hidrocoloide, en todas sus variantes de presentación, como sales de cationes metálicos o sales de compuestos nitrogenados o fosforados o sulfurados, así como de cualquiera de los productos de derivatización de los mencionados hidrocoloides.

6. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con las reivindicaciones precedentes, **caracterizado** porque el emulgente primario tiene un balance hidrofílico - lipofílico (HLB) en el rango 9-16, preferiblemente 12-14.

7. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con las reivindicaciones precedentes, **caracterizado** porque, en la primera emulsión formada, las gotas de agua en aceite (10) tienen un tamaño de partícula de entre 50-500 μm, preferiblemente entre 70-200 μm.

8. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con las reivindicaciones precedentes, **caracterizado** porque las microcápsulas formadas (7b) tienen un tamaño de partícula de en el rango 0.1-100 μm, preferiblemente en el rango 1-30 μm, mas preferiblemente en el rango 1-5 μm.

9. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con las reivindicaciones precedentes, **caracterizado** porque las microcápsulas formadas (7b) tienen un tamaño de partícula que varía con el tiempo, por agregación de microcápsulas, siendo el tamaño de partícula óptimo justamente cuando se va hacer uso de la formulación de microcápsulas.

10. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con las reivindicaciones precedentes, **caracterizado** porque las revoluciones por minuto del agitador usado para emulsionar y/o disminuir el tamaño de partícula permanecen en el rango de 3000 a 25000, siendo mayor éste valor durante la formación de la primera emulsión y menor cuando se añade el modificador de viscosidad.

11. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con cualquier combinación adecuada de las reivindicaciones precedentes, **caracterizado** porque la agitación se lleva a cabo mediante dos tipos de agitadores diferentes, uno de dientes y otro de tipo ancla.

12. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con las reivindicaciones precedentes, **caracterizado** porque al menos un hidrocoloide es substituido por un hidrogel, opcionalmente aquellos basados en albúmina, alginatos, policarboxilatos, poli-L-láctido, almidón, y derivados.

13. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con las reivindicaciones precedentes, **caracterizado** porque la solución o dispersión acuosa de hidrocoloide consiste en una mezcla binaria o ternaria de los hidrocoloides de acuerdo con la reivindicación 5 y/o hidrogeles de acuerdo con la reivindicación 12.

14. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con las reivindicaciones precedentes, **caracterizado** porque la solución o dispersión acuosa de coloide protector consiste en una mezcla binaria o ternaria de los hidrocoloides mencionados en la reivindicación 5.

15. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con las reivindicaciones precedentes, **caracterizado** porque además de compuestos biológicamente activos, las microcápsulas o la fase acuosa en la que están dispersas, contienen compuestos que facilitan o estabilizan la estructura de la microcápsula.

16. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con las reivindicaciones precedentes, **caracterizado** porque la fase acuosa continua en la que están dispersas las microcápsulas contiene materiales biológicamente activos, que han sido añadidos previamente en forma de disolución, dispersión o emulsión en algu-

na de las soluciones de; hidrocoloide(s), coloide(s) protectores, emulgente(s) primario(s), dichas soluciones siendo empleadas en el proceso de acuerdo con las reivindicaciones precedentes.

17. Proceso de microencapsulación de acuerdo con cualquier combinación adecuada de las reivindicaciones precedentes, **caracterizado** porque se realiza bajo al menos una de las siguientes condiciones: en vacío, presión reducida, en presencia de un gas inerte, preferentemente nitrógeno o helio, protegido de luz de cualquier longitud de onda, en condiciones estériles.

18. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con las reivindicaciones precedentes, **caracterizado** porque las soluciones o dispersiones acuosas son sustituidas por soluciones o dispersiones: (i) basadas en extractos acuosos, (ii) con un contenido en alcoholes (con peso molecular inferior a 144 unidades de masa atómica) no superior al 40%, siendo el resto agua (iii) de compuestos solubles o dispersables en agua.

19. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con las reivindicaciones precedentes, **caracterizado** porque la fase aceite es un aceite parcialmente hidrogenado o una cera, eventualmente miel.

20. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con las reivindicaciones precedentes, **caracterizado** porque la principal funcionalidad de la fase agua o la fase aceite es actuar de regulador térmico de la microcápsula, estabilizando las microcápsulas y compuesto(s) activo(s) presente(s) en ellas frente a cambios de temperatura, siendo posible añadir a la fase agua compuestos que disminuyan el punto de congelación, o que aumenten su punto de ebullición; asimismo, también se pueden añadir estos u otros materiales a la fase aceite, siempre con el objetivo de modificar las propiedades térmicas de la formulación y/o microcápsulas.

21. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con las reivindicaciones precedentes, **caracterizado** porque en alguna de los pasos del proceso se añade al menos un estabilizante microbiológico comprendido en el estado de la técnica, en al menos una de las fases agua o aceite.

22. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizado** porque en alguna de los pasos del proceso se añade al menos un estabilizante microbiológico comprendido en el estado de la técnica a una formulación seca de las microcápsulas (eventualmente liofilizadas, en forma de polvo, en forma de gránulos).

23. Proceso de microencapsulación de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizado** porque tras el secado de las microcápsulas, estas se reformulan y dispersan en una fase aceite o en un gel o en cualquier material semisólido o en una solución alcohólica o en un disolvente orgánico.

24. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizado** porque se aplican a cuales quiera alimentos y bebidas destinados a consumo humano, opcionalmente, pero no de modo limitante: cereales y derivados (opcionalmente muesli, cereales para leche), bollería y pastelería, azúcares y derivados (opcionalmente chocolates, dulces, turrones, mazapanes), dulces dietéticos (con bajo nivel de calorías), en alimentos de régimen y para diabéticos, aceites y derivados, lácteos y derivados, huevos, verduras y hortalizas, legumbres, frutas, tubérculos y derivados, tallos comestibles, snacks, aperitivos, raíces comestibles (opcionalmente regaliz), bayas y productos silvestres, conservas de frutas, frutos secos, carnes, embutidos, pescados, mariscos y crustáceos y sus conservas, bebidas alcohólicas y no alcohólicas, bebidas carbonatadas o no carbonatadas, zumos, jarabes, néctares, especias, condimentos, comidas precocinadas, alimentos pre-procesados (masa de pan congelada), pizzas, miel.

25. Proceso de microencapsulación de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizado** porque como materiales biológicamente activos se elige al menos un compuesto elegido entre el grupo de: semioquímicos, atrayentes, repelentes, insecticidas, esterilizantes, herbicidas, fungicidas, bactericidas, viricidas (o materiales que previenen las infecciones víricas), vectores de genes, aromas, pungentes indicadores de presencia de compuestos químicos inodoros, astringentes para evitar la ingestión de productos tóxicos (preferiblemente etanol, alcohol isopropílico, agua oxigenada, limpia muebles y otros productos similares de uso en el hogar).

26. Proceso de microencapsulación de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes **caracterizado** porque una o la principal funcionalidad de las microcápsulas producidas es prevenir la liberación de aromas indeseables para el consumidor (sea humano o animal), opcionalmente los aromas típicos del pescado u otras fuentes de materiales biológicamente activos.

27. Método de desarrollo de formulaciones de microcápsulas **caracterizado** porque el número de experimentos a realizar para seleccionar las variables independientes: tipo de compuestos de la pared, tamaño de partícula, emulgente (s), velocidad de rotación del agitador, tipo de agitador, modificador de viscosidad, tipo de compuesto a microencapsular -dependientes de una variable independiente que representa la calidad de la formulación o de las microcápsulas- se ve extraordinariamente reducido con el uso de análisis de varianza o múltiple análisis de varianza con diseño de fracciones factoriales, preferiblemente factorial en 2, 4, 8, 16, 32, y 64 bloques, media fracción saturada, diseño Box-Behnken, compuesto central, Plackett-Burman.

28. Microcápsulas producidas mediante un proceso continuo de microencapsulación **caracterizadas** porque (a)

contienen ingredientes activos beneficiosos para la salud humana; (b) la pared de las microcápsulas esta compuesta por una mezcla de al menos dos hidrocoloides, tal mezcla polimerizada y entrecruzada, tales hidocoloides son comestibles; (c) el grado de polimerización, entrecruzamiento y naturaleza de los hidrocoloides influye en la liberación controlada de los compuestos activos y la protección contra el oxígeno y/o luz y/o temperatura; (d) las microcápsulas contienen en su interior una emulsión de agua en aceite, existiendo opcionalmente ingredientes activos en la fase aceite, opcionalmente en la fase agua u opcionalmente en ambas fases y además, pueden contener microcápsulas más pequeñas (multi-encapsulación posible hasta, al menos, 5 grados de multi-encapsulación); (e) la media del tamaño de las microcápsulas se encuentra en el rango 0,1 μm - 100 μm, preferiblemente en el rango 1 μm - 10 μm (f) son producidas mediante un proceso continuo de multi- microencapsulación por polimerización interfacial *in situ*.

29. Microcápsulas producidas de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizadas** porque los materiales microencapsulados se liberan por motivo de al menos un factor elegido del grupo de: pH, temperatura, presión, fuerza fónica, osmosis, volatilización, presencia de compuestos que disuelven la pared de la microcápsula.

30. Formulación de microcápsulas de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes **caracterizada** porque se somete a operaciones pertenecientes al estado de la técnica concernientes a protección contra microorganismos, nocivos y/o no deseados tanto en la formulación recién terminada o posibles microorganismos colonizadores de la formulación o alimento al que se destina, siendo éstas operaciones eventualmente: esterilización, estabilización de microorganismos, pasteurización, UHT, ozonización, rayos UV, adición de productos antimicrobianos químicos (tanto de síntesis como naturales), irradiación con rayos gamma.

31. Formulación de microcápsulas de acuerdo con las reivindicaciones anteriores y especialmente de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizada** porque en el interior de las microcápsulas (opcionalmente en la fase aceite, o en la fase agua, o en ambas) y/o en la fase que contiene las microcápsulas, se encuentra un material estabilizador en términos de calidad microbiológica.

32. Formulación de microcápsulas conteniendo materiales biológicamente activos de acuerdo con reivindicaciones precedentes **caracterizada** porque se acompaña con un certificado de calidad en donde se analiza la inexistencia de metales pesados, productos nocivos de degradación de los materiales biológicamente activos, productos agroquímicos usados en la producción de los materiales biológicamente activos y demás compuestos que son nocivos para la salud.

33. Microcápsulas formadas de acuerdo con la reivindicación 1 y/o 2, **caracterizadas** porque son usadas para proporcionar anabolitos y/o nutrientes en medios de cultivo microbiológico de una manera continua o casi continua.

34.Microcápsulas formadas de acuerdo con la reivindicación 1 y/o 2, **caracterizadas** porque son usadas para proporcionar anabolitos y/o nutrientes en medios de cultivo microbiológico, y la liberación de al menos un ingrediente activo se produce cuando se alcanza cierto pH en el medio de cultivo.

35. Microcápsulas formadas de acuerdo con la reivindicación 1 y/o 2, **caracterizadas** porque son usadas para proporcionar anabolitos y/o nutrientes en medios de cultivo microbiológico, y la liberación de al menos un ingrediente activo se produce cuando se alcanza cierta concentración de al menos un enzima, en el medio de cultivo.

36. Microcápsulas formadas de acuerdo con cualquier combinación adecuada de las reivindicaciones precedentes, **caracterizadas** porque son usadas para proporcionar anabolitos y/o nutrientes en medios de cultivo microbiológico, y la liberación de al menos un ingrediente activo se produce cuando se alcanza cierta concentración de al menos un compuesto químico (preferiblemente etanol) en el cultivo.

37. Microcápsulas de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizadas** porque contienen al menos un ingrediente activo beneficioso y se añaden a edulcorantes naturales o artificiales, sal, pimienta, especias y condimentos en general, de tal forma que la adición de los citados condimentos a los alimentos hace que se incremente el valor nutritivo, o beneficio para la salud, de los alimentos.

38. Microcápsulas de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizadas** porque se les ha añadido al menos un protector o bloqueador y/o estabilizador y/o absorbente de rayos ultravioleta.

39. Formulación de microcápsulas de acuerdo con de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes **caracterizada** porque los ingredientes activo(s) se eligen entre el grupo: té verde, té negro, cacao, vino tinto o uvas tintas u orujos de unas tintas, sidra o manzana o zumo de manzana, germen o salvado de cereales, cariotas o zanahorias, chili, ajo, rábano (en especial, rábano picante).

40. Proceso de microencapsulación de materiales biológicamente activos beneficiosos para la salud humana y demás animales, de acuerdo con cualquier combinación adecuada de las reivindicaciones precedentes, **caracterizado** porque al menos uno de los compuestos biológicamente activos presentes en la formulación es preferiblemente escogido entre los grupos:

(a) flavonoides en general y sus derivados: antocianidinas, pro-antocianidinas, oligomero-procianidina, isoflavo-

nas, chalconas, catequina, epicatequina, epicatequina galato, epigalocatequina, epigalocatequina gallato, eriocitrina, narirutina, rutina, naringina, miricitrina, hesperidina, miricetina, eriodictiol, fisetina, quercetina, naringenina, luteolina, hesperitina, kaempferol, isorhamnetina, apigenina, rhamnetina, galangina, quercitrina, quercetina, diosmetina, taxifolina, galandina, biochanina A, genisteina, eriodictiol, chrysina, hidroxitirosol, oleuropeina, glabridina, licochalcona, daidzeina, matairesinol, secoisolariciresinol, enterodiol, enterolactona, equol, desmetilangolensina, luteoferol, luteolinidina, apiferol, apigenidina, leucocianidina, pelargonidina

(b) ácidos fenólicos en general y sus derivados (preferiblemente esteres, éteres, glicósidos, rutinósidos y aminas): gálico, sinápico, síringico, cafeico, clorogénico, ferúlico, (o-, m- or p-) cumárico, guaiacol, (o-, m- or p-) cresol, 4-etilfenol, 4-vinilguaicol, p-hidroxibenzoico, procatecuico, vainíllico, hidroxicinámico, taninos en general, elagiotaninos, galotaninos

(c) amidas estructuralmente combinadas comprendiendo ácidos hidroxicinámicos y ácidos antranílicos (avenantramidas), avenasterol, ácidos hidroxicinámicos estructuralmente combinados con ácidos grasos de cadena larga saturados o insaturados, ácidos hidroxicinámicos estructuralmente combinados con alcoholes, indoleaminas, melatonina, inulina; glutatión

(d) terpenoides en general y sus derivados, monoterpenos, diterpenos, sesquiterpenos, triterpenos, tetraterpenos, incluyendo los carotenoides, alfa-caroteno, fitotoeno, ciclo-artenol, beta-caroteno, ionona, zeaxantina, capsantina, astaxantina, cantaxantina, violaxantina, mutatoxantina, luteoxantina, auroxantina, neoxantina, apo-carotinal, xantofilas.

(e) antioxidantes usados comúnmente en la industria alimentaria (y sus derivados) del tipo de butilhidroxianisol, 2,6-di-ter-butilhidroxitolueno, ter-butilhidroquinona, 2,6-di-ter-butilhidroquinona, 2,6-diterbutyl-4-hidroximetilfenol, 2,4,5-trihidroxibutirofenona, tocoferoles y sus derivados, [alfa-, beta-, gamma- y delta-] tocoferol; tocotrienoles y sus derivados, [alfa-, beta-, gamma- y delta-] tocotrienoles; tococromanoles

(f) ácido alfa-lipoico; coenzima Q-10; yohimbina; escualeno; fitoestrógenos; clorofila; vitaminas; aminoácidos (preferiblemente L-arginina, cistina y cisteína) y sus correspondientes polímeros orgánicos como lo son los oligopeptidos, preferiblemente carnitina y carnosina, peptidos, enzimas; inhibidores enzimáticos, preferiblemente inibidores de las fenolasas, oxigenasas, lipooxigenasas, peroxidasas y lipasas

(g) así como minerales, oligoelementos, en especial aquellos que participan en procesos redox *in vivo* como el selenio, zinc y magnesio.

41. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizado** porque al menos uno de los materiales activos procede preferiblemente de: *Medicago sativa, Pimenal officinalis, Hibiscus abelmoschus, Angelica archangelica, Galipea officinalis, Catuaba, Pimpinella anisum, Ferula foetida, Ferula asafetida, Melissa officinalis, Myroxylon pereirae, Ocimum basilicum, Pimenta acris, Citrus aurantium bergamia, Prunus amygdalus, Citrus aurantium, Citrus aurantium amara, Piper nigrum, Prunus spinosa, Aniba rosaeodora, Camelia oleifera, Camelia sinensis, Carum carvi, Elettaria cardamomum, Ceratonia siliqua, Mate* (*Illex praguaiensis*), *Daucus carota, Dacus carota sativa, Cascarilla, Marapuama, Apium graveolens, Anthemis nobilis, Matricaria chamomilla, Anthemis nobilis, Anthriscus cerefolium, Cichorium intybus, Cinnamomum spp., Cinnamomum zeylanicum, Cymbopogon nardus, Salvia sclarea, Trifolium pratense, Theobroma cacao, Coffea arabica, Coriandrium sativum, Cuminum cyminum, Taraxacum officinale, Sambucus nigra, Elderweiss, Helichrysum italicum, Foeniculum vulgare, Trigonella foenumgraecum, Arabidopsis spp., Zingiber officinale, Citrus grandis, Psidium guajava, Humulus lupus, Marrubium vulgare, Monarda punctata, Hyssopus officinals, Jasminum officinale, Jasminum grandiflorum, Juniperus spp. Juniperus comunis, Eucaliptus officinalis, Cola acuminata, Laurus nobilis, Lavandula spp. Lavandula hybrida, Taxus baccata, Citrus medica limonum, Myristica fragans, Marjorana hortensis, Thymus spp., Thymus officinális, Thymus mastichina, Ilex paraguarensis, Chamomilla recutita, Saccharum officinarum, Myristica fragans, Allium cepa, Citrus aurantium dulcis, Carum petroselinum, Mentha pulegium, Mentha piperita, Pimenta officinalis, Chimaphila umbellate, Punica granatum, Pelargonium spp., Pelargonium graveolens, Rosmarinus officinalis, Crocus sativus, Salvia app., Salvia officinalis, Mentha spicata, Mentha viridis, Satureia hortensis, Satureja hortensis, Origanum majorana, Tamarindus indica, Citrus reticulata, Artemisia dracunculus, Thea sinensis, Thymus vulgaris, Polianthes tuberosa, Curcuma longa, Prunus serotina, Thymus serpillum, Satureja Montana, Cananga odorata, Curcuma zedoaria, Plantago major, Adansonia digitata, Ananas comosus, Artocarpus altilis, Carica papaya, Lycopersicon esculentum, Cephalophus spp., Vaccinium myrtillus, Thymus aragonensis, Thymus spp., Citrus aurantiifolia, Citrus paradisi, Cucumis melo, Cucurbita spp., Vitis spp., Vitis vinifera, Mangifera indica, Lamiaceae (Coleus, Hedeoma, Hyptis, Leonurus, Leucas, Lycopus, Marrubium, Mentha, Monarda, Perilla, Prunella, Salvia, Stachys, Teucrium, Thymus), Cannabis spp., Digitalis lanata, Adonis vernalis, Aesculus hippocastanum, Frazinus rhychophylla, Agrimonia supatoria, Rauvolfia sepentina, Andrographis paniculata, Areca catechu, Atropa belladonna, Berberis vulgaris, Ardisia japonica, Betula alba, Ananas comosus, Camellia sinensis, Cinnamomum camphora, Camptotheca acuminata, Potentilla fragarioides, Erythroxylum coca, Papaver somniferum, Colchicum autumnale, Claviceps purpurea, Digitalis purpurea, Digitalis lanata, Glaucium flavum, Papaver somniferum, Gossypium spp., Hyoscyamus niger, Camptotheca acuminata, Piper methysticum, Lobelia inflata, Crotalaria sessiliflora, Nicotiana tabacum, Physostigma venenosum, Ephedra sinica, Cinchona ledgeriana, Rhododendron molle, Datura spp., Taxus brevifolia, Strychnos nux-vomica, Stevia rebaudiana, Theobroma cacao, Valeriana officinalis, Pausinystalia yohimbe, Ephedra spp. Crataegus oxyacantha, Hamamelis virginiana, Hydrastis Canadensis, Hypericum perforatum, Potentilla erectra, Ledum palustre, Salvia officinalis, Chamomilla recutita, Arctostaphylos uva,*

Eucommia ulmoides, Mytilus galloprovincialis, Diplazium esculentum, Manihot utillissima, Sauropous androgynus, Terminalia arjuna, Iberis amara, Crataegus spp., Arbutus unedo, Cynara scolymus, Amaranthus caudatus, Alchornea laxiflora, Alpinia officinarum, Xanthophyllomyces dendrorhous, Crataegus monogyna, Taxus yunnanensis, Bacopa monniera, Cistus albidus, Ocimum basilicum, Rosmarinus officinalis, Thymus vulgaris, Bixa orellana, Centella asiatica, Urtica dioica, Agrocybe aegerita, Crataegus laevigata, Satureja hortensis, Crocus sativus, Coccinia indica, Brugia malayi, Rubus spp., Silybum marianum, Cannabis spp., Cannabis sativa, Hypericum perforatum, Rhus coriaria, Olea europaea, Cyclopia intermedia, Ginkgo biloba, Lentinus lepideus, Pseudomonas putida, Sargassum micracanthum, Pinus radiata, Pinus sp., Phaseoulus mungo, Cicer arietinum, Vigna sinensis, Phaseolus aureus, Dolichos lablab, Cajanus tajan, Vicia faba, Dolichos biflorus, Phaseolus lunatus, Phaseolus aconitifolius, Pisum sativum, Psophocarpus tetragonolobus, Arachis hypoagea, Brassica spp., Brassica campestris, Brassica napus, Valeriana officinalis, Echinacea purpurea, Echinacea pallida, Echinacea angustifolia, Glcyrrhiza glabra, Seronea repens, Vaccinium macrocarpon, Tancetum parthenuum, Tancetum parthenuum, Vaccinium macrocarpon, cereales, frutales de hueso, bayas silvestres, legumbres, té verde, té negro y microorganismos productores de ácidos grasos de cadena larga insaturados.

42. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con cualquier combinación adecuada de las reivindicaciones precedentes, **caracterizado** porque al menos uno de los materiales biológicamente activos presentes en la formulación consiste en bacterias probióticas.

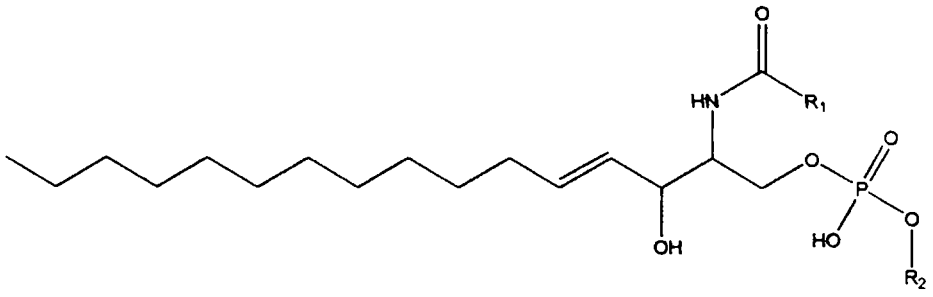
43. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizado** porque al menos uno de los materiales biológicamente activos presentes en la formulación consiste en bacterias probióticas, opcionalmente bacterias ácido-lácticas y más preferiblemente elegidos entre el grupo de: *Lactobacillus casei., L. acidophillus, L. rhamnosus, L. paracasei, L. gasseri, L. fermentum, L. plantarum, L. salivarius, L. crispatus, L. bulgaricus, L. fermentum, L. reuteri, Bifidobacterium infantis, B. bifidum, Streptococcus termophilus, S. bovis, Enterococcus durans, E. faecalis, E. Gallinarum, Escherichia coli, Propionibacterium freudenreicheii*, o bacterias u hongos o levaduras modificadas genéticamente en las que se han insertado genes propios beneficiosos de las bacterias probióticas.

44. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizado** porque al menos uno de los materiales biológicamente activos presentes en la formulación consiste en levaduras probióticas, preferiblemente elegidas entre el grupo de: *Saccharomyces cerevisiae, Kluyveromyces marxianus, Rhodotorula rubra, Sporobolomyces puniceus, Aureobasidium pullulans, Leucosporidium scotti*.

45. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizado** porque al menos uno de los materiales biológicamente activos presentes en la formulación consiste en hongos probióticos, preferiblemente aquellos hongos presentes en, o coincidentes con, o provenientes de, quesos.

46. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizado** porque al menos uno de los compuestos activos se elige entre el grupo de compuestos que corresponden a las siguientes estructuras moleculares (A) y (B), en todas sus variantes estereoisoméricas, y/o isoméricas:

Compuesto(s) A

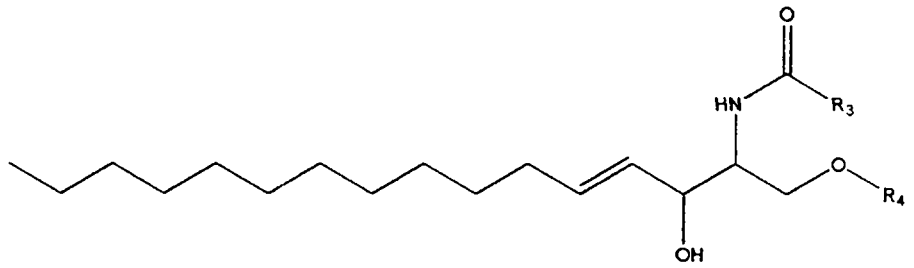


en donde,

R_1 es un éster de un ácido graso omega-3 o de un ácido graso omega-6

R_2 es un éster de un ácido graso omega-3 o de un ácido graso omega-6

Compuesto(s) B



en donde,

R_3 es un éster de un ácido graso omega-3 o de un ácido graso omega-6 o de un ácido graso w-9

R_4 es un éster de un ácido graso omega-3 o de un ácido graso omega-6 o de un ácido graso w-9 o de un oligosacárido unido covalentemente.

47. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizado** porque al menos uno de los materiales biológicamente activos presentes en la formulación consiste preferiblemente en, al menos, un ácido graso de cadena larga (al menos 6 carbonos) insaturado, en cualquier configuración isomérica y/o estereoquímica, así como derivados de él (los) mismo (s) -preferiblemente ésteres, éteres, glicéridos, fosfolípidos, esfingolípidos y más con mayor preferencia, diglicéridos, triglicéridos, fosfolípidos, compuestos (A) y/o (B)- estereadiónico, eicosapentaenoico, docosahexaenoico, docosapentaenoico, linoleico -y ácidos linoleicos conjugados-, linolénico, gamma-linolénico, alfa-linoléico, dihomogamma-linolénico, araquidónico, oléico.

48. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizado** porque los ácidos grasos se eligen preferiblemente del grupo de ácidos: oleico, estereadiónico, eicosapentaenoico, docosahexaenoico, docosapentaenoico, linoleico, linoleicos conjugados, linolénico, gamma-linolénico, alfa-linolénico, dihomogamma-linolénico, araquidónico.

49. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizado** porque los ácidos grasos de cadena larga (al menos 6 carbonos) insaturados están preferiblemente conjugados, manteniendo o no manteniendo intactas todas o parte de las insaturaciones, y/o unidos covalentemente con glicéridos -más con mayor preferencia, ésteres monoglicéridos, diglicéridos y/o triglicéridos-; fosofolípidos; esfingolípidos; mielina; aminas; amidas; éteres; azúcares, oligosacáridos, polisacáridos; heterociclos nitrogenados, fosforados, oxigenados, sulfurados; anillos aromáticos sustituidos.

50. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizado** porque los ácidos grasos de cadena larga (al menos 6 carbonos) son elegidos por sus virtudes medicinales.

51. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes **caracterizado** porque los ácidos grasos insaturados de cadena larga (de más de 6 carbonos) provienen de fuentes naturales, o de organismos genéticamente modificados de las siguientes fuentes naturales, preferiblemente de:

(e) origen vegetal: con mayor preferencia de las familias: *Boraginaceae*, en especial (*Borago spp*. y en especial *Borago officinalis*); *Linaceae* (*Linum usitatissimum, Linum arvense, Linum sativum*); *Onograceae* (*Oenothera biennis*); *Grossulariaceae* (*Ribes nigrum*), *Zea Mais, Gossypium hirsutum, Carthamus tinctorius, Glycine max.*

(f) algas, con mayor preferencia de las familias: *Graciliariceae* (*Gracilaria spp*); *Gigartinaceae* (*Iridaea spp*.); *Kallymeniaceae* (*Callopyllis variegata*); *Durvillaceae* (*Durvillaea antartica*); *Solieriaceae* (*Euchema cottoni*); *Gelidiaceae* (*Gelidium spp*); *Lossoniaceae* (*Lesonia nigrescens*); *Gigantinaceae* (*Gigartina spp*.); *Lessoniaceae* (*Macrocystis spp*.); *Bangiaceae* (*Porphyra spp*.) y *Crypthecodinium spp*.

(g) origen animal, generalmente de aceites de pescado, con mayor preferencia de las familias -entre paréntesis, géneros y/o especies especialmente preferidas)-: *Engaulidae* (*Lycengraulis olidus*); *Clupeidae* (*Sardina pilchardus*); *Scomberesocidae* (*Scomberesox saurus scombroides*); *Berycidae* (*Beryx splendens*); *Engraulidae* (*Engraulis ringens*); *Ophichthyidae* (*Ophichthus spp*.); *Serranidae* (*Hemilutjanus macrophthalmus*); *Scombridae* (*Thunnus spp*., en especial, *Thunnus albacares, Thunnus alalunga, Thunnus obesus*); *Sciaenidae* (*Cynoscion analis*); *Carcharhinidae* (*Prionace glauca*); *Normanichthyidae* (*Normanichthys crockeri*); *Percichthyidae* (*Polyprion oxygeneios*); *Nototheniidae* (*Dissostichus eleginoides*); *Apogonidae* (*Epigonus crassicaudus*); *Branchiostegidae* (*Prolatilus jugularis*); *Scombridae* (*Thunnus spp., Thunnus albacares, Thunnus alalunga, Thunnus obesus, Sarda spp., Sarda chiliensis, Scomber*

japonicus peruanus), *Sciaenidae* (*Cynoscion analis*), *Carcharhinidae, Normanichthyidae* (*Normanichthys crockeri*); *Percichthyidae* (*Polyprion oxygeneios*); *Nototheniidae* (Bacalao de profundidad); *Apogonidae* (*Epigonus crassicaudus*); *Branchiostegidae* (*Prolatilus jugularis*); *Cheilodactylidae* (*Cheilodactylus gayi*); *Gadidae* (*Salilota australis*); *Pomadasyidae;; Scorpaenidae; Serranidae; Cyprinidae; Monacanthidae; Centrolophidae; Ophidiidae; Scorpaenidae; Coryphaenidae; Channichthydae; Sciaenidae; Aplodactylidae; Carangidae* (*Trachurus symetricus murphyi*); *Bothidae* (*Paralichthys microps*); *Mugilidae; Clupeidae; Priacathidae; Merlucciidae* (*Merluccius gayi gayi, Merluccius australis*); *Macruronidae* (*Macruronus magellanicus*); *Gadidae* (*Micromesistius australis*); *Girellidae; Trachichthyidae; Carangidae; Kyphosidae; Callorhynchidae; Labridae; Macrouridae; Atherinidae; Gobiesocidae; Alopiidae; Galaxiidae; Rajidae; Bramidae; Carangidae; Nototheniidae; Scianidae; Mugiloididae; Salmonidae* (*Salmo spp., Salmo salar, Oncorhynchus spp., Oncorhynchus kisutch, Oncorhynchus mykiss, Oncorhynchus tshawytscha*); *Clupeidae* (*Sardinops spp., Sardinops sagax, Clupea bentincki*); *Pomadasyidae; Gempylidae; Lamnidae* (*Isurus spp., Isurus oxyrinchus*);*Triakidae; Clinidae; Scophthalmidae; Labridae*.

◦ De especial preferencia son las especies *Atlantic mackerel, Engraulis encrasicholus, Pomatomus saltatrix, Sarda sarda, Sardina pilchardus, Brevoortia tyrannus, Brevoortia patronus, Chloroscombrus chrysurus, Auxis thazard, Scomber scombrus, Scomber japonicus, Alosa aestivalis, Clupea harengus, Etrumeus teres, Argentina silus, Ictalurus punctatus*.

(h) de origen microbiano, con mayor preferencia: *Saccharomices cerevisiae, Escherichia coli, Schizochytrium spp., Thraustochytrium aureum, Thraustochytrium roseum, Thraustochytrium striatum, Mortiriella spp., Phytium spp., Aspergillus spp. Aspergillus nidulans, Aspergillus sydowi, Fusarium spp., Fusarium equiseti, Fusarium oxysporum*

52. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizado** porque los ácidos grasos insaturados omega-3 y/o omega-6 y/o omega-9 que se incorporan a la formulación referida en la reivindicación 1 ó 2, proceden de productos comerciales destinados a ser incorporados en alimentos, basados en aceites de pescado o de plantas o de origen microbiano o sus mezclas.

53. Proceso de microencapsulación de materiales biológicamente activos de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizado** porque se combinan compuestos omega-3, omega-6, cerebrósidos y, opcionalmente, omega-9 para mejorar el desarrollo o mantenimiento o recuperación del cortex cerebral.

54. Formulación consistente en una suspensión de microcápsulas producidas de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizada** porque contiene como compuesto activo, o como un compuesto activo adicional, ácido linoleico, ácidos linoleicos conjugados, ácido araquidónico, ácido docosahexenoico, ácido eicosapentenoico, ácido esteradiónico, ácido alfa-linolénico, ácido dihomogamma-linolénico, ácido oleico, ácido linolénico, en todas sus configuraciones estereoquímicas y/o isoméricas.

55. Formulación microencapsulada destinada a incrementar el desarrollo neuronal, en especial del cerebro y más especialmente en fetos, recién nacidos, lactantes y niños **caracterizada** porque al menos existe un compuesto **caracterizado** por las formulas B y/o A.

56. Formulación microencapsulada destinada a incrementar la inteligencia potencial en fetos y bebés lactantes de leche materna -mediante el consumo materno con un vehículo alimentario apropiado en el que se añade la formulación microencapsulada-, en formulaciones de leche para lactantes y en niños, de acuerdo con las reivindicaciones anteriores, **caracterizada** porque contiene ácidos grasos omega-3 y omega-6 en una proporción entre 0.5 y 10.0, preferiblemente entre 1.4 y 5.7 y además contiene cerebrósidos en un porcentaje entre el 0,005% y 1% o/y opcionalmente compuestos A y/o B, también opcionalmente ácidos grasos omega-9.

57. Formulación microencapsulada para su empleo en fórmulas infantiles, de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizada** porque opcionalmente se prescinde de cualquier ácido graso omega-6 e, independientemente, opcionalmente se añade ácido gamma-linolénico en una proporción del 1.25%.

58. Formulación microencapsulada destinada a incrementar el desarrollo del córtex cerebral y la inteligencia, **caracterizada** porque contiene ácidos grasos omega-3 y omega-6 en una proporción entre 0.5 y 10.0, preferiblemente entre 1.4 y 5.7 y además contiene cerebrósidos en un porcentaje entre el 0,005% y 1% y opcionalmente compuestos A y/o B.

59. Bebida refrescante conteniendo una formulación de microcápsulas, producida de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizada** porque dicha bebida contiene microcápsulas, y estas a su vez contienen en su fase aceite ácidos omega-6 y/o omega-3, opcionalmente con antioxidantes añadidos en la fase discontinúa acuosa de la microcápsula o en la fase continua hidrofóbica de la microcápsula o en ambas, y la bebida contiene aromas o extractos de: uva, piña y al menos algún cítrico, preferiblemente entre el grupo de tangerina, naranja, mandarina, limón, lima, y los ácidos grasos omega-3 y/o omega-6 permanecen estables en la bebida, una vez finalizado todo el proceso industrial (incluyendo procesos usuales de estabilización microbiológica como la pasteurización), al menos durante un mes (pérdida de omega-3 menor al 7%).

60. Microcápsulas formadas de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizadas** porque permanecen estables a pH superior a 3.5.

61. Microcápsulas formadas de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizadas** porque la pared de las microcápsulas y la consiguiente liberación de su contenido ocurre rápidamente a pH inferior a 3.

62. Microcápsulas formadas de acuerdo con el proceso descrito en la reivindicación 1, **caracterizadas** porque la pared de las microcápsulas y la consiguiente liberación de su contenido ocurre en condiciones del estómago humano.

63. Uso de microcápsulas formadas de acuerdo con cualquier combinación adecuada de las reivindicaciones precedentes, **caracterizado** porque dichas microcápsulas están formadas con hidrocoloides biocompatibles con un tamaño de microcápsula igual o inferior a 2 μm, y permiten ser utilizadas en xenotransplantes, autotransplantes de tejidos dérmicos, transplante de islotes de Langherns, de células productoras de insulina en general -tanto de origen animal, vegetal, microbiano; natural o modificado genéticamente-.

64. Microcápsulas formadas de acuerdo con el proceso descrito en la reivindicación 1, **caracterizadas** porque la pared de las microcápsulas y la consiguiente liberación de su contenido ocurre en condiciones del estómago de animales, siendo los materiales de la pared de la cápsula adecuadamente escogidos para el rango de pH del animal en cuestión o su capacidad de digestión por enzimas.

65. Microcápsulas adecuadas para su ingestión, conteniendo ingredientes activos del tipo omega-3 y/o omega-6 y/o omega 9 y/o esfingolípidos, realizadas de acuerdo con el proceso descrito en cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizadas** porque las microcápsulas se incluyen en una formulación infantil en una proporción de acuerdo con recomendaciones médicas públicas, nacionales o internacionales, estabilizadas con vitamina E y/o vitamina C, así como derivados de ambas vitaminas (en especial aquellos derivados que inciden en el grado de lipofilidad o hidrofilidad).

66. Microcápsulas de acuerdo con cualquier combinación apropiada de la reivindicaciones precedentes, **caracterizadas** porque los ingredientes activos son hormonas resistentes a las condiciones del estomago de cualquier animal respectivo.

67. Microcápsulas de acuerdo con cualquier combinación apropiada de la reivindicaciones precedentes, **caracterizadas** porque los hidrocoloides y coloides protectores son elegidos en función del rango de pH del estómago del animal que lo ingiere, entendiendo que los animales de un mismo género y especie tienen un mismo rango de pH en el estómago.

68. Microcápsulas de acuerdo con cualquier combinación apropiada de la reivindicaciones precedentes, **caracterizadas** porque los hidrocoloides y coloides protectores son elegidos en función del rango de pH del estómago del animal, incluyendo el hombre, que lo ingiere, entendiendo que los animales de un mismo género y especie tienen un mismo rango de pH en el estómago, liberándose pues al menos un ingrediente activo en el estómago.

69. Microcápsulas de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizadas** porque se emplean en productos alimenticios ácidos, tales como yogures, zumos, bebidas refrescantes, etc.

70. Microcápsulas de acuerdo con cualquier combinación apropiada de la reivindicaciones precedentes **caracterizadas** porque la ruptura de la pared de las microcápsulas sucede por el ataque de al menos un enzima, eventualmente activado por un determinado pH.

71. Microcápsulas de acuerdo con cualquier combinación apropiada de la reivindicaciones precedentes **caracterizadas** porque la ruptura de la pared, total o parcial, se produce por enzimas, eventualmente por el pH, presente(s) en la cavidad bucal del animal, incluyendo el hombre, que las ingiere.

72. Formulación de microcápsulas de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes **caracterizada** porque todos los ingredientes activos, y opcionalmente todos los componentes de la formulación, proceden de agricultura (término que incluye actividades agropecuarias y piscícolas) "biológica" y/o "ecológica".

73. Formulación de microcápsulas de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes **caracterizada** porque se han empleado, para la obtención de ingredientes activos, organismos genéticamente modificados, variedades vegetales híbridas u obtenidas mediante selección humana, así como cultivos microbiológicos seleccionados mediante cualquier técnica.

74. Microcápsulas de acuerdo con cualquier combinación apropiada de las reivindicaciones precedentes, **caracterizadas** porque se aplican a alimentos destinados a consumo animal, en especial en ganadería, (opcionalmente avicultura), piscicultura, cría de animales domésticos y mascotas.

75. Microcápsulas de acuerdo con cualquier combinación adecuada de las reivindicaciones anteriores, **caracterizadas** porque se incluyen en formulaciones medicinales, sean en combinación con principios activos no presentes en

las microcápsulas o siendo los ingredientes activos presentes en las microcápsulas o formulación de las microcápsulas los únicos ingredientes activos de la preparación medicinal, incluyendo bajo el término preparación medicinal también materiales de contraste en radiología, semillas para radioterapia oncológica, termoterapia o terapia por irradiación de luz de cualquier longitud de onda.

76. Microcápsulas de acuerdo con cualquier combinación adecuada de las reivindicaciones anteriores, **caracterizadas** porque se añaden a productos para-farmacéuticos de cualquier composición, estando presentes los ingredientes activos de las microcápsulas en cualquier porcentaje en el producto para-farmacéutico.

77. Formulación alimentaria conteniendo microcápsulas creadas con materiales aptos para uso alimentario conteniendo ingredientes activos aceptables para uso alimentario, **caracterizada** porque se las microcápsulas se añaden a la formulación alimentaria (cualquier tipo de alimento o nutracéutico sólido o líquido) justo en el momento del consumo, por medio de una separación física de las microcápsulas y el resto del alimento.

78. Formulación alimentaria conteniendo microcápsulas conteniendo ingredientes activos, **caracterizada** porque las microcápsulas se añaden a la formulación alimentaria (cualquier tipo de alimento o nutracéutico sólido o líquido) justo en el momento del consumo, por medio de una separación física -durante el almacenamiento del alimento- de las microcápsulas y el resto del alimento por una barrera o membrana; produciéndose una adición de las microcápsulas al alimento por rotura de la membrana que las separa del alimento, en el momento previo a su consumo o en un intervalo de tiempo prudencial para permitir una correcta dispersión o disolución de las microcápsulas en el alimento; en el caso de bebidas, dichas microcápsulas están preferiblemente encerradas en un receptáculo y son disueltas o dispersadas en la bebida mediante presión externa del mencionado receptáculo y rotura de una membrana que las separa del resto del contenido de la bebida, preferiblemente dicho receptáculo presente en la tapa o chapa de la bebida.

79. Microcápsulas producidas de acuerdo con las reivindicaciones precedentes, **caracterizadas** porque los materiales de la pared de las microcápsulas se disuelven o degradan o liberan los materiales activos cuando se encuentran en la boca del consumidor (sea humano u otro animal), siendo capaz de apreciar las cualidades organolépticas de al menos un material microencapsulado.

80. Microcápsulas producidas de acuerdo con la reivindicación 53, **caracterizadas** porque al menos uno de los hidrocoloides presentes en la pared, o el único componente de la pared, es un hidrogel o un polímero altamente soluble y/o gelificable con la humedad presente en la boca del consumidor (sea humano u otro animal).

81. Formulación de microcápsulas de acuerdo con cualquier combinación posible de las reivindicaciones anteriores **caracterizada** porque todos los materiales usados y presentes en la formulación final de microcápsulas son de uso alimentario.

82. Formulación de microcápsulas de acuerdo con cualquier combinación posible de las reivindicaciones anteriores **caracterizada** porque todos los materiales usados y presentes en la formulación final de microcápsulas son de uso alimentario, dependiendo este último término de la legislación correspondiente a la región o país en donde se consuma y/o fabrique dicha formulación de microcápsulas.

83. Zumo conteniendo microcápsulas producidas de acuerdo con cualquier combinación adecuada de las reivindicaciones anteriores **caracterizado** porque (a) las microcápsulas contienen ácidos omega-3 provenientes de una formulación comercial basada en aceite comestible de lino; (b) la fase aceite contiene el aceite de lino y un emulgente basado en compuestos de soja (c) la fase agua contiene una mezcla de diferentes subclases de hidrocoloides del tipo de los alginatos y/o goma arábiga y/o kappa-carragenato y/o goma guar, además de un emulgente primario alimentario de HLB entre 10 y 14, y un modificador de viscosidad alimentario (d) y el pH de la formulación de microcápsulas esta en el rango de 3-6, el tamaño en el percentil 50 de las microcápsulas recién producidas está en el rango 1-10 μm. (e) el componente mayoritario del zumo es zumo de naranja.

84. Zumo de acuerdo con la reivindicación 83 **caracterizado** porque las frutas originarias del zumo se eligen del grupo: cítricos, piña, uva.

85. Zumo de acuerdo con las reivindicaciones 83 y 84 **caracterizado** porque contiene (todos los datos referidos a 150 mL de zumo) omega-3 en el rango 20-200 mg, omega-6 en el rango 10-100 mg, y w-9 en el rango 5-50 mg; con un ratio de omega-3 / omega-6 de alrededor de 3 / 1.

86. Formulación consistente en una dispersión de microcápsulas de acuerdo con cualquier combinación adecuada de las reivindicaciones anteriores, **caracterizada** porque los ingredientes activos que son fácilmente oxidables, en especial los ácidos grasos insaturados, se protegen por medio de otros ingredientes activos que pueden tener estructuras químicas determinadas o bien ser extractos o zumos con propiedades antioxidantes, estando los antioxidantes, independientemente de su lipofilicidad o hidrofilicidad, en la fase acuosa o en la fase aceite, preferiblemente en la fase en donde se encuentra el material fácilmente oxidable.

Fig. 1

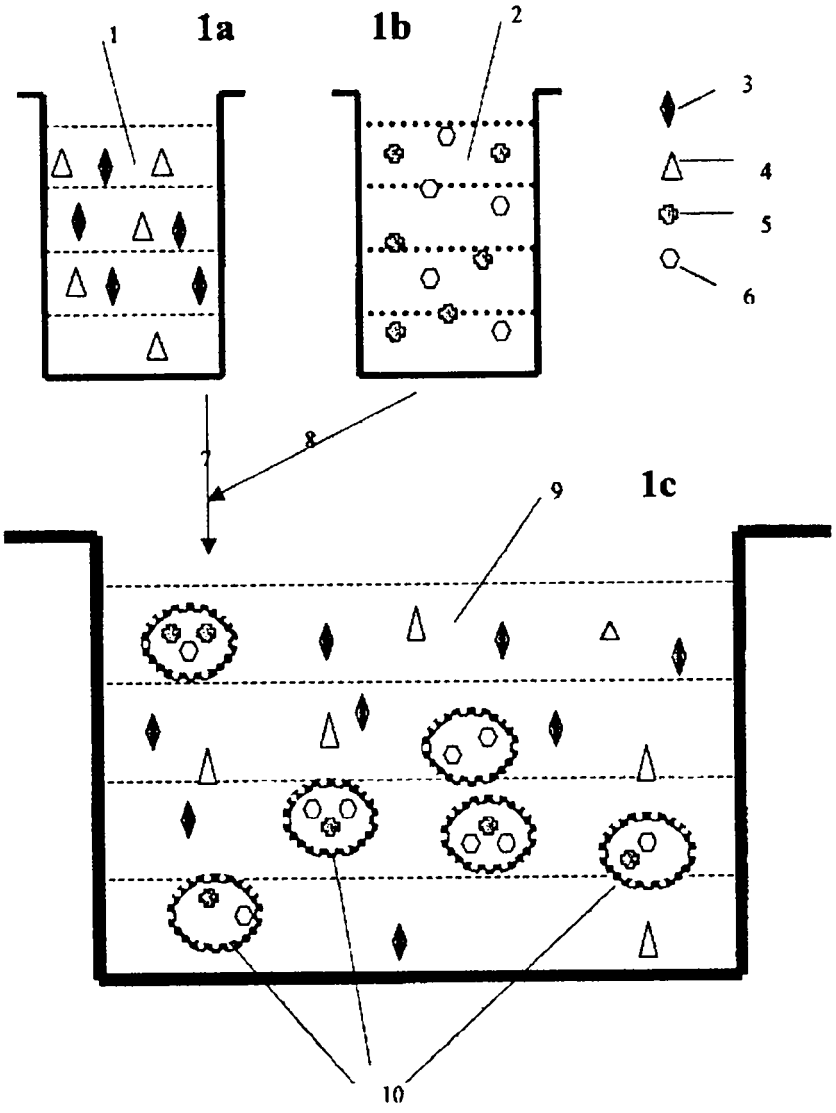


Fig. 2

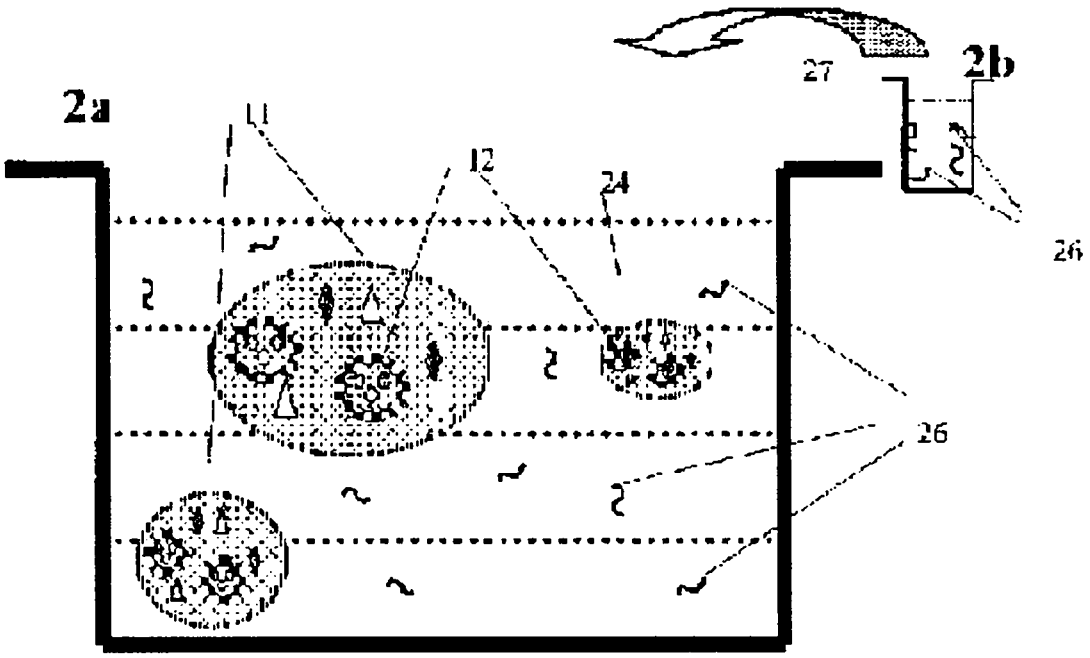


Fig. 3

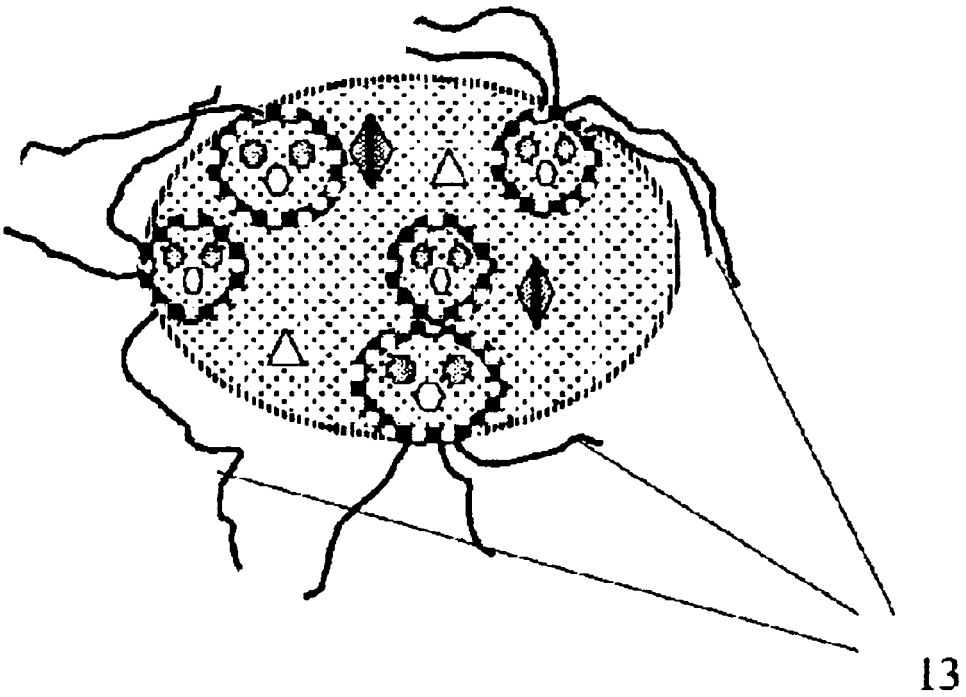


Fig.4

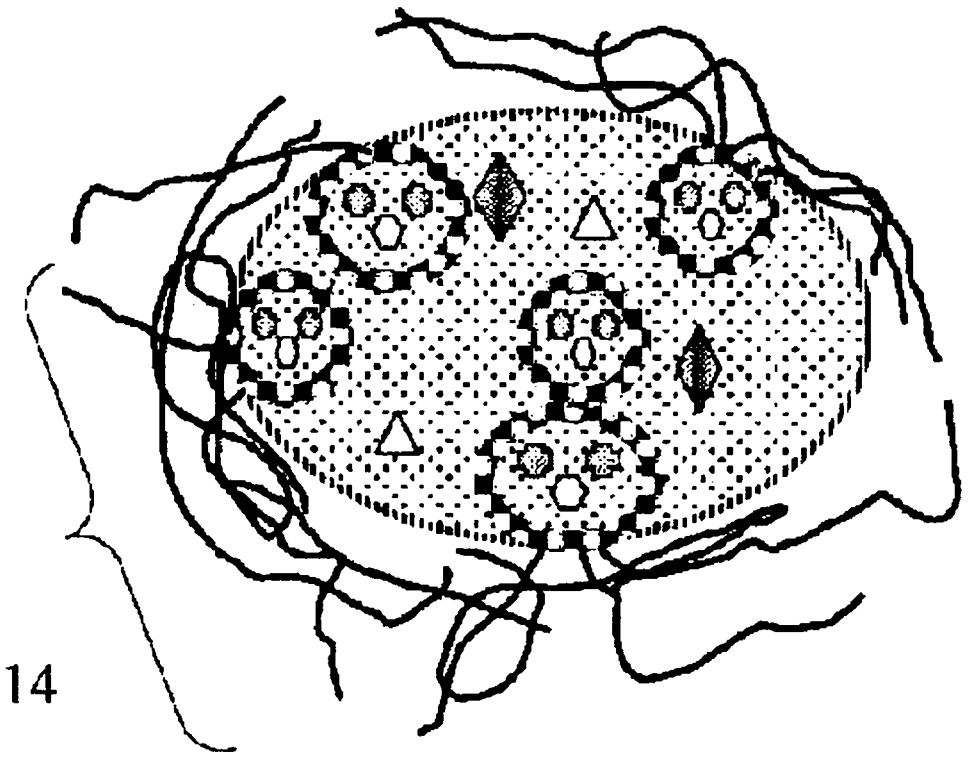


Fig. 5

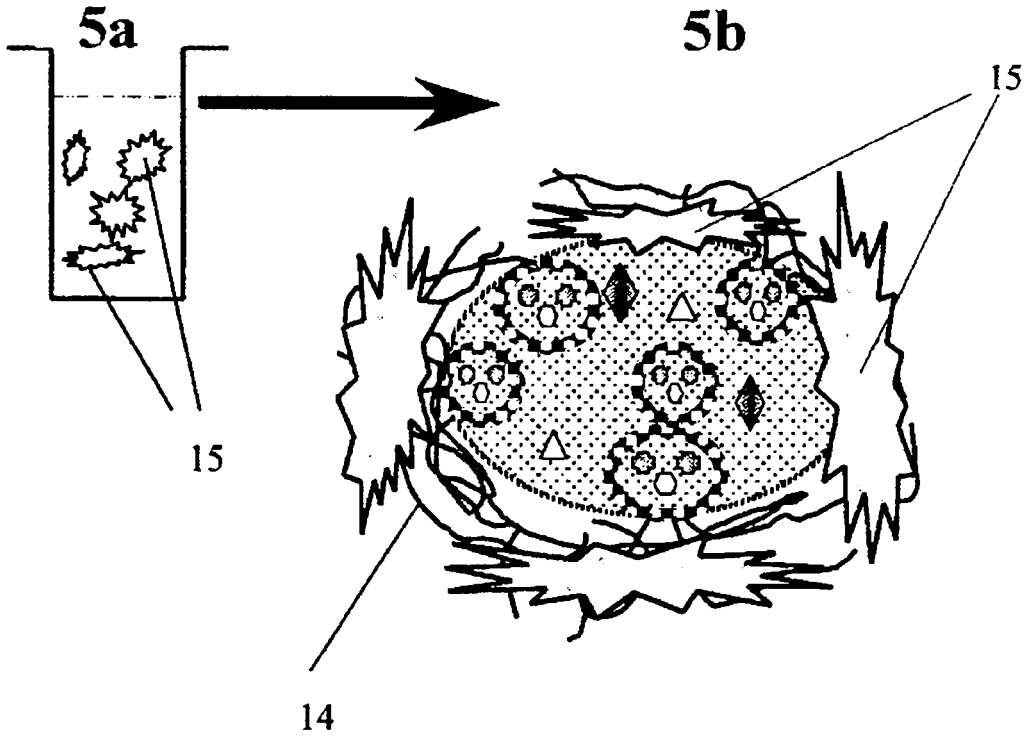


Fig.6

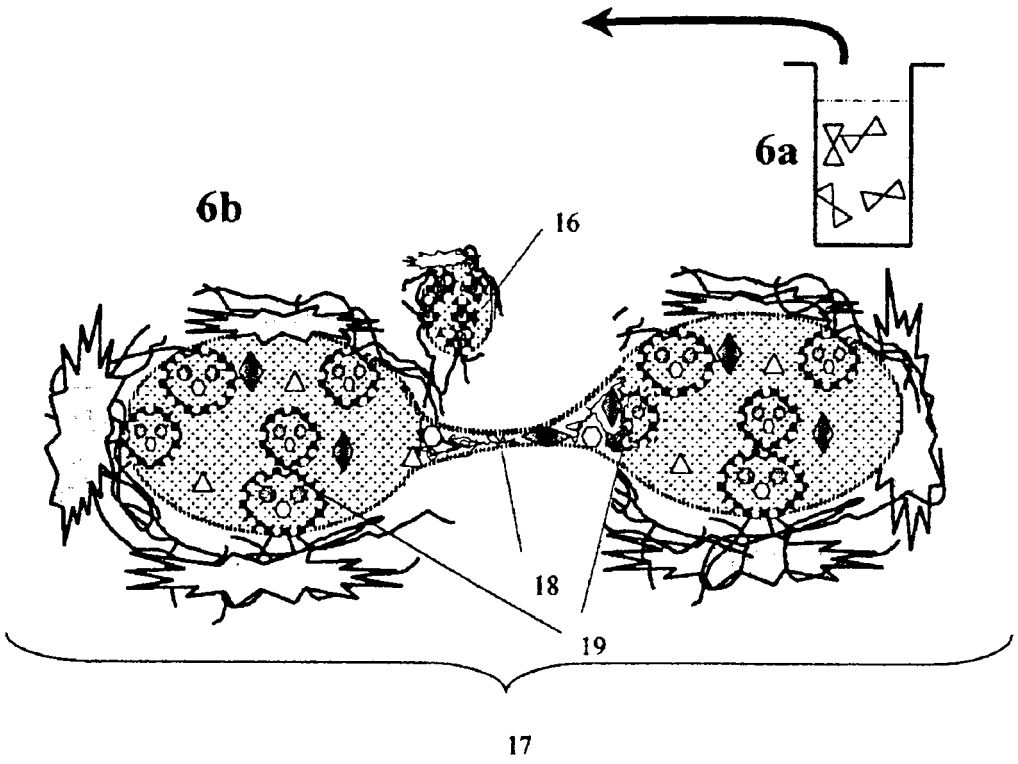


Fig. 7

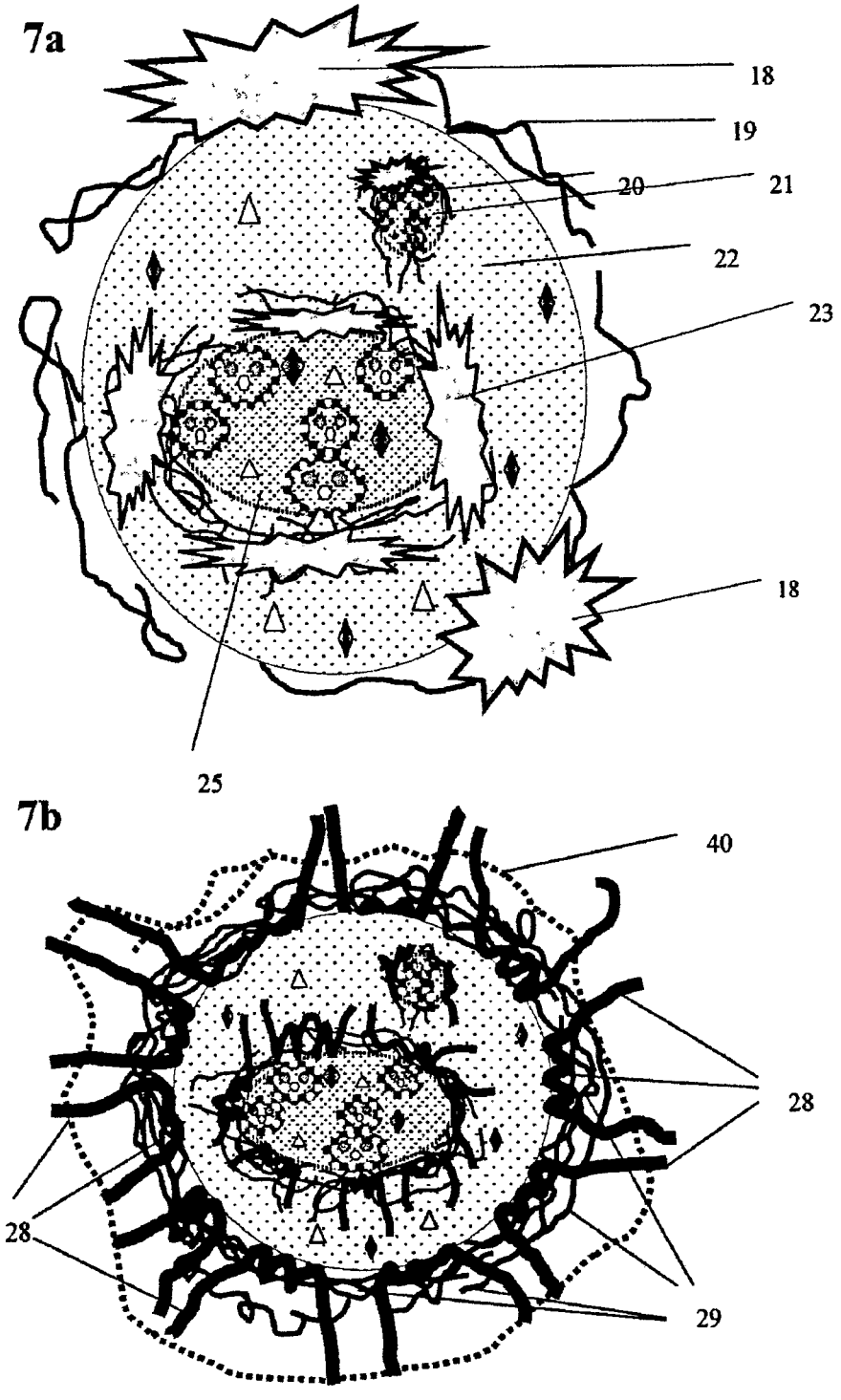
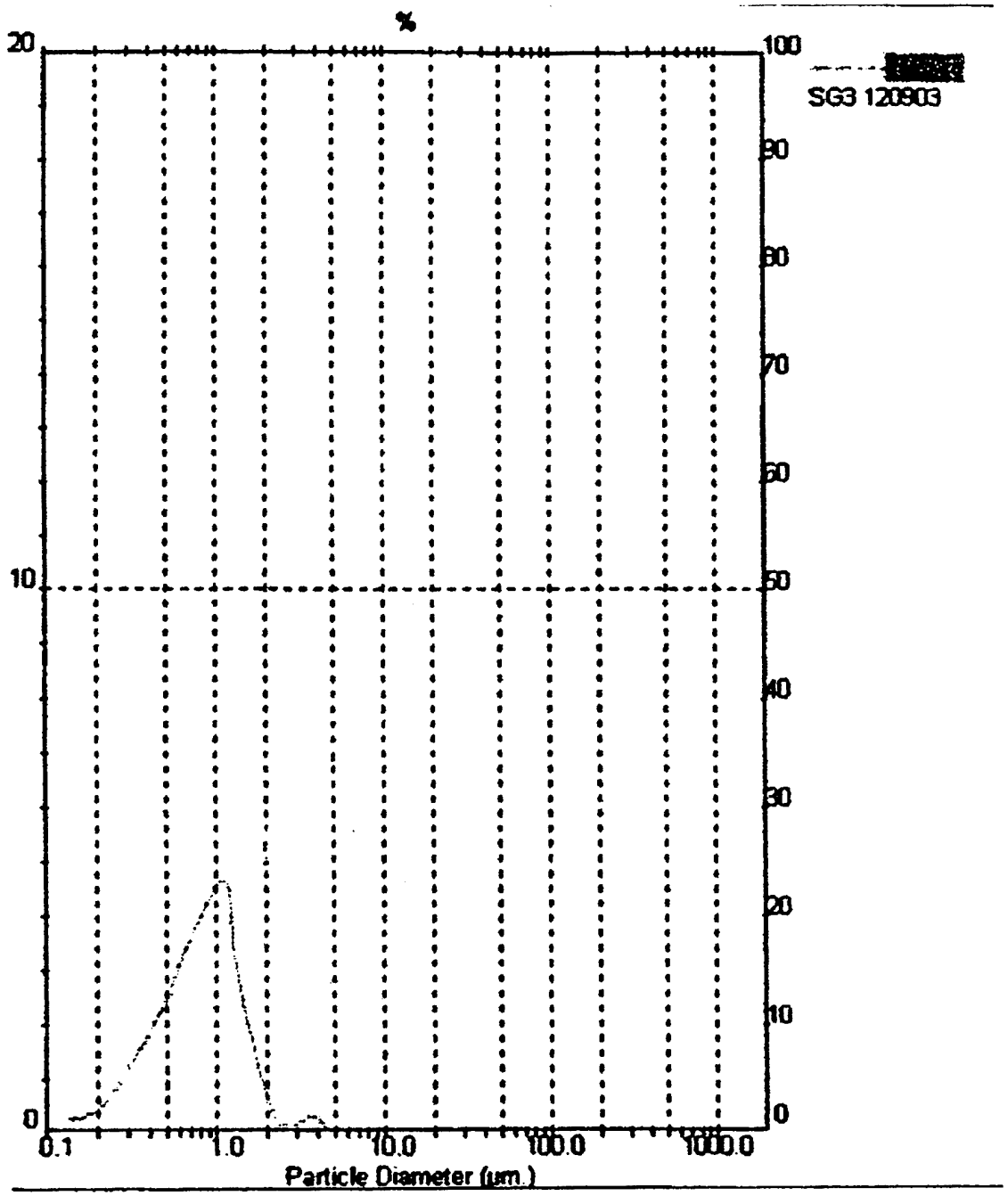


Fig.8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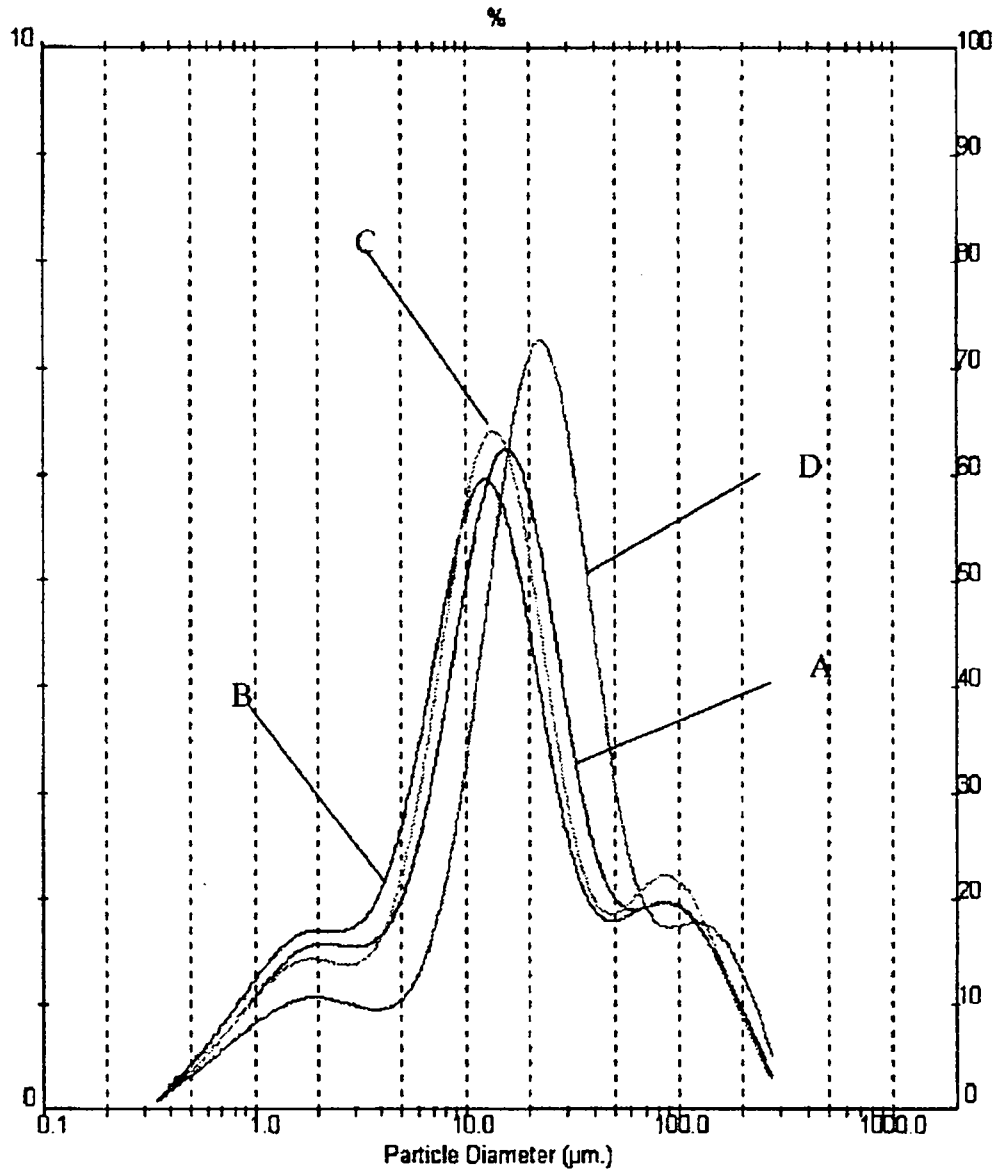
1000.0
Particle Diameter (µm.)

Fig. 9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Particle Diameter (µm.)

Fig. 10

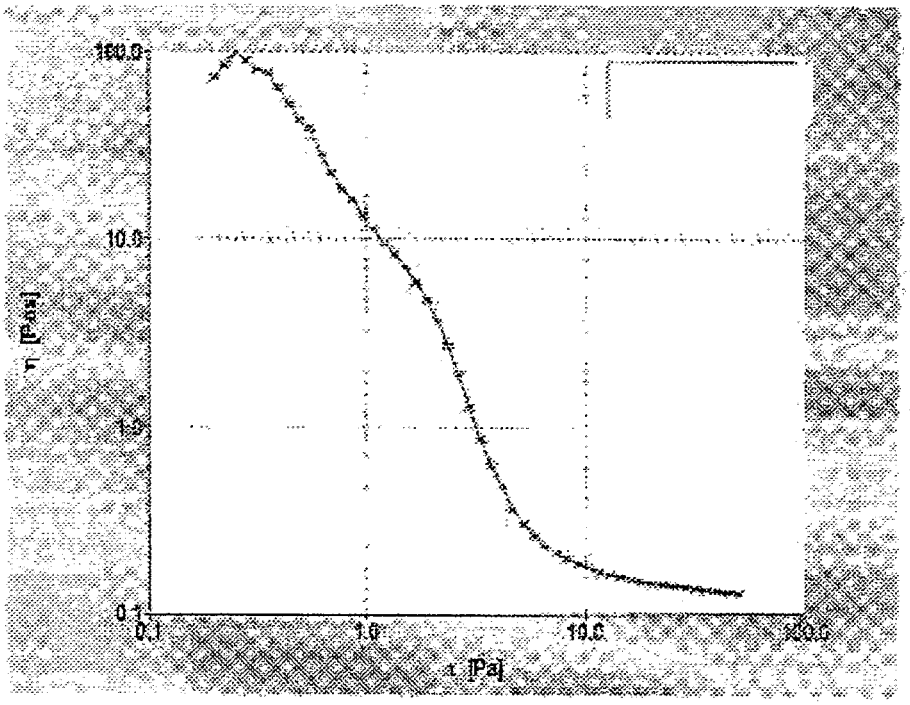
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Fig. 11

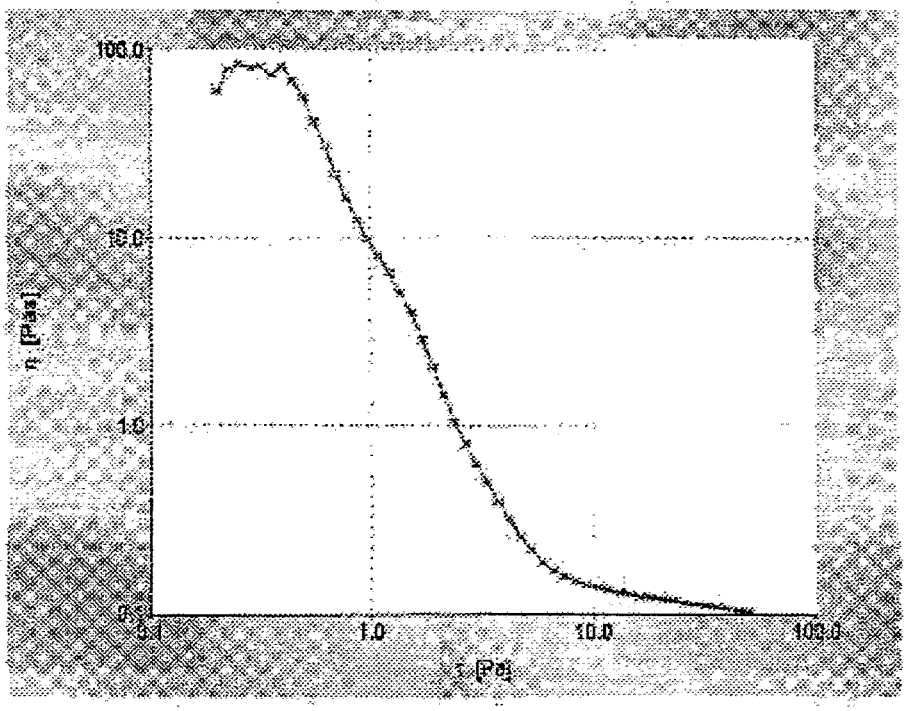
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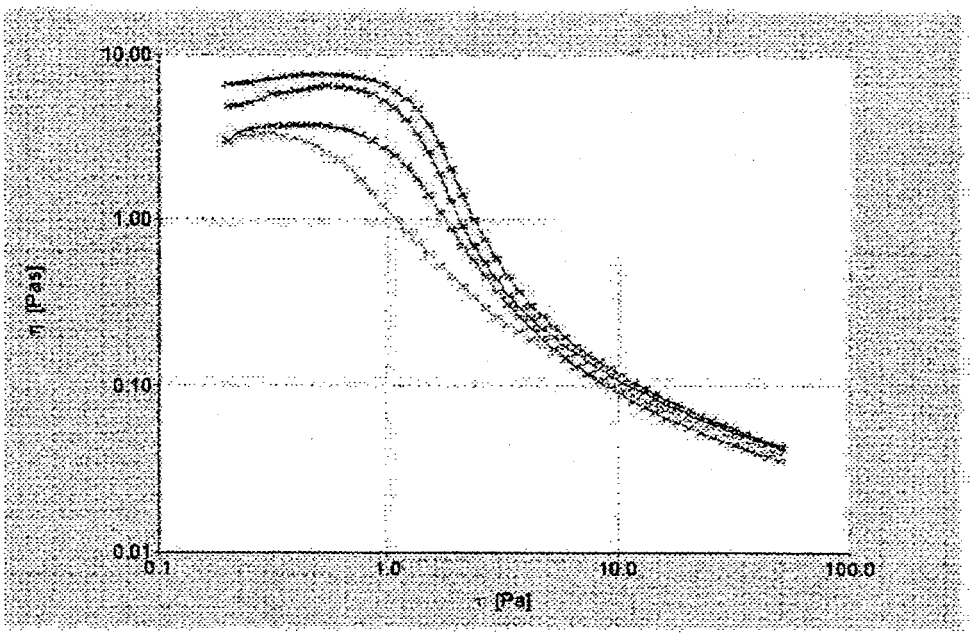
Fig. 12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τ [Pa]

Fig. 13

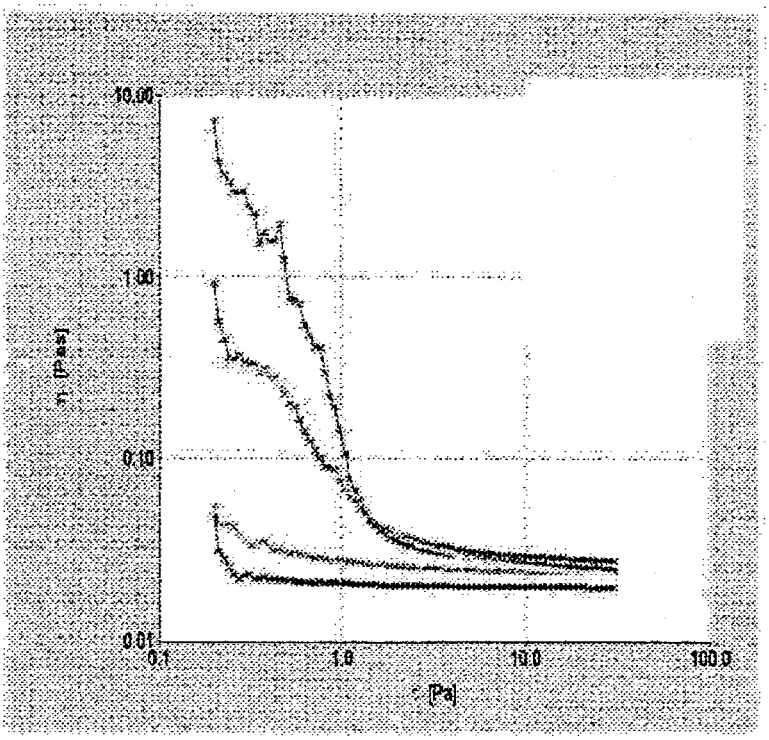

10.00
1.00
0.10
0.01
η [Pas]
0.1
1.0
10.0
100.0
τ [Pa]

Fig.14

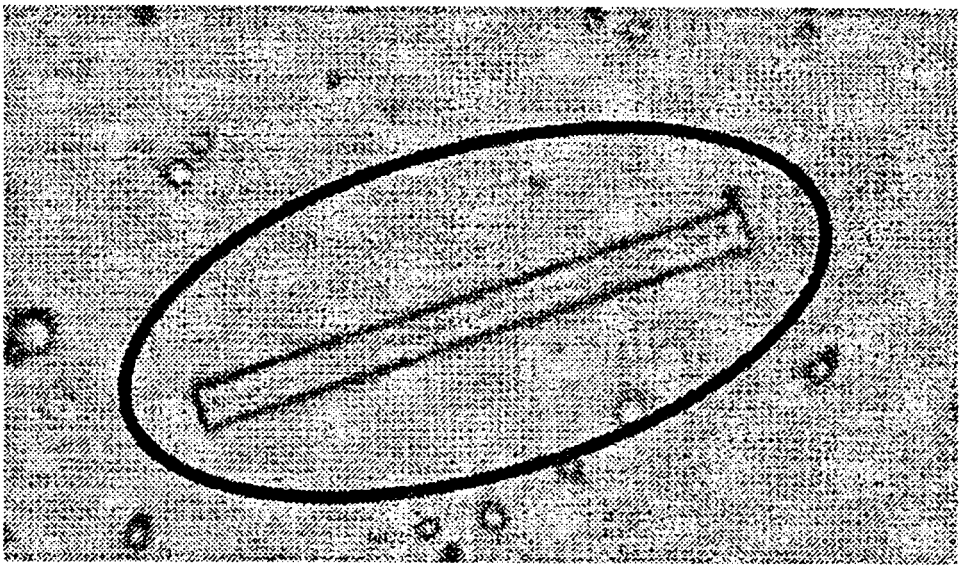


Fig. 15

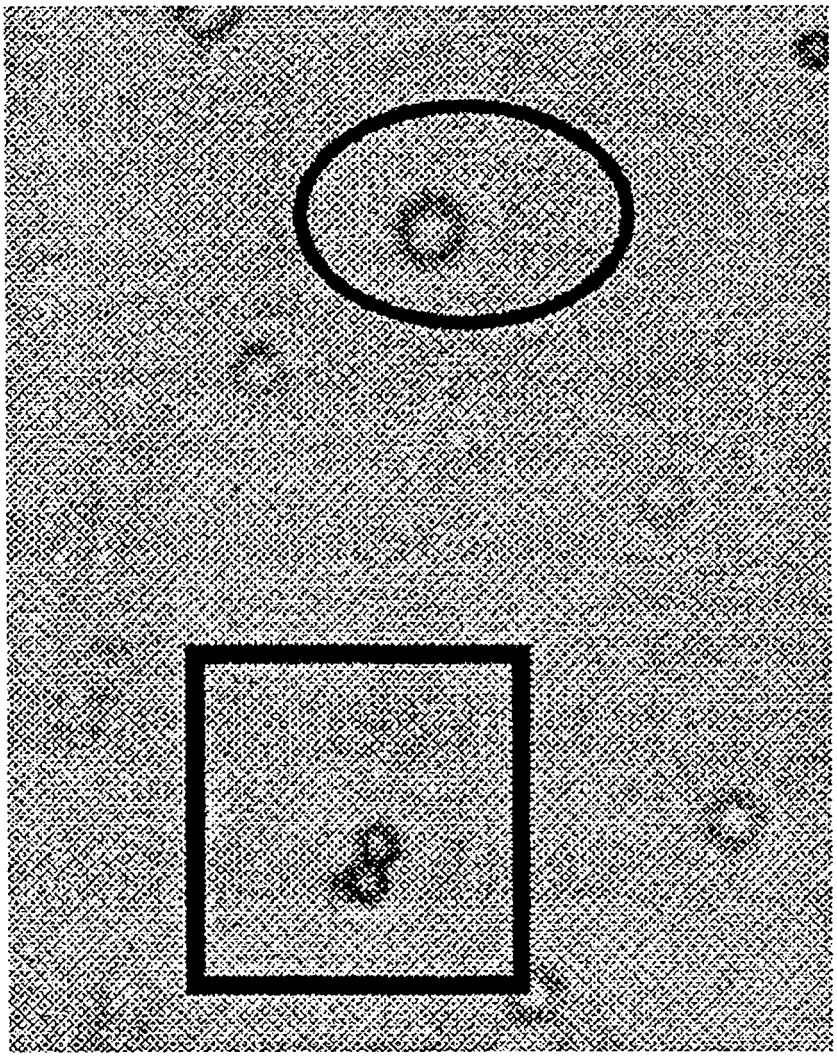


Fig. 16

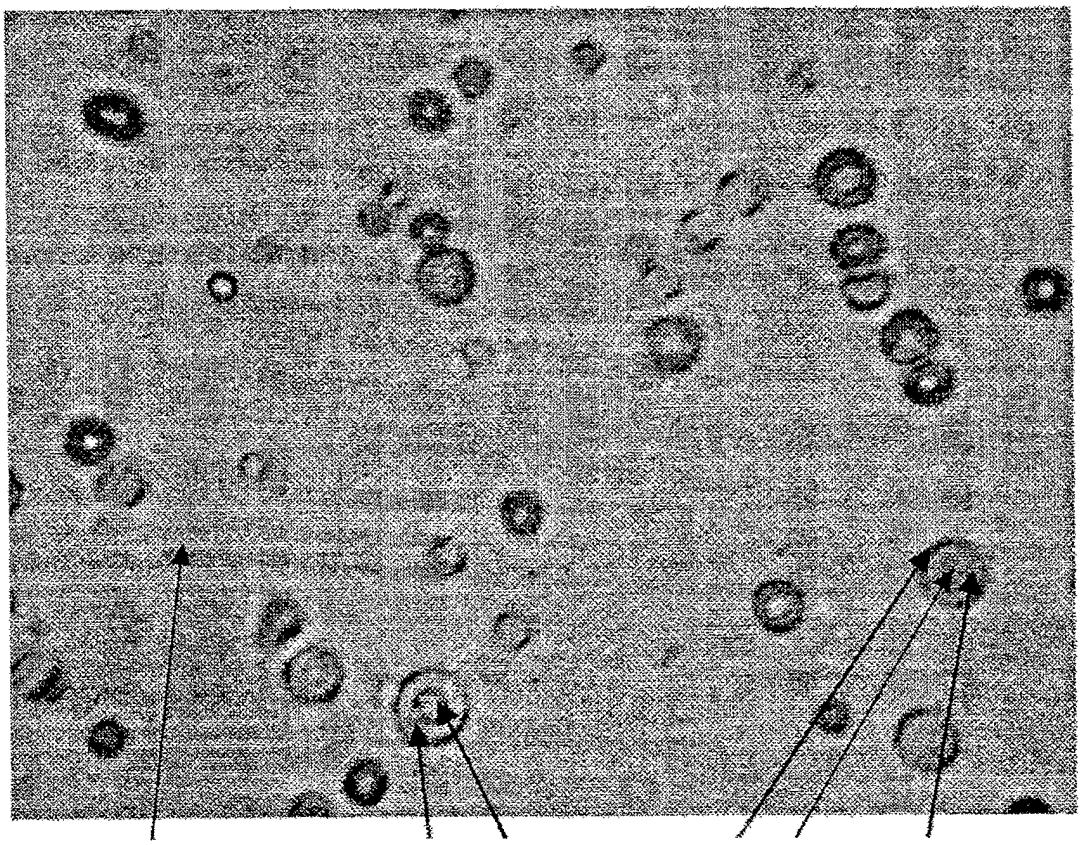


Fig.17

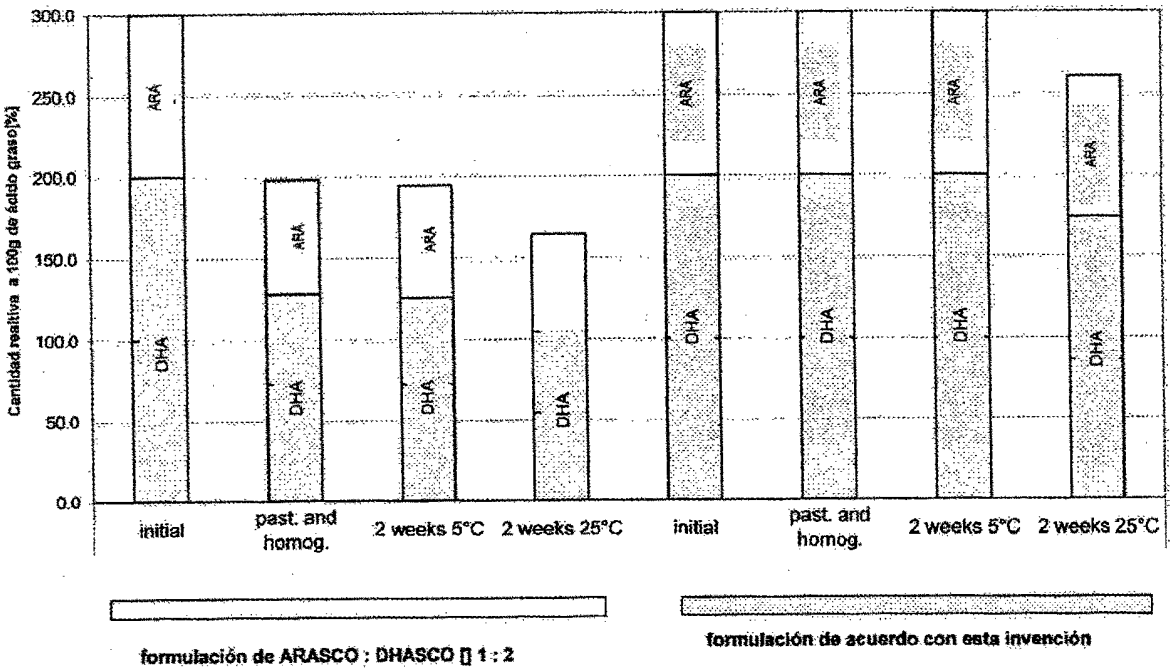

Cantidad relativa a 100g de ácido graso[%]
300.0
250.0
200.0
150.0
100.0
50.0
0.0
ARA
DHA
initial
past. and homog.
2 weeks 5°C
2 weeks 25°C
initial
past. and homog.
2 weeks 5°C
2 weeks 25°C
formulación de ARASCO : DHASCO [] 1 : 2
formulación de acuerdo con esta invención

Fig.18

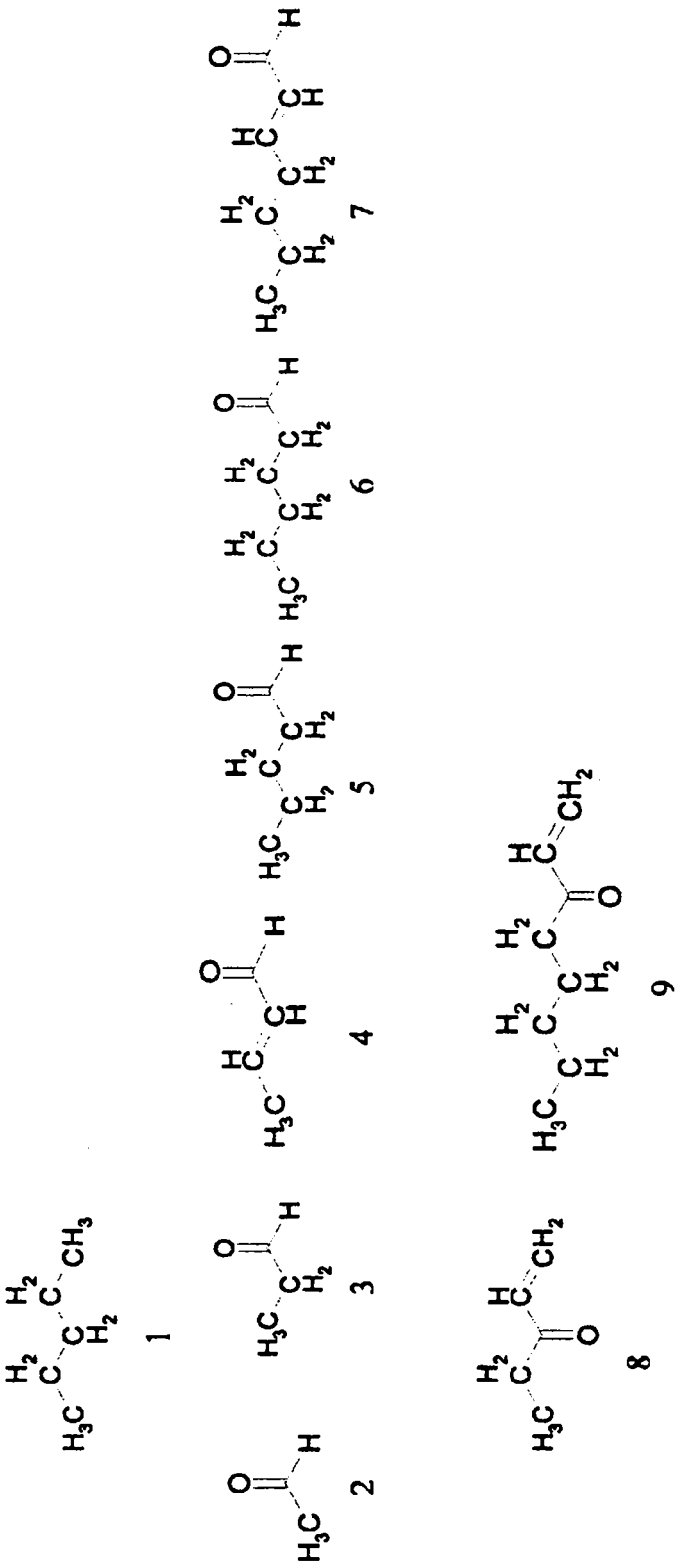
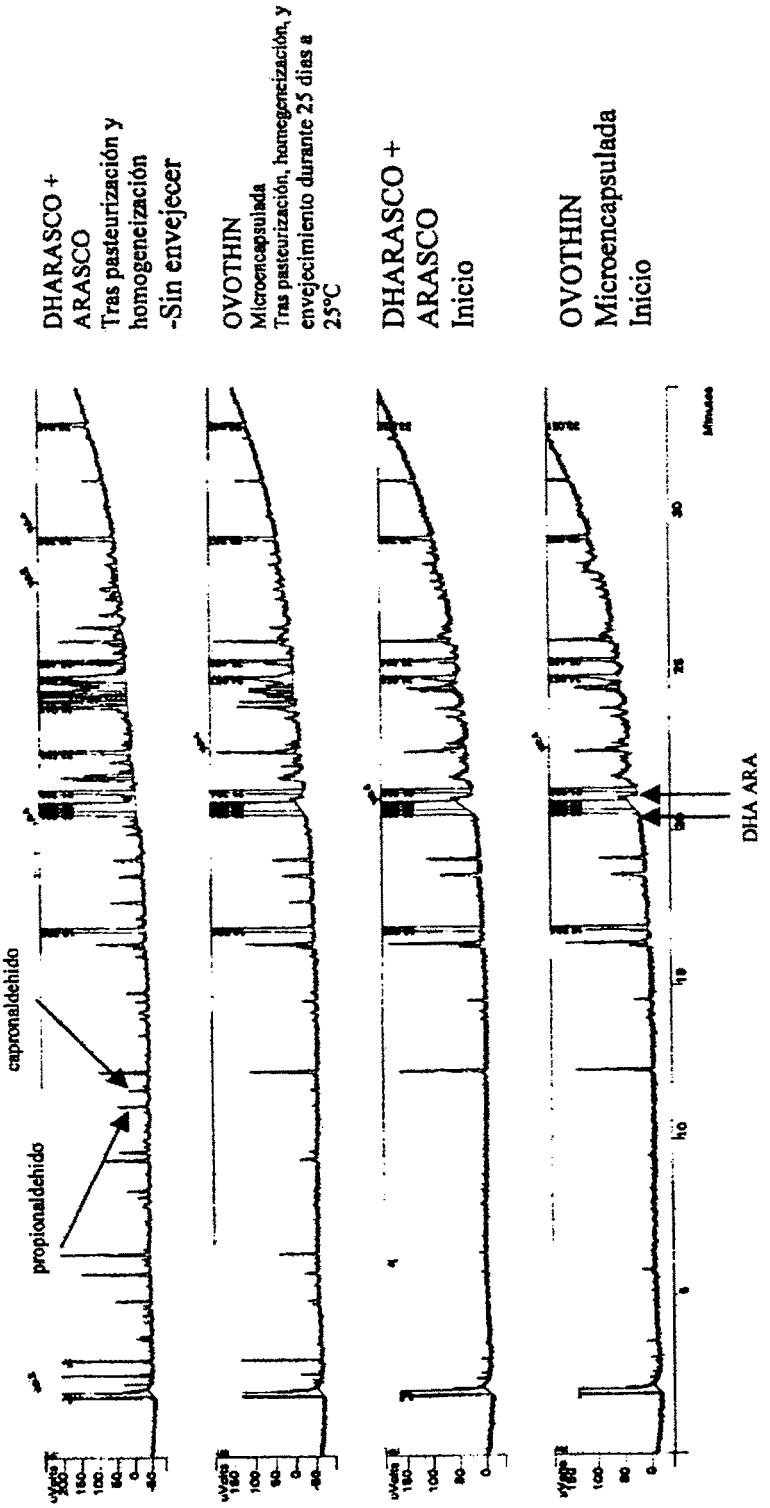


Fig.19


DHARASCO +
ARASCO
Tras pasteurización y
homogeneización
-Sin envejecer
OVOTHIN
Microencapsulada
Tras pasteurización, homegeneización, y
envejecimiento durante 25 dias a
25ºC
DHARASCO +
ARASCO
Inicio
OVOTHIN
Microencapsulada
Inicio
capronaldehido
propionaldehido
DHA ARA

OFICINA ESPAÑOLA DE PATENTES Y MARCAS

ESPAÑA

(11) ES 2 235 642

(21) Nº de solicitud: 200302998

(22) Fecha de presentación de la solicitud: **18.12.2003**

(32) Fecha de prioridad:

INFORME SOBRE EL ESTADO DE LA TÉCNICA

(51) **Int. Cl.**[7]**:** B01J 13/16

DOCUMENTOS RELEVANTES

Categoría	Documentos citados	Reivindicaciones afectadas
A	US 4308165 A (ANTHONY E. VASSILIADES et al.) 29.12.1981, todo el documento.	1-83
A	US 3875074 A (ANTHONY E. VASSILIADES et al.) 01.04.1975, todo el documento.	1-83
A	EP 1344516 A1 (COGNIS IBERIA SL) 17.09.2003, resumen EPOC.	1-83
A	ES 2066044 T3 (MILUPA AKTIENGESELLSCHAFT) 01.03.1995, todo el documento.	1-83

Categoría de los documentos citados

X: de particular relevancia
Y: de particular relevancia combinado con otro/s de la misma categoría
A: refleja el estado de la técnica
O: referido a divulgación no escrita
P: publicado entre la fecha de prioridad y la de presentación de la solicitud
E: documento anterior, pero publicado después de la fecha de presentación de la solicitud

El presente informe ha sido realizado

☒ para todas las reivindicaciones ☐ para las reivindicaciones nº:

Fecha de realización del informe	Examinador	Página
27.04.2005	M. Ybarra Fernández	1/1

Project no. : COOP-CT-2003-508649

Acronym : PARADOX

Project title : French Paradox
Red wine extract food additives

Instrument : Special Research Project for SME`s
Cooperative Research (CRAFT)

MANAGEMENT REPORT

Period covered : from 15. 02. 2005 to 14. 02 2006 Date of Preparation :30.03.2006

Start date of project : 15.02.2004 Duration : 24 months

Project coordinator name : Dr. Barbara Gimeno

Project coordinator organisation name : GAT Formulation GmbH

Revision : Draft

PARADOX PROJECT

EU CONTRACT COOP-CT-2003-508649

From the lab to the shelf

We are proud to announce the completion of the EU Project PARADOX, the achievement of all targets within the difficult task of bringing the results from basic research to an Industrial success in a short timeframe. We would like to express our thanks to the CRAFT initiative of the European Commission in the 6. Framework programme and to the officers responsible for the implementation.

The PARADOX project finished in a REAL tangible practical result as a novel technology of Functional Food Ingredient is arriving to the industrial circuit with commercial products. This project is NOT what some of the EU-projects finally are condemned to be: a final report kept in the drawers of the European Commission (also in some Scientific Publications) that never arrive to show an industrially significant impact.

What was the Objective of the Project?

Research based on the agricultural residues of red wine making (grape pomace) in order to develop a clinically proven Ingredient for Functional Foods.

What was the Result of the Project?

Full achievement of the Objectives. Launch of commercial competitive Functional Food Ingredients.

What has been done to arrive to the Objective?

0.- EVALUATION PRIOR TO PROJECT SUBMISSION

Before even applying for the project we have evaluated the economic implications of getting involved in a strong effort of collaborative R&D with unknown results (balance success/failure) and reviewed the Intellectual Property status in the field of research in order to take appropriate measures to patent-protect the outcome of the project, from an early stage of the project, considering the European Laws on Food Ingredients. The evaluation was focused on the goal to develop a product that eventually can be successfully sold.

1.- FIND PARTNERS

Establish a collaborative network of partners carefully selected to ensure the achievement of goals in each Project Phase.

2.- GET SCIENTIFIC EXPERTISE

Involve groups from the areas of basic research, applied research, and industrial research. In our case we included clinical trial facilities to ensure the quality of the product for human consumption.

3.- INSISTANT COORDINATION

Good coordination and timely reporting is absolutely required to ensure the achievement of the Objectives of all groups involved.

4.- KEEP ASKING

We tried to maintain continuous feedback in between the partners to redirect the research and the needs of each downhill and uphill steps in the development.

5.- KEEP TALKING

Numerous consultations and discussions requiring flexibility of all partners to achieve common positions, even in those situations where a partner may raise objections (but the project must go on).

6.- COMMIT TO SEARCH

Scientific spirit and eagerness to discover to overcome the lack of economic support or time in certain stages of the project.

What has been research and developed in the Project?

The aim was to develop a Functional Food Ingredient with antioxidant and anticarcinogenic properties, already attributed to some compounds present in wine and in grapes, in a format that protects from early oxidation before consumption thanks to micro-encapsulation.

First, we have chosen as a source of these antioxidants a product that has i) low/no commercial value ii) represents an environmental issue. Indeed the grape pomace is a product with very low value for the wine-making industry, and at it may constitute a contaminant, with the difficulties of getting rid of this agricultural waste.

We included grape pomace from different climates, varieties, wine-making processes and countries in order to assess the suitability of different sources of grape pomace.

For the extraction method, we have performed a combined solution in order to extract the antioxidants from the grape pomace at a high yield while maintaining the requirements of an economically viable extraction in an industrial process. However, also high-yield techniques of extraction (but not industrially applicable) were included in the protocol to compare our results with those of previous literature.

For this purpose, we tried different techniques of extraction, with different solvents (ethanol, methanol, ethyl acetate, acetone, water, trichloromethane, tetra-hydrofurane, tert-methylbutyl ether) and also the most preferred extraction solvent (a fully inert solvent) supercritical CO2. Various pre-extraction steps, such as crushing the whole pomace, the seeds or the skins were tested. Further ways to concentrate the desired antioxidants of the most successful extraction methods were developed, such as column chromatography separation (Sephadex column as the most representative), and precipitation of polar solvent extract with non-polar solvent (to crystallize the antioxidants of interest).

Methods of analysis for the extracts, in order to scan the yield of each technique were developed, such as HPLC-DAD, HPLC-UV, HPLC-derivatization-UV, HPLC-MS, GC-FID, GC-MS, Colorimetry, Fluorometry and RedOx reactions.

Once the “coupling” of the analytical techniques and the extraction techniques allowed to identify the optimal ways of extraction (in between of dozens tried), samples were ready to be evaluated in vitro on cell cultures for their biological activity.

It is worthy to mention that this project has triggered the thorough research of a type of molecule that, even being antioxidant, is suspected to act in the human body as beneficial for atherosclerosis prevention not due to its antioxidant ability, but rather due to its ability of inhibiting the enzyme ET-1. This scientific finding introduces a novel approach in the field of beneficial compounds for its use in Functional Foods. The molecular compound (a tetrameric epicatechine) has been the object of detailed analysis.

Tests in vitro were performed in a thorough way incorporating the newest state of the art techniques to assess the beneficial effect of the grape pomace extract at the cellular level: Reactive Oxygen Species, actin cytoskeleton modifications, cell proliferation (anticarcinogenity tests) in different cell lines as markers of prostate cancer, breast cancer and liver carcinoma, antioxidant activity. Extensive and complicated analyses relating to the activity in vitro (in cell-lines) of the pomace extract as inhibitor of the enzyme Endothelin 1 were performed.

Once determined unambiguously the beneficial effects of the pomace extract obtained by our process, it was brought into a stable and easy to handle galenic format. A particular microencapsulation with an edible wall material and all food-approved ingredients for the pomace extracts was developed, in order: i) to avoid oxidation of the extract in the final Functional Food (during processing, transport and shelf live) ii) to allow that once ingested (inside the body) all the grape pomace extract is released and made bioavailable.

The next crucial aspect has been to microencapsulate the grape pomace extract fulfilling the conditions above mentioned. We finally were able to apply a new microencapsulation method that surpasses in technological benefits the state of the art of encapsulation technology: we arrived to a full protection of the antioxidants against O2, temperature, pressure (homogenization processes) and light.

The ready microencapsulated pomace extract was stabilized as a ready to market Functional Food Ingredient and branded as PARADOX. The product was added to orange juice for consumption by a group of volunteers in a clinical study.

Final clinical data have shown that consumption of our product results in a decreased oxidation level in blood plasma of the volunteers, which is the biological marker for the bioavailability of the healthy and beneficial properties of the product we have developed.

At last, all these extreme efforts in basic and applied research have ended up in a tangible and physical result: currently the European consumer may acquire Functional Foods containing our microencapsulated GAT Food Essential PARADOX.

Cost Follow-up Table

Contract No.:	COOP-CT-2003-508649	Acronym :	PARADOX		Date :	21.03.2006	
PARTICIPANT	TYPE of EXPENDITURE	BUDGET	ACTUAL COSTS (EUR)			Pct. Spent	Remaining
No.: Short Name	(as defined by participant)	EUR	Period 1	Period 2	Total	Total	Budget EUR
1 GAT	Total Person-month	37	30	13	43	116,22%	-6
	Personnel costs	226.473	173.846	62.764	236.610	104,48%	-10.137
	Other costs	53.600	23.215	12.577	35.792	66,78%	17.808
	Flat rate	49.220	32.285	11.435	43.720	88,83%	5.500
	Total costs	329.293	229.346	86.776	316.122	96,00%	13.171
2 RODA	Total Person-month	3,6	1,4	3	4	119,44%	-0,7
	Personnel costs	19.644	6.803	12.246	19.049	96,97%	595
	Other costs	9.600	217	1.724	1.941	20,22%	7.659
	Flat rate	4.811	1.404	1.548	2.952	61,36%	1.859
	Total costs	34.055	8.424	15.518	23.942	70,30%	10.113
4 CHAPOUTIER	Total Person-month	3,6	0,1	0	0	2,78%	3,5
	Personnel costs	19.644	544	0	544	2,77%	19.100
	Other costs	4.800	72	0	72	1,50%	4.728
	Flat rate	4.889	122	0	122	2,50%	4.767
	Total costs	29.333	738	0	738	2,52%	28.595
5 HEINRICH	Total Person-month	3,6		5	5	138,89%	-1
	Personnel costs	19.644	0	15.891	15.891	80,89%	3.753
	Other costs	4.800	0	3.369	3.369	70,19%	1.431
	Flat rate	4.889	0	3.752	3.752	76,74%	1.137
	Total costs	29.333	0	23.012	23.012	78,45%	6.321
6 TILIA	Total Person-month	3,6			0	0,00%	4
	Personnel costs	19.644	0		0	0,00%	19.644
	Other costs	4.800	0		0	0,00%	4.800
	Flat rate	4.889	0		0	0,00%	4.889
	Total costs	29.333	0	0	0	0,00%	29.333
7 VINCELLER	Total Person-month	3,6		3,8	3,8	105,56%	-0,2
	Personnel costs	19.644	0	6.643	6.643	33,82%	13.001
	Other costs	4.800	0	16.300	16.300	339,58%	-11.500
	Flat rate	4.889	0	4.429	4.429	90,59%	460
	Total costs	29.333	0	27.372	27.372	93,31%	1.961
8 CINS	Total Person-month	9	7,1	1	8	90,00%	0,9
	Personnel costs	46.710	42.420	4.305	46.725	100,03%	-15
	Other costs	9.500	9.809	3.314	13.123	138,14%	-3.623
	Flat rate	11.242	10.446	924	11.370	101,14%	-128
	Total costs	67.452	62.675	8.543	71.218	105,58%	-3.766
9 NATEX	Total Person-month	9	3,5	2,7	6	68,89%	2,8
	Personnel costs	46.710	30.376	13.153	43.529	93,19%	3.181
	Other costs	14.300	15.167	20.691	35.858	250,76%	-21.558
	Flat rate	11.164	9.109	5.510	14.619	130,95%	-3.455
	Total costs	72.174	54.652	39.354	94.006	130,25%	-21.832
10 CHIROBLOCK	Total Person-month	13	5,4	2,2	8	58,46%	5,4
	Personnel costs	62.280	12.475	6.400	18.875	30,31%	43.405
	Other costs	23.800	9.327	4.774	14.101	59,25%	9.699
	Flat rate	16.178	4.361	2.235	6.596	40,77%	9.582
	Total costs	102.258	26.163	13.409	39.572	38,70%	62.686
11 CAMPI	Total Person-month	1,5		3	3	166,67%	-1
	Personnel costs	7.785	0		0	0,00%	7.785
	Other costs	14.600	0	23.460	23.460	160,68%	-8.860
	Flat rate		0	4.592	4.592		-4.592
	Total costs	22.385	0	28.052	28.052	125,32%	-5.667
12 VALMAR	Total Person-month	1,5	0	0	0	0,00%	1,5
	Personnel costs	7.785	0	0	0	0,00%	7.785
	Other costs	7.800	0	0	0	0,00%	7.800
	Flat rate		0	0	0		0
	Total costs	15.585	0	0	0	0,00%	15.585
13 KUK	Total Person-month	1,5	0,4	0,5	0,9	60,00%	0,6
	Personnel costs	7.785	1.195	2.389	3.584	46,04%	4.201
	Other costs	7.800	685	915	1.600	20,51%	6.200
	Flat rate		376	661	1.037		-1.037
	Total costs	15.585	2.256	3.965	6.221	39,92%	9.364
14 ATYS	Total Person-month	1,5	0	0	0	0,00%	2
	Personnel costs	7.785	0	0	0	0,00%	7.785
	Other costs	7.800	0	0	0	0,00%	7.800
	Flat rate		0	0	0		0
	Total costs	15.585	0	0	0	0,00%	15.585
15 QMUL	Total Person-month	23	2	4	6	26,09%	17
	Personnel costs	108.990	10.476	23.716	34.192	31,37%	74.798
	Other costs	37.800		12.365	12.365	32,71%	25.435
	Flat rate	29.358	2.095	7.217	9.312	31,72%	20.046
	Total costs	176.148	12.571	43.298	55.869	31,72%	120.279
16 CERIELLO	Total Person-month	18		29	29	161,11%	-11
	Personnel costs	93.030	0	66.179	66.179	71,14%	26.851
	Other costs	31.800	0	25.133	25.133	79,03%	6.667
	Flat rate	24.966	0	18.262	18.262	73,15%	6.704
	Total costs	149.796	0	109.574	109.574	73,15%	40.222
17 CASTANAS	Total Person-month	30	7,5	20	28	91,67%	2,5
	Personnel costs	157.820	15.054	117.946	133.000	84,27%	24.820
	Other costs	57.600	74.470	1.160	75.630	131,30%	-18.030
	Flat rate	39.970	17.905	23.589	41.494	103,81%	-1.524
	Total costs	255.390	107.429	142.695	250.124	97,94%	5.266
TOTAL	Total Person-month	163	57	87	144	88,34%	19
	Personnel costs	871.373	293.189	331.632	624.821	71,71%	246.552
	Other costs	295.200	132.962	125.782	258.744	87,65%	36.456
	Flat rate	206.465	78.103	84.154	162.257	78,59%	44.208
	Total costs	1.373.038	504.254	541.568	1.045.822	76,17%	327.216

Person-Month Status Table

CONTRACT N° : COOP-CT-2003-508649

ACRONYM : PARADOX

PERIOD : 15.2.2004 - 14.2.2006

Partner - Person - Month per Workpackage

		TOTALS	Coordinator	Part. 1	Partic. 2	Partic.4	Partic.5	Partic.6	Partic 7	Partic.8	Partic.9	Partic.10	Partic.11	Partic.12	Partic.13	Partic.14	Partic.15	Partic.16	Partic.17
Workpackage 1 :	**Actual WP total :**	13			4,3	0,1	5		3,8										
Raw material / red wine residues	**Planned WP total :**	18			3,6	4	4	3,6	3,6										
Workpackage 2 :	**Actual WP total :**	11								7,1	3,5								
EXTRACTION	**Planned WP total :**	18								9	9								
Workpackage 3 :	**Actual WP total :**	20		13								7,0							
ANALYTICAL CHARACTERISATION	**Planned WP total :**	25		12								13							
Workpackage 4 :	**Actual WP total :**	13		13															
FORMULATION	**Planned WP total :**	9		9															
Workpackage 5 :	**Actual WP total :**	62															6	29	27
EFFICIACY TESTING	**Planned WP total :**	71															23	18	30
Workpackage 6 :	**Actual WP total :**	6,5		7															
PROCESS DEVELOPMENT	**Planned WP total :**	6		6															
Workpackage 7 :	**Actual WP total :**	4		4															
TRAINING	**Planned WP total :**	4		4															
Workpackage 8 :	**Actual WP total :**	3,3											2,5	0	0,8	0			
DISSEMINATION	**Planned WP total :**	6											1,5	1,5	1,5	1,5			
Workpackage 9 :	**Actual WP total :**	5,9	5,9																
PROJECT MANAGEMENT	**Planned WP total :**	6	6																
	Actual total :	139	6	37	4,3	0,1	5	0	3,8	7,1	3,5	7	2,5	0	0,8	0	6	29	27,0
Total Project Person-month	**Planned total :**	163	6	31	3,6	4	4	3,6	3,6	9	9	13	1,5	1,5	1,5	1,5	23	18	30

PARADOX - MANAGEMENTREPORT

Justification of major cost items and resources - Description

No.:	Participant Name	declared costs 2. reporting period Research/techn. Dev Innvoation	Management	20% Flat Rate	Total
1	**GAT**	**57.175,08**	**18.166,00**	**11.434,92**	**86.776,00**

Research/Development :

WP 3 (Development of analytical methods)
WP 4 (Development of formulation and microencapsulation)
WP 6 (Process Development)
WP 7 (Training)

Personnel costs for 1.832 working hours :	49.557,00
Transportation Materials	200,08
Costs for 1.526 maschine hours (GC,HPLC, GC/MS) incl. analytical and lab materials	7.418,00
	57.175,08

Management of the consortium :
WP 9 (Project Management)

Personnel costs for 333 working hours (communication, information,meetings,reporting)	13.207,00
Travel expenses and accommodation (Meeting London,Seminar Brussels)	1.267,00
Internet platform "PARADOX" project communication , licence and rental fees	342,00
Management costs (external Audit costs)	3.350,00
	18.166,00

No.:	Participant Name	declared costs 2. reporting period Research/techn. Dev Innvoation	Management	20% Flat Rate	Total

2	**RODA**	**7.740,00**	**6.230,00**	**1.548,00**	**15.518,00**

Research/Development :

WP 1 (Raw material providing)

Personnel costs for 496 working hours (Harvest,analysis,sampling,vinification process		
Management, filling,sending,control and coordination)	6.916,00	
Material and transport	824,00	
	7.740,00	
Management of the project (Personnel costs)		5.330,00
External Audit costs		900,00
		6.230,00

No.:	Participant Name	declared costs 2. reporting period Research/techn. Dev Innvoation	Management	20% Flat Rate	Total
4	**CHAPOUTIER**	**0,00**	**0,00**	**0,00**	**0,00**

No costs claimed

No.:	Participant Name	declared costs 2. reporting period Research/techn. Dev Innvoation	Management	20% Flat Rate	Total
5	**HEINRICH**	**18.759,72**	**500,00**	**3.751,94**	**23.011,66**

Research/Development :

WP 1 (Raw material providing)

Personnel costs 876 working hours,Harvest 2004+2005 (preparation, administration)	15.891,10	
Travel expenses	1.903,52	
costs of material	965,10	
	18.759,72	
Management costs (external Audit costs)		500,00

No.:	Participant Name	declared costs 2. reporting period Research/techn. Dev Innvoation	Management	20% Flat Rate	Total
6	**TILIA**	**0,00**			**0,00**

Research/Development :

WP 1 (Raw material providing)
Personnel costs ### working hours (preparation, administration)
costs of material

0,00

No.:	Participant Name	declared costs 2. reporting period Research/techn. Dev Innvoation	Management	20% Flat Rate	Total
7	**VINCELLER**	**9.412,73**	**800,00**	**1.882,55**	**27.372,00**

Research/Development :

WP 1 (Raw material providing)
Personnel costs 307 working hours (preparation, administration) 4.634,40
costs of material and equipment 4.778,33
9.412,73

Adjustments previous periods :
Personnel costs 345 working hours (preparation, administration) 2.008,80
costs of material and equipment 10.722,54
sub total 12.731,34
incl. 20% flat rate 15.277,61
Management costs (external Audit costs) 800,00

No.:	Participant Name	declared costs 2. reporting period Research/techn. Dev Innvoation	Management	20% Flat Rate	Total
8	**CINS**	**4.619,26**	**3.000,00**	**923,85**	**8.543,11**

Research/Development :

WP 2 (Extraction experiments, development of extraction process and optimization)

Personnel costs for 148 working hours	4.305,00
Travel expenses and accommodation	314,26
	4.619,26

Management costs (external Audit costs) 3.000,00

No.:	Participant Name	declared costs 2. reporting period Research/techn. Dev Innvoation	Management	20% Flat Rate	Total
9	**NATEX**	**31.343,88**	**2.500,00**	**5.509,97**	**39.353,85**

Research/Development :

WP 2 (Extraction experiments, development extraction techniques and optimization)

Personnel costs for 473 working hours	13.152,78
Consumables (Chemicals, Analytical materials, Lab materials)	8.865,13
Rental fee for lab equipment	9.200,00
Travel expenses and accommodation (kick-off meeting Heraklion)	125,97
	31.343,88

Management costs (external Audit costs) 2.500,00

No.:	Participant Name	declared costs 2. reporting period Research/techn. Dev Innvoation	Management	20% Flat Rate	Total

10	**CHIROBLOCK**	**9.973,84**	**1.200,00**	**2.234,77**	**13.408,61**

Research/Development :

WP 3 (Analytical Characterisation,identification of polyphenol structures)

Personnel costs for 281 working hours	6.399,91
Consumables (Chemicals, Synthesis- and Analytical material, Lab materials)	1.538,41
Costs for machine hours (HPLC, NMR,Parallelsynthesizer,Kapillarzonenelektrophorese)	2.035,53
	9.973,84

Management costs (external Audit costs) 1.200,00

No.:	Participant Name	declared costs 2. reporting period Research/techn. Dev Innvoation	Management	20% Flat Rate	Total
11	**CAMPI Y JOVE**	**22.960,00**	**500,00**	**4.592,00**	**28.052,00**

Research/Development :

Marketing Activities

Travel expenses	22.960,00
Travel activities invested time 432 hours	
	22.960,00

Management costs (external Audit costs) 500,00

No.:	Participant Name	declared costs 2. reporting period Research/techn. Dev Innvoation	Management	20% Flat Rate	Total

12	VALMAR	0,00	0,00	0,00	0,00

No costs claimed

No.:	Participant Name	declared costs 2. reporting period Research/techn. Dev Innvoation	Management	20% Flat Rate	Total
13	KUK	3.303,91		660,79	3.964,70

Research/Development :

Personnel costs for 93 working hours (prep. Meeting, Meeting + Trainingclients)	2.389,41
Travel expenses and accommodation	914,50
	3.303,91

No.:	Participant Name	declared costs 2. reporting period Research/techn. Dev Innvoation	Management	20% Flat Rate	Total
14	ATYS	0,00	0,00	0,00	0,00

No costs claimed

No.:	Participant Name	declared costs 2. reporting period Research/techn. Dev Innvoation	Management	20% Flat Rate	Total
15	QMUL	35.641,00	440,00	7.217,00	43.298,00

Research/Development :

WP 5 (Efficiacy Testing, In vitro experiments on endothelian cells, reporting and elaboration of papers for publication)		
Personnel costs for 700 working hours	23.242,00	
Consumables (Lab materials)	12.365,00	
Travel expenses	34,00	
	35.641,00	
Cost Audit Certificate		440,00

No.:	Participant Name	declared costs 2. reporting period Research/techn. Dev Innvoation	Management	20% Flat Rate	Total
16	**CERIELLO**	**90.999,80**	**312,00**	**18.262,36**	**109.574,16**

Research/Development :

WP 5 (Efficiacy Testing, In vitro experiments on endothelian cells, reporting and elaboration of papers for publication)	66.178,90	
Personnel costs for 5062 working hours		
travel and subsistence (Meetings in Vienna,London)	2.041,49	
Consumables (cell culture media and regents,biochemical regents,lba materials)	22.779,41	
	90.999,80	
Management costs (external Audit costs)		312,00

No.:	Participant Name	declared costs 1. reporting period Research/techn. Dev Innvoation	Management	20% Flat Rate	Total
17	**CASTANAS**	**117.945,94**	**1.160,00**	**23.589,19**	**142.695,13**

Research/Development :

WP 5 (Efficiacy Testing,In vitro and in vivo experiments on cancer cell lines, reporting and elaboration of papers for publication

Personnel costs for 3.403 working hours	43.045,64			
Consumables (cell culture media and regents,biochemical regents,Radioactive tracers etc.)	63.141,01			
Lab equipment (Incubator for cell cultures)	10.826,00			
Travel expenses	933,29			
	117.945,94			
Management costs (external Audit costs)		1.160,00		
TOTAL	**409.875,16**	**34.808,00**	**81.607,34**	**541.567,23**

Form C - Model of Financial Statement per Activity for a Specific Cooperative Research for SMEs

(to be completed by each contractor)

Type of instrument	**Specific Research Project for SMEs**	**Type of Action (if necessary)**	**Cooperative Research (CRAFT)**
Project Title (or Acronym)	**PARADOX**	**Contract n°**	**COOP-CT-2003-508649**

Contractors's legal name	**GAT Formulation GmbH**		
Legal Type	**Small and medium enterprise**		
Contact Person	**Dr. Barbara Gimeno**	**Telephone**	+ 43 2624 53922-0
Telecopy	**+43 2624 53922 38**	**E-mail**	gat@gat-formulation.com

Cost model used (AC/FC or FCF)	**FCF**	**Indirect costs (Real or Flat Rate of 20% of Direct costs, except subcontracting)**	**20 % Flat Rate**
Period from	**15.02.2005**	**TO**	**14.02.2006**

1- Resources (Third party(ies))

Are there any resources made available on the basis of a prior agreement with third parties identified in Annex I of the contract? (Yes / No)	No
If Yes, please provide the following information	

Third Party 1 (Y1) Legal name		**Cost model used**	
Third Party 2 (Y2) Legal name		**Cost model used**	
Third Party 3 (Y3) Legal name		**Cost model used**	
Third Party 4 (Y4) Legal name		**Cost model used**	

If necessary add another Form C

2- Declaration of eligible costs (in €)

Please complete only the activity covered by the relevant instrument (and type of action) indicated above and as mentioned in Article II.25 and/or in Annexes I and III of the contract.

If you are a contractor using the additional cost model (AC):

- indicate only your additional eligible costs, except for Management of the Consortium Activity for which you may indicate your full eligible costs;

do not declare eligible direct additional costs specifically covered by contributions from third parties as mentioned in Articles II.20 and II.23.a and b of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate your full eligible costs
The costs declared should distinguish between direct and indirect costs
If necessary, adjustments to previous period(s) may be included where appropriate

	Type of Activity											
	Research and Technological Development / Innovation (A)		Demonstration (B)		Training (C)		Management of the Consortium (D)		Other Specific Activities (E)		Total (F) = (A)+(B)+(C)+(D)+(E)	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Direct costs	57.175						18.472				75.647	
Of which subcontracting												
Indirect costs	11.435										11.435	
Adjustments to previous period(s)												
Total costs	68.610						18.472				87.082	

3- Declaration of receipts (in €)

If you are a contractor using the additional cost model (AC), indicate only receipts covered by Article II.23.c of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate receipts covered by Article II.23 of the contract.

	Type of Activity											
	Research and Technological Development / Innovation (A')		Demonstration (B')		Training (C')		Management of the Consortium (D')		Other Specific Activities (E')		Total (F') = (A')+(B')+(C')+(D')+(E')	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Total receipts												

4- Declaration of interest generated by the pre-financing (in €)	
To be completed only by the coordinator.	
Did the pre-financing (advance) you received by the Commission for this period earn interest? (Yes / No)	Yes
If yes, please indicate the amount (in €)	
5- Request of FP6 Financial Contribution (in €)	
For this period, the FP6 Community financial contribution resuested is equal to (amount in €)	49.144

6- Audit certificates				
According to the contract, does this Financial Statement need an audit certificate (or several in case of Third party(ies)) delivered by independent auditor(s)? (Yes / No)				YES
If Yes, does this(those) audit certificate(s) cover only this Financial Statement per Activity? (Yes / No)				NO
If No, what are the periods covered by this(those) audit certificate(s) ?		From -to	15.2.04 - 14.02.06	
What is the total cost of this(those) audit certificate(s) (in €) per independent auditor(s) ? € 3.350,00				

Audit certificate of the contractor (X)			
Legal name of the audit firm	Ecovis Austria Wirtschaftsprüfungs GmbH	**Cost of the certificate**	€ 3.350,00
Audit certificate(s) of the third party(ies) (Ys) ***(if necessary)***			
Y1 : Legal name of the audit firm		**Cost of the certificate**	
Y2 : Legal name of the audit firm		**Cost of the certificate**	
Y3 : Legal name of the audit firm		**Cost of the certificate**	
Y4 : Legal name of the audit firm		**Cost of the certificate**	
If necessary add another Form C.		**Total (Z) = (X) + (Ys)**	

Reminders:
The cost of an audit certificate is included in the costs declared under the activity "Management of the Consortium".The required audit certificate (s) is (are) attached to this Financial Statement

7- Conversion rates	
Costs incurred in currencies other than EURO shall be reported in EURO.	
Please mention the conversion rate used (only one choice is possible) – Please note that the same principle applies for receipts.	
Contractor	
- Conversion rate of the date of incurred actual costs? (YES / NO)	No
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	No

Third Party(ies) *(if necessary)*	
Third Party 1 (Y1)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 2 (Y2)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 3 (Y3)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 4 (Y4)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	

If necessary add another Form C.

8- Contractor's Certificate

We certify that:

- the costs declared above are directly related to the resources used to reach the objectives of the project ;
- the receipts declared above are directly related to the resources used to reach the objectives of the project ;
- the costs declared above fall within the definition of eligible costs specified in Articles II.19, II.20, II.21, II.22 and II.25 of the contract, and, if relevant, in Annex III and Article 9 (special clauses) of the contract ;
- the receipts declared above fall within the definition of receipts specified in Article II.23 of the contract ;
- the interest generated by the pre-financing declared above falls within the definition of Article II.27 of the contract ; incorporated in the above Statement ;
- the above information declared is complete and true ;
- there is full supporting documentation to justify the information hereby declared. It will be made available at the request of the Commission and in the event of an audit by the Commission and/or by the Court of Auditors and/or their authorised representatives.

Contractor's Stamp	Name of the Person responsible for the work	Name of the duly authorised Financial Officer
	Dr. Barbara Gimeno	**Dr. Barbara Gimeno**
	Date	**Date**
	22.03.2006	**22.03.2006**
	Signature	**Signature**

Form C - Model of Financial Statement per Activity for a Specific Cooperative Research for SMEs

(to be completed by each contractor)

Type of instrument	**Specific Research Project for SMEs**	**Type of Action (if necessary)**	**Cooperative Research (CRAFT)**
Project Title (or Acronym)	**PARADOX**	**Contract n°**	**COOP-CT-2003-508649**

Contractors's legal name	**Bodegas RODA S.A.**		
Legal Type	**Small and medium enterprise**		
Contact Person	**Esperanza TOMAS**	**Telephone**	34941303001
Telecopy	**34941312703**	**E-mail**	estomas@roda.es

Cost model used (AC/FC or FCF)	**FCF**	**Indirect costs (Real or Flat Rate of 20% of Direct costs, except subcontracting)**	20 % Flatrate
Period from	**15.02.2005**	**TO**	**14.02.2006**

1- Resources (Third party(ies))

Are there any resources made available on the basis of a prior agreement with third parties identified in Annex I of the contract? (Yes / No)	No

If Yes, please provide the following information

Third Party 1 (Y1) Legal name		**Cost model used**	
Third Party 2 (Y2) Legal name		**Cost model used**	
Third Party 3 (Y3) Legal name		**Cost model used**	
Third Party 4 (Y4) Legal name		**Cost model used**	

If necessary add another Form C

2- Declaration of eligible costs (in €)

Please complete only the activity covered by the relevant instrument (and type of action) indicated above and as mentioned in Article II.25 and/or in Annexes I and III of the contract.

If you are a contractor using the additional cost model (AC):

- indicate only your additional eligible costs, except for Management of the Consortium Activity for which you may indicate your full eligible costs;

do not declare eligible direct additional costs specifically covered by contributions from third parties as mentioned in Articles II.20 and II.23.a and b of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate your full eligible costs
The costs declared should distinguish between direct and indirect costs
If necessary, adjustments to previous period(s) may be included where appropriate

	Type of Activity											
	Research and Technological Development / Innovation (A)		Demonstration (B)		Training (C)		Management of the Consortium (D)		Other Specific Activities (E)		Total (F) = (A)+(B)+(C)+(D)+(E)	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Direct costs	7.740,00						6.230,00				13.970,00	
Of which subcontracting												
Indirect costs	1.548,00										1.548,00	
Adjustments to previous period(s)												
Total costs	9.288,00						6.230,00				15.518,00	

3- Declaration of receipts (in €)

If you are a contractor using the additional cost model (AC), indicate only receipts covered by Article II.23.c of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate receipts covered by Article II.23 of the contract.

	Type of Activity											
	Research and Technological Development / Innovation (A')		Demonstration (B')		Training (C')		Management of the Consortium (D')		Other Specific Activities (E')		Total (F') = (A')+(B')+(C')+(D')+(E')	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Total receipts											0	0

4- Declaration of interest generated by the pre-financing (in €)	
To be completed only by the coordinator.	
Did the pre-financing (advance) you received by the Commission for this period earn interest? (Yes / No)	
If yes, please indicate the amount (in €)	
5- Request of FP6 Financial Contribution (in €)	
For this period, the FP6 Community financial contribution resuested is equal to (amount in €)	10.874,00

6- Audit certificates				
According to the contract, does this Financial Statement need an audit certificate (or several in case of Third party(ies)) delivered by independent auditor(s)? (Yes / No)				Yes
If Yes, does this(those) audit certificate(s) cover only this Financial Statement per Activity? (Yes / No)				No
If No, what are the periods covered by this(those) audit certificate(s) ?		From -to	**15.2.04-14.02.06**	
What is the total cost of this(those) audit certificate(s) (in €) per independent auditor(s) ? € 900,00				

Audit certificate of the contractor (X)			
Legal name of the audit firm		**Cost of the certificate**	€ 900
Audit certificate(s) of the third party(ies) (Ys) ***(if necessary)***			
Y1 : Legal name of the audit firm		**Cost of the certificate**	
Y2 : Legal name of the audit firm		**Cost of the certificate**	
Y3 : Legal name of the audit firm		**Cost of the certificate**	
Y4 : Legal name of the audit firm		**Cost of the certificate**	
If necessary add another Form C.		**Total (Z) = (X) + (Ys)**	

Reminders:
The cost of an audit certificate is included in the costs declared under the activity "Management of the Consortium".The required audit certificate (s) is (are) attached to this Financial Statement

7- Conversion rates	
Costs incurred in currencies other than EURO shall be reported in EURO.	
Please mention the conversion rate used (only one choice is possible) – Please note that the same principle applies for receipts.	
Contractor	
- Conversion rate of the date of incurred actual costs? (YES / NO)	No
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	No

Third Party(ies) *(if necessary)*	
Third Party 1 (Y1)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 2 (Y2)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 3 (Y3)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 4 (Y4)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	

If necessary add another Form C.

8- Contractor's Certificate

We certify that:

- the costs declared above are directly related to the resources used to reach the objectives of the project ;
- the receipts declared above are directly related to the resources used to reach the objectives of the project ;
- the costs declared above fall within the definition of eligible costs specified in Articles II.19, II.20, II.21, II.22 and II.25 of the contract, and, if relevant, in Annex III and Article 9 (special clauses) of the contract ;
- the receipts declared above fall within the definition of receipts specified in Article II.23 of the contract ;
- the interest generated by the pre-financing declared above falls within the definition of Article II.27 of the contract ;

the above Statement ;

- the above information declared is complete and true ;
- there is full supporting documentation to justify the information hereby declared. It will be made available at the request of the Commission and in the event of an audit by the Commission and/or by the Court of Auditors and/or their authorised representatives.

Contractor's Stamp	Name of the Person responsible for the work	Name of the duly authorised Financial Officer
	Esperanza Tomás	**Alba Torquemada**
	Date	**Date**
	19.04.2006	**19,04.2006**
	Signature	**Signature**

Form C - Model of Financial Statement per Activity for a Specific Cooperative Research for SMEs

(to be completed by each contractor)

Type of instrument	**Specific Research Project for SMEs**	**Type of Action (if necessary)**	**Cooperative Research (CRAFT)**
Project Title (or Acronym)	**PARADOX**	**Contract n°**	**COOP-CT-2003-508649**

Contractors's legal name	**M. Chapoutier**		
Legal Type	**Small and medium enterprise**		
Contact Person	**Albéric Mazoyer**	**Telephone**	+33 4 75 08 28 65
Telecopy		**E-mail**	ypinot@chapoutier.com

Cost model used (AC/FC or FCF)	**FCF**	**Indirect costs (Real or Flat Rate of 20% of Direct costs, except subcontracting)**	**20% Flat Rate**
Period from	**15.02.2005**	**TO**	**14.02.2006**

1- Resources (Third party(ies))

Are there any resources made available on the basis of a prior agreement with third parties identified in Annex I of the contract? (Yes / No)	No

If Yes, please provide the following information

Third Party 1 (Y1) Legal name		**Cost model used**	
Third Party 2 (Y2) Legal name		**Cost model used**	
Third Party 3 (Y3) Legal name		**Cost model used**	
Third Party 4 (Y4) Legal name		**Cost model used**	

If necessary add another Form C

2- Declaration of eligible costs (in €)

Please complete only the activity covered by the relevant instrument (and type of action) indicated above and as mentioned in Article II.25 and/or in Annexes I and III of the contract.

If you are a contractor using the additional cost model (AC):

- indicate only your additional eligible costs, except for Management of the Consortium Activity for which you may indicate your full eligible costs;

do not declare eligible direct additional costs specifically covered by contributions from third parties as mentioned in Articles II.20 and II.23.a and b of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate your full eligible costs
The costs declared should distinguish between direct and indirect costs
If necessary, adjustments to previous period(s) may be included where appropriate

	Type of Activity											
	Research and Technological Development / Innovation (A)		Demonstration (B)		Training (C)		Management of the Consortium (D)		Other Specific Activities (E)		Total (F) = (A)+(B)+(C)+(D)+(E)	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Direct costs	0										0	
Of which subcontracting												
Indirect costs	0										0	
Adjustments to previous period(s)												
Total costs	0										0	

3- Declaration of receipts (in €)

If you are a contractor using the additional cost model (AC), indicate only receipts covered by Article II.23.c of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate receipts covered by Article II.23 of the contract.

	Type of Activity											
	Research and Technological Development / Innovation (A')		Demonstration (B')		Training (C')		Management of the Consortium (D')		Other Specific Activities (E')		Total (F') = (A')+(B')+(C')+(D')+(E')	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Total receipts												

4- Declaration of interest generated by the pre-financing (in €)	
To be completed only by the coordinator.	
Did the pre-financing (advance) you received by the Commission for this period earn interest? (Yes / No)	
If yes, please indicate the amount (in €)	
5- Request of FP6 Financial Contribution (in €)	
For this period, the FP6 Community financial contribution resuested is equal to (amount in €)	0

6- Audit certificates				
According to the contract, does this Financial Statement need an audit certificate (or several in case of Third party(ies)) delivered by independent auditor(s)? (Yes / No)				No costs
If Yes, does this(those) audit certificate(s) cover only this Financial Statement per Activity? (Yes / No)				
If No, what are the periods covered by this(those) audit certificate(s) ?		From -to	15.2.04 - 14.02.06	
What is the total cost of this(those) audit certificate(s) (in €) per independent auditor(s) ?				

Audit certificate of the contractor (X)			
Legal name of the audit firm		**Cost of the certificate**	
Audit certificate(s) of the third party(ies) (Ys) ***(if necessary)***			
Y1 : Legal name of the audit firm		**Cost of the certificate**	
Y2 : Legal name of the audit firm		**Cost of the certificate**	
Y3 : Legal name of the audit firm		**Cost of the certificate**	
Y4 : Legal name of the audit firm		**Cost of the certificate**	
If necessary add another Form C.		**Total (Z) = (X) + (Ys)**	

Reminders:

The cost of an audit certificate is included in the costs declared under the activity "Management of the Consortium".The required audit certificate (s) is (are) attached to this Financial Statement

7- Conversion rates	
Costs incurred in currencies other than EURO shall be reported in EURO.	
Please mention the conversion rate used (only one choice is possible) – Please note that the same principle applies for receipts.	
Contractor	
- Conversion rate of the date of incurred actual costs? (YES / NO)	No
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	No

Third Party(ies) *(if necessary)*	
Third Party 1 (Y1)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 2 (Y2)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 3 (Y3)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 4 (Y4)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	

If necessary add another Form C.

8- Contractor's Certificate

We certify that:

- the costs declared above are directly related to the resources used to reach the objectives of the project ;
- the receipts declared above are directly related to the resources used to reach the objectives of the project ;
- the costs declared above fall within the definition of eligible costs specified in Articles II.19, II.20, II.21, II.22 and II.25 of the contract, and, if relevant, in Annex III and Article 9 (special clauses) of the contract ;
- the receipts declared above fall within the definition of receipts specified in Article II.23 of the contract ;
- the interest generated by the pre-financing declared above falls within the definition of Article II.27 of the contract ; incorporated in the above Statement ;
- the above information declared is complete and true ;
- there is full supporting documentation to justify the information hereby declared. It will be made available at the request of the Commission and in the event of an audit by the Commission and/or by the Court of Auditors and/or their authorised representatives.

Contractor's Stamp	Name of the Person responsible for the work	Name of the duly authorised Financial Officer
	Albéric Mazoyer	**Albéric Mazoyer**
	Date	**Date**
	27.03.2006	**27.03.2006**
	Signature	**Signature**

Form C - Model of Financial Statement per Activity for a Specific Cooperative Research for SMEs

(to be completed by each contractor)

Type of instrument	Specific Research Project for SMEs	Type of Action (if necessary)	Cooperative Research (CRAFT)
Project Title (or Acronym)	PARADOX	Contract n°	COOP-CT-2003-508649

Contractors's legal name	Weingut J. Heinrich & Mitges.		
Legal Type	Other		
Contact Person	Silvia Heinrich-Kanyak	Telephone	+43 2613 89615
Telecopy	+43 2613 89615 4	E-mail	silvia@weingut-heinrich.at

Cost model used (AC/FC or FCF)	FCF	Indirect costs (Real or Flat Rate of 20% of Direct costs, except subcontracting)	20% Flat Rate
Period from	15.02.2005	TO	14.02.2006

1- Resources (Third party(ies))

Are there any resources made available on the basis of a prior agreement with third parties identified in Annex I of the contract? (Yes / No)	No

If Yes, please provide the following information

Third Party 1 (Y1) Legal name		Cost model used	
Third Party 2 (Y2) Legal name		Cost model used	
Third Party 3 (Y3) Legal name		Cost model used	
Third Party 4 (Y4) Legal name		Cost model used	

If necessary add another Form C

2- Declaration of eligible costs (in €)

Please complete only the activity covered by the relevant instrument (and type of action) indicated above and as mentioned in Article II.25 and/or in Annexes I and III of the contract.

If you are a contractor using the additional cost model (AC):

- indicate only your additional eligible costs, except for Management of the Consortium Activity for which you may indicate your full eligible costs;

do not declare eligible direct additional costs specifically covered by contributions from third parties as mentioned in Articles II.20 and II.23.a and b of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate your full eligible costs
The costs declared should distinguish between direct and indirect costs
If necessary, adjustments to previous period(s) may be included where appropriate

	Type of Activity											
	Research and Technological Development / Innovation (A)		Demonstration (B)		Training (C)		Management of the Consortium (D)		Other Specific Activities (E)		Total (F) = (A)+(B)+(C)+(D)+(E)	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Direct costs	13.190,20						500				13.690,20	
Of which subcontracting												
Indirect costs	2.638,04										2.638,04	
Adjustments to previous period(s)	6.683,42										6.683,42	
Total costs	22.511,66						500				23.011,66	

3- Declaration of receipts (in €)

If you are a contractor using the additional cost model (AC), indicate only receipts covered by Article II.23.c of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate receipts covered by Article II.23 of the contract.

	Type of Activity											
	Research and Technological Development / Innovation (A')		Demonstration (B')		Training (C')		Management of the Consortium (D')		Other Specific Activities (E')		Total (F') = (A')+(B')+(C')+(D')+(E')	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Total receipts												

4- Declaration of interest generated by the pre-financing (in €)	
To be completed only by the coordinator.	
Did the pre-financing (advance) you received by the Commission for this period earn interest? (Yes / No)	
If yes, please indicate the amount (in €)	
5- Request of FP6 Financial Contribution (in €)	
For this period, the FP6 Community financial contribution resuested is equal to (amount in €)	11.755,83

6- Audit certificates				
According to the contract, does this Financial Statement need an audit certificate (or several in case of Third party(ies)) delivered by independent auditor(s)? (Yes / No)				**Yes**
If Yes, does this(those) audit certificate(s) cover only this Financial Statement per Activity? (Yes / No)				**No**
If No, what are the periods covered by this(those) audit certificate(s) ?		From -to	**15.2.04-14.2.06**	
What is the total cost of this(those) audit certificate(s) (in €) per independent auditor(s) ? € 600,00				

Audit certificate of the contractor (X)			
Legal name of the audit firm	Steuerberater Mag. Ottilie Reinfeld	**Cost of the certificate**	€ 600,00
Audit certificate(s) of the third party(ies) (Ys) ***(if necessary)***			
Y1 : Legal name of the audit firm		**Cost of the certificate**	
Y2 : Legal name of the audit firm		**Cost of the certificate**	
Y3 : Legal name of the audit firm		**Cost of the certificate**	
Y4 : Legal name of the audit firm		**Cost of the certificate**	
If necessary add another Form C.		**Total (Z) = (X) + (Ys)**	

Reminders:
The cost of an audit certificate is included in the costs declared under the activity "Management of the Consortium".The required audit certificate (s) is (are) attached to this Financial Statement

7- Conversion rates	
Costs incurred in currencies other than EURO shall be reported in EURO.	
Please mention the conversion rate used (only one choice is possible) – Please note that the same principle applies for receipts.	
Contractor	
- Conversion rate of the date of incurred actual costs? (YES / NO)	No
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	No

Third Party(ies) *(if necessary)*	
Third Party 1 (Y1)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 2 (Y2)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 3 (Y3)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 4 (Y4)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	

If necessary add another Form C.

8- Contractor's Certificate

We certify that:

- the costs declared above are directly related to the resources used to reach the objectives of the project ;
- the receipts declared above are directly related to the resources used to reach the objectives of the project ;
- the costs declared above fall within the definition of eligible costs specified in Articles II.19, II.20, II.21, II.22 and II.25 of the contract, and, if relevant, in Annex III and Article 9 (special clauses) of the contract ;
- the receipts declared above fall within the definition of receipts specified in Article II.23 of the contract ;
- the interest generated by the pre-financing declared above falls within the definition of Article II.27 of the contract ; incorporated in the above Statement ;
- the above information declared is complete and true ;
- there is full supporting documentation to justify the information hereby declared. It will be made available at the request of the Commission and in the event of an audit by the Commission and/or by the Court of Auditors and/or their authorised representatives.

Contractor's Stamp	Name of the Person responsible for the work	Name of the duly authorised Financial Officer
	Silvia Heinrich-Kanyak	**Silvia Heinrich-Kanyak**
	Date	**Date**
	27.03.2006	**27.03.2006**
	Signature	**Signature**

Form C - Model of Financial Statement per Activity for a Specific Cooperative Research for SMEs

(to be completed by each contractor)

Type of instrument	**Specific Research Project for SMEs**	**Type of Action (if necessary)**	**Cooperative Research (CRAFT)**
Project Title (or Acronym)	**PARADOX**	**Contract n°**	**COOP-CT-2003-508649**

Contractors's legal name	**Vinceller Mps Kkt.**		
Legal Type	**Small and medium enterprise**		
Contact Person	**Istvan SASDI**	**Telephone**	+36 99 357602
Telecopy	**+ 36 20 957 1594**	**E-mail**	vinceller.mps@axelero.hu

Cost model used (AC/FC or FCF)	**FCF**	**Indirect costs (Real or Flat Rate of 20% of Direct costs, except subcontracting)**	20 % Flatrate
Period from	**15.02.2005**	**TO**	**14.02.2006**

1- Resources (Third party(ies))

Are there any resources made available on the basis of a prior agreement with third parties identified in Annex I of the contract? (Yes / No)	No

If Yes, please provide the following information

Third Party 1 (Y1) Legal name		**Cost model used**	
Third Party 2 (Y2) Legal name		**Cost model used**	
Third Party 3 (Y3) Legal name		**Cost model used**	
Third Party 4 (Y4) Legal name		**Cost model used**	

If necessary add another Form C

2- Declaration of eligible costs (in €)

Please complete only the activity covered by the relevant instrument (and type of action) indicated above and as mentioned in Article II.25 and/or in Annexes I and III of the contract.

If you are a contractor using the additional cost model (AC):

- indicate only your additional eligible costs, except for Management of the Consortium Activity for which you may indicate your full eligible costs;

do not declare eligible direct additional costs specifically covered by contributions from third parties as mentioned in Articles II.20 and II.23.a and b of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate your full eligible costs
The costs declared should distinguish between direct and indirect costs
If necessary, adjustments to previous period(s) may be included where appropriate

	Type of Activity											
	Research and Technological Development / Innovation (A)		Demonstration (B)		Training (C)		Management of the Consortium (D)		Other Specific Activities (E)		Total (F) = (A)+(B)+(C)+(D)+(E)	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Direct costs	9.412,00						800,00				10.212,00	
Of which subcontracting												
Indirect costs	1.882,00										1.882,00	
Adjustments to previous period(s)	15.278,00										15.278,00	
Total costs	26.572,00						800,00				27.372,00	

3- Declaration of receipts (in €)

If you are a contractor using the additional cost model (AC), indicate only receipts covered by Article II.23.c of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate receipts covered by Article II.23 of the contract.

	Type of Activity											
	Research and Technological Development / Innovation (A')		Demonstration (B')		Training (C')		Management of the Consortium (D')		Other Specific Activities (E')		Total (F') = (A)'+(B')+(C')+(D')+(E')	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Total receipts											0	0

4- Declaration of interest generated by the pre-financing (in €)	
To be completed only by the coordinator.	
Did the pre-financing (advance) you received by the Commission for this period earn interest? (Yes / No)	
If yes, please indicate the amount (in €)	
5- Request of FP6 Financial Contribution (in €)	
For this period, the FP6 Community financial contribution resuested is equal to (amount in €)	14.086,00

6- Audit certificates				
According to the contract, does this Financial Statement need an audit certificate (or several in case of Third party(ies)) delivered by independent auditor(s)? (Yes / No)				YES
If Yes, does this(those) audit certificate(s) cover only this Financial Statement per Activity? (Yes / No)				NO
If No, what are the periods covered by this(those) audit certificate(s) ?		From -to	15.2.04-14.2.06	
What is the total cost of this(those) audit certificate(s) (in €) per independent auditor(s) ?				

Audit certificate of the contractor (X)			
Legal name of the audit firm	L es B Könyvvizsgalo Adotanacsado es Konyvvezetö BT		€ 800
Audit certificate(s) of the third party(ies) (Ys) ***(if necessary)***			
Y1 : Legal name of the audit firm		**Cost of the certificate**	
Y2 : Legal name of the audit firm		**Cost of the certificate**	
Y3 : Legal name of the audit firm		**Cost of the certificate**	
Y4 : Legal name of the audit firm		**Cost of the certificate**	
If necessary add another Form C.		**Total (Z) = (X) + (Ys)**	

Reminders:
The cost of an audit certificate is included in the costs declared under the activity "Management of the Consortium".The required audit certificate (s) is (are) attached to this Financial Statement

7- Conversion rates	
Costs incurred in currencies other than EURO shall be reported in EURO.	
Please mention the conversion rate used (only one choice is possible) – Please note that the same principle applies for receipts.	
Contractor	
- Conversion rate of the date of incurred actual costs? (YES / NO)	YES
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	No

Third Party(ies) *(if necessary)*	
Third Party 1 (Y1)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 2 (Y2)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 3 (Y3)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 4 (Y4)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	

If necessary add another Form C.

8- Contractor's Certificate

We certify that:

- the costs declared above are directly related to the resources used to reach the objectives of the project ;
- the receipts declared above are directly related to the resources used to reach the objectives of the project ;
- the costs declared above fall within the definition of eligible costs specified in Articles II.19, II.20, II.21, II.22 and II.25 of the contract, and, if relevant, in Annex III and Article 9 (special clauses) of the contract ;
- the receipts declared above fall within the definition of receipts specified in Article II.23 of the contract ;
- the interest generated by the pre-financing declared above falls within the definition of Article II.27 of the contract ;

the above Statement ;

- the above information declared is complete and true ;
- there is full supporting documentation to justify the information hereby declared. It will be made available at the request of the Commission and in the event of an audit by the Commission and/or by the Court of Auditors and/or their authorised representatives.

Contractor's Stamp	Name of the Person responsible for the work	Name of the duly authorised Financial Officer
	Istvan Sásdi	**Istvan Sásdi**
	Date	**Date**
	31.03.2006	**31.03.2006**
	Signature	**Signature**

Form C - Model of Financial Statement per Activity for a Specific Cooperative Research for SMEs

(to be completed by each contractor)

Type of instrument	**Specific Research Project for SMEs**	**Type of Action (if necessary)**	**Cooperative Research (CRAFT)**
Project Title (or Acronym)	**PARADOX**	**Contract n°**	**COOP-CT-2003-508649**

Contractors's legal name	**CINS, Center for isolation of natural substances d.o.o.**		
Legal Type	**Small and medium enterprise**		
Contact Person	**Marijana KNEZ**	**Telephone**	+386 2 320 3000
Telecopy	**+386 2 320 3001**	**E-mail**	marijana.knez@guest.arnes.si

Cost model used (AC/FC or FCF)	**FCF**	**Indirect costs (Real or Flat Rate of 20% of Direct costs, except subcontracting)**	Flat Rate 20%
Period from	**15.02.2005**	**TO**	**14.02.2006**

1- Resources (Third party(ies))

Are there any resources made available on the basis of a prior agreement with third parties identified in Annex I of the contract? (Yes / No)	No

If Yes, please provide the following information

Third Party 1 (Y1) Legal name		**Cost model used**	
Third Party 2 (Y2) Legal name		**Cost model used**	
Third Party 3 (Y3) Legal name		**Cost model used**	
Third Party 4 (Y4) Legal name		**Cost model used**	

If necessary add another Form C

2- Declaration of eligible costs (in €)

Please complete only the activity covered by the relevant instrument (and type of action) indicated above and as mentioned in Article II.25 and/or in Annexes I and III of the contract.

If you are a contractor using the additional cost model (AC):

- indicate only your additional eligible costs, except for Management of the Consortium Activity for which you may indicate your full eligible costs;

do not declare eligible direct additional costs specifically covered by contributions from third parties as mentioned in Articles II.20 and II.23.a and b of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate your full eligible costs
The costs declared should distinguish between direct and indirect costs
If necessary, adjustments to previous period(s) may be included where appropriate

	Type of Activity											
	Research and Technological Development / Innovation (A)		Demonstration (B)		Training (C)		Management of the Consortium (D)		Other Specific Activities (E)		Total (F) = (A)+(B)+(C)+(D)+(E)	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Direct costs	4619						3000				7619	
Of which subcontracting												
Indirect costs	924										924	
Adjustments to previous period(s)												
Total costs	5543						3000				8543	

3- Declaration of receipts (in €)

If you are a contractor using the additional cost model (AC), indicate only receipts covered by Article II.23.c of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate receipts covered by Article II.23 of the contract.

	Type of Activity											
	Research and Technological Development / Innovation (A')		Demonstration (B')		Training (C')		Management of the Consortium (D')		Other Specific Activities (E')		Total (F') = (A')+(B')+(C')+(D')+(E')	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Total receipts											0	0

4- Declaration of interest generated by the pre-financing (in €)	
To be completed only by the coordinator.	
Did the pre-financing (advance) you received by the Commission for this period earn interest? (Yes / No)	
If yes, please indicate the amount (in €)	
5- Request of FP6 Financial Contribution (in €)	
For this period, the FP6 Community financial contribution resuested is equal to (amount in €)	5.772,00

6- Audit certificates				
According to the contract, does this Financial Statement need an audit certificate (or several in case of Third party(ies)) delivered by independent auditor(s)? (Yes / No)				Yes
If Yes, does this(those) audit certificate(s) cover only this Financial Statement per Activity? (Yes / No)				No
If No, what are the periods covered by this(those) audit certificate(s) ?		From -to	15.2.04-15.2.06	
What is the total cost of this(those) audit certificate(s) (in €) per independent auditor(s) 3000				

Audit certificate of the contractor (X)			
Legal name of the audit firm	Revizija d.o.o.	**Cost of the certificate**	€ 3.600,00 inkl. VAT
Audit certificate(s) of the third party(ies) (Ys) ***(if necessary)***			
Y1 : Legal name of the audit firm		**Cost of the certificate**	
Y2 : Legal name of the audit firm		**Cost of the certificate**	
Y3 : Legal name of the audit firm		**Cost of the certificate**	
Y4 : Legal name of the audit firm		**Cost of the certificate**	
If necessary add another Form C.		**Total (Z) = (X) + (Ys)**	

Reminders:
The cost of an audit certificate is included in the costs declared under the activity "Management of the Consortium".The required audit certificate (s) is (are) attached to this Financial Statement

7- Conversion rates	
Costs incurred in currencies other than EURO shall be reported in EURO.	
Please mention the conversion rate used (only one choice is possible) – Please note that the same principle applies for receipts.	
Contractor	
- Conversion rate of the date of incurred actual costs? (YES / NO)	Yes
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	No

Third Party(ies) *(if necessary)*	
Third Party 1 (Y1)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 2 (Y2)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 3 (Y3)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 4 (Y4)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	

If necessary add another Form C.

8- Contractor's Certificate

We certify that:

- the costs declared above are directly related to the resources used to reach the objectives of the project ;
- the receipts declared above are directly related to the resources used to reach the objectives of the project ;
- the costs declared above fall within the definition of eligible costs specified in Articles II.19, II.20, II.21, II.22 and II.25 of the contract, and, if relevant, in Annex III and Article 9 (special clauses) of the contract ;
- the receipts declared above fall within the definition of receipts specified in Article II.23 of the contract ;
- the interest generated by the pre-financing declared above falls within the definition of Article II.27 of the contract ; incorporated in the above Statement ;
- the above information declared is complete and true ;
- there is full supporting documentation to justify the information hereby declared. It will be made available at the request of the Commission and in the event of an audit by the Commission and/or by the Court of Auditors and/or their authorised representatives.

Contractor's Stamp	Name of the Person responsible for the work	Name of the duly authorised Financial Officer
	Prof.Dr.ŽeljkoKnez	**MarijanaKnez prof.**
	Date	**Date**
	March 13th 2006	**March 13th 2006**
	Signature	**Signature**

Form C - Model of Financial Statement per Activity for a Specific Cooperative Research for SMEs

(to be completed by each contractor)

Type of instrument	Specific Research Project for SMEs	Type of Action (if necessary)	Cooperative Research (CRAFT)
Project Title (or Acronym)	PARADOX	Contract n°	COOP-CT-2003-508649

Contractors's legal name	NATEX PROZESSTECHNOLOGIE GESMBH		
Legal Type	Small and medium enterprise		
Contact Person	Dr. Eduard Lack	Telephone	0043/2630/32120-17
Telecopy	0043/2630/38163	E-mail	office@natex.at

Cost model used (AC/FC or FCF)	FCF	Indirect costs (Real or Flat Rate of 20% of Direct costs, except subcontracting)	20 % Flat Rate

Period from	15.02.2005	TO	14.02.2006

1- Resources (Third party(ies))			
Are there any resources made available on the basis of a prior agreement with third parties identified in Annex I of the contract? (Yes / No)			No
If Yes, please provide the following information			
Third Party 1 (Y1) Legal name		**Cost model used**	
Third Party 2 (Y2) Legal name		**Cost model used**	
Third Party 3 (Y3) Legal name		**Cost model used**	
Third Party 4 (Y4) Legal name		**Cost model used**	
If necessary add another Form C			

2- Declaration of eligible costs (in €)

Please complete only the activity covered by the relevant instrument (and type of action) indicated above and as mentioned in Article II.25 and/or in Annexes I and III of the contract.

If you are a contractor using the additional cost model (AC):

- indicate only your additional eligible costs, except for Management of the Consortium Activity for which you may indicate your full eligible costs;

do not declare eligible direct additional costs specifically covered by contributions from third parties as mentioned in Articles II.20 and II.23.a and b of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate your full eligible costs

The costs declared should distinguish between direct and indirect costs

If necessary, adjustments to previous period(s) may be included where appropriate

	Type of Activity											
	Research and Technological Development / (A)		Demonstration (B)		Training (C)		Management of the Consortium (D)		Other Specific Activities (E)		Total (F) = (A)+(B)+(C)+(D)+(E)	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Direct costs	27.549,85						2.500,00				27.549,85	
Of which subcontracting												
Indirect costs	5.509,97										5.509,97	
Adjustments to previous period(s)	3.794,03										3.794,03	
Total costs	36.853,85						2.500,00				39.353,85	

3- Declaration of receipts (in €)

If you are a contractor using the additional cost model (AC), indicate only receipts covered by Article II.23.c of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate receipts covered by Article II.23 of the contract.

	Type of Activity											
	Research and Technological Development / Innovation (A')		Demonstration (B')		Training (C')		Management of the Consortium (D')		Other Specific Activities (E')		Total (F') = (A')+(B')+(C')+(D') +(E')	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Total receipts											0	0

4- Declaration of interest generated by the pre-financing (in €)	
To be completed only by the coordinator.	
Did the pre-financing (advance) you received by the Commission for this period earn interest? (Yes / No)	
If yes, please indicate the amount (in €)	
5- Request of FP6 Financial Contribution (in €)	
For this period, the FP6 Community financial contribution resuested is equal to (amount in €)	**€ 20.926,93**

6- Audit certificates

According to the contract, does this Financial Statement need an audit certificate (or several in case of Third party(ies)) delivered by independent auditor(s)? (Yes / No)				YES
If Yes, does this(those) audit certificate(s) cover only this Financial Statement per Activity? (Yes / No)				NO
If No, what are the periods covered by this(those) audit certificate(s) ?	15.02.2004	From -to	14.02.2006	
What is the total cost of this(those) audit certificate(s) (in €) per independent auditor(s) ? **€ 2500**				

Audit certificate of the contractor (X)			
Legal name of the audit firm	LBG Wirtschaftstreuhand GesmbH	**Cost of the certificate**	**€ 2.500**
Audit certificate(s) of the third party(ies) (Ys) ***(if necessary)***			
Y1 : Legal name of the audit firm		**Cost of the certificate**	
Y2 : Legal name of the audit firm		**Cost of the certificate**	
Y3 : Legal name of the audit firm		**Cost of the certificate**	
Y4 : Legal name of the audit firm		**Cost of the certificate**	
If necessary add another Form C.		**Total (Z) = (X) + (Ys)**	

Reminders:
The cost of an audit certificate is included in the costs declared under the activity "Management of the Consortium".The required audit certificate (s) is (are) attached to this Financial Statement

7- Conversion rates	
Costs incurred in currencies other than EURO shall be reported in EURO. Please mention the conversion rate used (only one choice is possible) – Please note that the same principle applies for receipts.	
Contractor	
- Conversion rate of the date of incurred actual costs? (YES / NO)	No
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	No
Third Party(ies) ***(if necessary)***	
Third Party 1 (Y1)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 2 (Y2)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 3 (Y3)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 4 (Y4)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	

If necessary add another Form C.

8- Contractor's Certificate

We certify that:

- the costs declared above are directly related to the resources used to reach the objectives of the project ;
- the receipts declared above are directly related to the resources used to reach the objectives of the project ;
- the costs declared above fall within the definition of eligible costs specified in Articles II.19, II.20, II.21, II.22 and II.25 of the contract, and, if relevant, in Annex III and Article 9 (special clauses) of the contract ;
- the receipts declared above fall within the definition of receipts specified in Article II.23 of the contract ;
- the interest generated by the pre-financing declared above falls within the definition of Article II.27 of the contract ;
- the necessary adjustments, especially to costs reported in previous Financial Statement(s) per Activity, have been incorporated in the above Statement ;
- the above information declared is complete and true ;
- there is full supporting documentation to justify the information hereby declared. It will be made available at the request of the Commission and in the event of an audit by the Commission and/or by the Court of Auditors and/or their authorised representatives.

Contractor's Stamp	Name of the Person responsible for the work	Name of the duly authorised Financial Officer
	Dr. Lack Eduard	**Ing. Lang Franz**
	Date	**Date**
	24.03.2006	**24.03.2006**
	Signature	**Signature**

Form C - Model of Financial Statement per Activity for a Specific Cooperative Research for SMEs

(to be completed by each contractor)

Type of instrument	**Specific Research Project for SMEs**	**Type of Action (if necessary)**	**Cooperative Research (CRAFT)**
Project Title (or Acronym)	**PARADOX**	**Contract n°**	**COOP-CT-2003-508649**

Contractors's legal name	**ChiroBlock GmbH, Andresenstraße 1a, 06766 Wolfen**		
Legal Type			
Contact Person	**Oliver Seidelmann**	**Telephone**	+49-3494-638323
Telecopy	**+49-3494-638324**	**E-mail**	contact@chiroblock.de

Cost model used (AC/FC or FCF)		**Indirect costs (Real or Flat Rate of 20% of Direct costs, except subcontracting)**	**20% Flat Rate**
Period from	**15.02.2005**	**TO**	**14.02.2006**

1- Resources (Third party(ies))

Are there any resources made available on the basis of a prior agreement with third parties identified in Annex I of the contract? (Yes / No)	No

If Yes, please provide the following information

Third Party 1 (Y1) Legal name		**Cost model used**	
Third Party 2 (Y2) Legal name		**Cost model used**	
Third Party 3 (Y3) Legal name		**Cost model used**	
Third Party 4 (Y4) Legal name		**Cost model used**	

If necessary add another Form C

2- Declaration of eligible costs (in €)

Please complete only the activity covered by the relevant instrument (and type of action) indicated above and as mentioned in Article II.25 and/or in Annexes I and III of the contract.

If you are a contractor using the additional cost model (AC):

- indicate only your additional eligible costs, except for Management of the Consortium Activity for which you may indicate your full eligible costs;

do not declare eligible direct additional costs specifically covered by contributions from third parties as mentioned in Articles II.20 and II.23.a and b of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate your full eligible costs
The costs declared should distinguish between direct and indirect costs
If necessary, adjustments to previous period(s) may be included where appropriate

	Type of Activity											
	Research and Technological Development / Innovation (A)		Demonstration (B)		Training (C)		Management of the Consortium (D)		Other Specific Activities (E)		Total (F) = (A)+(B)+(C)+(D)+(E)	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Direct costs	9.973,84						1.200,00				11.173,84	
Of which subcontracting												
Indirect costs	1.994,77						240,00				2.234,77	
Adjustments to previous period(s)												
Total costs	11.968,61						1.440,00				13.408,61	

3- Declaration of receipts (in €)

If you are a contractor using the additional cost model (AC), indicate only receipts covered by Article II.23.c of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate receipts covered by Article II.23 of the contract.

	Type of Activity											
	Research and Technological Development / Innovation (A')		Demonstration (B')		Training (C')		Management of the Consortium (D')		Other Specific Activities (E')		Total (F') = (A')+(B')+(C')+(D')+(E')	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Total receipts											0	0

4- Declaration of interest generated by the pre-financing (in €)	
To be completed only by the coordinator.	
Did the pre-financing (advance) you received by the Commission for this period earn interest? (Yes / No)	
If yes, please indicate the amount (in €)	
5- Request of FP6 Financial Contribution (in €)	
For this period, the FP6 Community financial contribution resuested is equal to (amount in €)	7.424,31

6- Audit certificates				
According to the contract, does this Financial Statement need an audit certificate (or several in case of Third party(ies)) delivered by independent auditor(s)? (Yes / No)				**Yes**
If Yes, does this(those) audit certificate(s) cover only this Financial Statement per Activity? (Yes / No)				**No**
If No, what are the periods covered by this(those) audit certificate(s) ?		From -to	**15.2.04-14.2.06**	
What is the total cost of this(those) audit certificate(s) (in €) per independent auditor(s) ?				

Audit certificate of the contractor (X)			
Legal name of the audit firm	Steuerkanzlei Schmidt	**Cost of the certificate**	€ 1.956
Audit certificate(s) of the third party(ies) (Ys) ***(if necessary)***			
Y1 : Legal name of the audit firm		**Cost of the certificate**	
Y2 : Legal name of the audit firm		**Cost of the certificate**	
Y3 : Legal name of the audit firm		**Cost of the certificate**	
Y4 : Legal name of the audit firm		**Cost of the certificate**	
If necessary add another Form C.		**Total (Z) = (X) + (Ys)**	

Reminders:
The cost of an audit certificate is included in the costs declared under the activity "Management of the Consortium".The required audit certificate (s) is (are) attached to this Financial Statement

7- Conversion rates	
Costs incurred in currencies other than EURO shall be reported in EURO.	
Please mention the conversion rate used (only one choice is possible) – Please note that the same principle applies for receipts.	
Contractor	
- Conversion rate of the date of incurred actual costs? (YES / NO)	No
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	No

Third Party(ies) *(if necessary)*	
Third Party 1 (Y1)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 2 (Y2)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 3 (Y3)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 4 (Y4)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	

If necessary add another Form C.

8- Contractor's Certificate

We certify that:

- the costs declared above are directly related to the resources used to reach the objectives of the project ;
- the receipts declared above are directly related to the resources used to reach the objectives of the project ;
- the costs declared above fall within the definition of eligible costs specified in Articles II.19, II.20, II.21, II.22 and II.25 of the contract, and, if relevant, in Annex III and Article 9 (special clauses) of the contract ;
- the receipts declared above fall within the definition of receipts specified in Article II.23 of the contract ;
- the interest generated by the pre-financing declared above falls within the definition of Article II.27 of the contract ; incorporated in the above Statement ;
- the above information declared is complete and true ;
- there is full supporting documentation to justify the information hereby declared. It will be made available at the request of the Commission and in the event of an audit by the Commission and/or by the Court of Auditors and/or their authorised representatives.

Contractor's Stamp	Name of the Person responsible for the work	Name of the duly authorised Financial Officer
	Dr. Volkmar Wendisch	**Dr. Oliver Seidelmann**
	Date	**Date**
	13.03.2006	**13.03.2006**
	Signature	**Signature**

Form C - Model of Financial Statement per Activity for a Specific Cooperative Research for SMEs

(to be completed by each contractor)

Type of instrument	**Specific Research Project for SMEs**	**Type of Action (if necessary)**	**Cooperative Research (CRAFT)**
Project Title (or Acronym)	**PARADOX**	**Contract n°**	**COOP-CT-2003-508649**

Contractors's legal name	**CAMPI Y JOVE S.A. (now Barentz Campi y Jove S.A.)**		
Legal Type	**Other**		
Contact Person	**Mr. Santiago Lanchas**	**Telephone**	+ 34 93 4766666
Telecopy	**+ 34 93 2073707**	**E-mail**	slanchas@cyjsa.com

Cost model used (AC/FC or FCF)	**FC**	**Indirect costs (Real or Flat Rate of 20% of Direct costs, except subcontracting)**	20 % Flat Rate
Period from	**15.02.2005**	**TO**	**14.02.2006**

1- Resources (Third party(ies))

Are there any resources made available on the basis of a prior agreement with third parties identified in Annex I of the contract? (Yes / No)	No
If Yes, please provide the following information	

Third Party 1 (Y1) Legal name		**Cost model used**	
Third Party 2 (Y2) Legal name		**Cost model used**	
Third Party 3 (Y3) Legal name		**Cost model used**	
Third Party 4 (Y4) Legal name		**Cost model used**	

If necessary add another Form C

2- Declaration of eligible costs (in €)

Please complete only the activity covered by the relevant instrument (and type of action) indicated above and as mentioned in Article II.25 and/or in Annexes I and III of the contract.

If you are a contractor using the additional cost model (AC):

- indicate only your additional eligible costs, except for Management of the Consortium Activity for which you may indicate your full eligible costs;

do not declare eligible direct additional costs specifically covered by contributions from third parties as mentioned in Articles II.20 and II.23.a and b of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate your full eligible costs
The costs declared should distinguish between direct and indirect costs
If necessary, adjustments to previous period(s) may be included where appropriate

	Type of Activity											
	Research and Technological Development / Innovation (A)		Demonstration (B)		Training (C)		Management of the Consortium (D)		Other Specific Activities (E)		Total (F) = (A)+(B)+(C)+(D)+(E)	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Direct costs	22.960						500				23.460	
Of which subcontracting												
Indirect costs	4.592										4.592	
Adjustments to previous period(s)												
Total costs	27.552						500				28.052	

3- Declaration of receipts (in €)

If you are a contractor using the additional cost model (AC), indicate only receipts covered by Article II.23.c of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate receipts covered by Article II.23 of the contract.

	Type of Activity											
	Research and Technological Development / Innovation (A')		Demonstration (B')		Training (C')		Management of the Consortium (D')		Other Specific Activities (E')		Total (F') = (A')+(B')+(C')+(D')+(E')	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Total receipts											0	

4- Declaration of interest generated by the pre-financing (in €)	
To be completed only by the coordinator.	
Did the pre-financing (advance) you received by the Commission for this period earn interest? (Yes / No)	
If yes, please indicate the amount (in €)	
5- Request of FP6 Financial Contribution (in €)	
For this period, the FP6 Community financial contribution resuested is equal to (amount in €)	13.788

6- Audit certificates				
According to the contract, does this Financial Statement need an audit certificate (or several in case of Third party(ies)) delivered by independent auditor(s)? (Yes / No)				Yes
If Yes, does this(those) audit certificate(s) cover only this Financial Statement per Activity? (Yes / No)				No
If No, what are the periods covered by this(those) audit certificate(s) ?		From -to	15.2.04 - 14.02.06	
What is the total cost of this(those) audit certificate(s) (in €) per independent auditor(s) ? € 500,00				

Audit certificate of the contractor (X)			
Legal name of the audit firm		**Cost of the certificate**	€ 500
Audit certificate(s) of the third party(ies) (Ys) ***(if necessary)***			
Y1 : Legal name of the audit firm		**Cost of the certificate**	
Y2 : Legal name of the audit firm		**Cost of the certificate**	
Y3 : Legal name of the audit firm		**Cost of the certificate**	
Y4 : Legal name of the audit firm		**Cost of the certificate**	
If necessary add another Form C.		**Total (Z) = (X) + (Ys)**	

Reminders:
The cost of an audit certificate is included in the costs declared under the activity "Management of the Consortium".The required audit certificate (s) is (are) attached to this Financial Statement

7- Conversion rates	
Costs incurred in currencies other than EURO shall be reported in EURO.	
Please mention the conversion rate used (only one choice is possible) – Please note that the same principle applies for receipts.	
Contractor	
- Conversion rate of the date of incurred actual costs? (YES / NO)	No
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	No

Third Party(ies) *(if necessary)*	
Third Party 1 (Y1)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 2 (Y2)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 3 (Y3)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 4 (Y4)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	

If necessary add another Form C.

8- Contractor's Certificate

We certify that:

- the costs declared above are directly related to the resources used to reach the objectives of the project ;
- the receipts declared above are directly related to the resources used to reach the objectives of the project ;
- the costs declared above fall within the definition of eligible costs specified in Articles II.19, II.20, II.21, II.22 and II.25 of the contract, and, if relevant, in Annex III and Article 9 (special clauses) of the contract ;
- the receipts declared above fall within the definition of receipts specified in Article II.23 of the contract ;
- the interest generated by the pre-financing declared above falls within the definition of Article II.27 of the contract ; incorporated in the above Statement ;
- the above information declared is complete and true ;
- there is full supporting documentation to justify the information hereby declared. It will be made available at the request of the Commission and in the event of an audit by the Commission and/or by the Court of Auditors and/or their authorised representatives.

Contractor's Stamp	Name of the Person responsible for the work	Name of the duly authorised Financial Officer
	Santiago Lanchas	
	Date	**Date**
	19.04.2006	**19.04.2006**
	Signature	**Signature**

Form C - Model of Financial Statement per Activity for a Specific Cooperative Research for SMEs

(to be completed by each contractor)

Type of instrument	Specific Research Project for SMEs	Type of Action (if necessary)	Cooperative Research (CRAFT)
Project Title (or Acronym)	PARADOX	Contract n°	COOP-CT-2003-508649

Contractors's legal name	KUK HandelsgesmbH, Auleiten, A-4910 Ried im Innkreis		
Legal Type	OTHER		
Contact Person	Maria Haslinger	Telephone	07762 85 805 16
Telecopy		E-mail	maria.haslinger@kuk.com

Cost model used (AC/FC or FCF)	FC	Indirect costs (Real or Flat Rate of 20% of Direct costs, except subcontracting)	20% Flat Rate
Period from	15.02.2005	TO	14.02.2006

1- Resources (Third party(ies))

Are there any resources made available on the basis of a prior agreement with third parties identified in Annex I of the contract? (Yes / No)	No

If Yes, please provide the following information

Third Party 1 (Y1) Legal name		Cost model used	
Third Party 2 (Y2) Legal name		Cost model used	
Third Party 3 (Y3) Legal name		Cost model used	
Third Party 4 (Y4) Legal name		Cost model used	

If necessary add another Form C

2- Declaration of eligible costs (in €)

Please complete only the activity covered by the relevant instrument (and type of action) indicated above and as mentioned in Article II.25 and/or in Annexes I and III of the contract.

If you are a contractor using the additional cost model (AC):

- indicate only your additional eligible costs, except for Management of the Consortium Activity for which you may indicate your full eligible costs;

do not declare eligible direct additional costs specifically covered by contributions from third parties as mentioned in Articles II.20 and II.23.a and b of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate your full eligible costs
The costs declared should distinguish between direct and indirect costs
If necessary, adjustments to previous period(s) may be included where appropriate

	Type of Activity											
	Research and Technological Development / Innovation (A)		Demonstration (B)		Training (C)		Management of the Consortium (D)		Other Specific Activities (E)		Total (F) = (A)+(B)+(C)+(D)+(E)	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Direct costs	3303,91										3303,91	
Of which subcontracting												
Indirect costs	660,79										660,79	
Adjustments to previous period(s)												
Total costs	3964,7										3964,7	

3- Declaration of receipts (in €)

If you are a contractor using the additional cost model (AC), indicate only receipts covered by Article II.23.c of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate receipts covered by Article II.23 of the contract.

	Type of Activity											
	Research and Technological Development / Innovation (A')		Demonstration (B')		Training (C')		Management of the Consortium (D')		Other Specific Activities (E')		Total (F') = (A')+(B')+(C')+(D')+(E')	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Total receipts												

4- Declaration of interest generated by the pre-financing (in €)	
To be completed only by the coordinator.	
Did the pre-financing (advance) you received by the Commission for this period earn interest? (Yes / No)	
If yes, please indicate the amount (in €)	
5- Request of FP6 Financial Contribution (in €)	
For this period, the FP6 Community financial contribution resuested is equal to (amount in €)	1.982

6- Audit certificates				
According to the contract, does this Financial Statement need an audit certificate (or several in case of Third party(ies)) delivered by independent auditor(s)? (Yes / No)				**Yes**
If Yes, does this(those) audit certificate(s) cover only this Financial Statement per Activity? (Yes / No)				**No**
If No, what are the periods covered by this(those) audit certificate(s) ?		From -to	**15.2.04-14.2.06**	
What is the total cost of this(those) audit certificate(s) (in €) per independent auditor(s) ? € 200				

Audit certificate of the contractor (X)			
Legal name of the audit firm	Mag. Kreil, Wirtschaftsprüfer	**Cost of the certificate**	€ 200
Audit certificate(s) of the third party(ies) (Ys) ***(if necessary)***			
Y1 : Legal name of the audit firm		**Cost of the certificate**	
Y2 : Legal name of the audit firm		**Cost of the certificate**	
Y3 : Legal name of the audit firm		**Cost of the certificate**	
Y4 : Legal name of the audit firm		**Cost of the certificate**	
If necessary add another Form C.		**Total (Z) = (X) + (Ys)**	

Reminders:
The cost of an audit certificate is included in the costs declared under the activity "Management of the Consortium".The required audit certificate (s) is (are) attached to this Financial Statement

7- Conversion rates	
Costs incurred in currencies other than EURO shall be reported in EURO.	
Please mention the conversion rate used (only one choice is possible) – Please note that the same principle applies for receipts.	
Contractor	
- Conversion rate of the date of incurred actual costs? (YES / NO)	No
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	No

Third Party(ies) *(if necessary)*	
Third Party 1 (Y1)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 2 (Y2)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 3 (Y3)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 4 (Y4)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	

If necessary add another Form C.

8- Contractor's Certificate

We certify that:

- the costs declared above are directly related to the resources used to reach the objectives of the project ;
- the receipts declared above are directly related to the resources used to reach the objectives of the project ;
- the costs declared above fall within the definition of eligible costs specified in Articles II.19, II.20, II.21, II.22 and II.25 of the contract, and, if relevant, in Annex III and Article 9 (special clauses) of the contract ;
- the receipts declared above fall within the definition of receipts specified in Article II.23 of the contract ;
- the interest generated by the pre-financing declared above falls within the definition of Article II.27 of the contract ;

incorporated in the above Statement ;

- the above information declared is complete and true ;
- there is full supporting documentation to justify the information hereby declared. It will be made available at the request of the Commission and in the event of an audit by the Commission and/or by the Court of Auditors and/or their authorised representatives.

Contractor's Stamp	Name of the Person responsible for the work	Name of the duly authorised Financial Officer
	Maria Haslinger	**Dr. Anton Kirchtag**
	Date	**Date**
	27.03.2006	**27.03.2006**
	Signature	**Signature**

Form C - Model of Financial Statement per Activity for a Specific Cooperative Research for SMEs

(to be completed by each contractor)

Type of instrument	**Specific Research Project for SMEs**	**Type of Action (if necessary)**	**Cooperative Research (CRAFT)**
Project Title (or Acronym)	**PARADOX**	**Contract n°**	**COOP-CT-2003-508649**

Contractors's legal name	**Queen Mary & Westfield College, University of London**		
Legal Type			
Contact Person	**Lindsay Warren**	**Telephone**	020 7882 7264
Telecopy	**020 7882 7264**	**E-mail**	l.a.warren@qmul.ac.uk

Cost model used (AC/FC or FCF)	**AC**	**Indirect costs (Real or Flat Rate of 20% of Direct costs, except subcontracting)**	**20% Flat Rate**
Period from	**15.02.2005**	**TO**	**14.02.2006**

1- Resources (Third party(ies))

Are there any resources made available on the basis of a prior agreement with third parties identified in Annex I of the contract? (Yes / No)	No
If Yes, please provide the following information	

Third Party 1 (Y1) Legal name		**Cost model used**	
Third Party 2 (Y2) Legal name		**Cost model used**	
Third Party 3 (Y3) Legal name		**Cost model used**	
Third Party 4 (Y4) Legal name		**Cost model used**	

If necessary add another Form C

2- Declaration of eligible costs (in €)

Please complete only the activity covered by the relevant instrument (and type of action) indicated above and as mentioned in Article II.25 and/or in Annexes I and III of the contract.

If you are a contractor using the additional cost model (AC):

- indicate only your additional eligible costs, except for Management of the Consortium Activity for which you may indicate your full eligible costs;

do not declare eligible direct additional costs specifically covered by contributions from third parties as mentioned in Articles II.20 and II.23.a and b of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate your full eligible costs
The costs declared should distinguish between direct and indirect costs
If necessary, adjustments to previous period(s) may be included where appropriate

	Type of Activity											
	Research and Technological Development / Innovation (A)		Demonstration (B)		Training (C)		Management of the Consortium (D)		Other Specific Activities (E)		Total (F) = (A)+(B)+(C)+(D)+(E)	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Direct costs	36081										36081	
Of which subcontracting												
Indirect costs	7217										7217	
Adjustments to previous period(s)												
Total costs	43298										43298	

3- Declaration of receipts (in €)

If you are a contractor using the additional cost model (AC), indicate only receipts covered by Article II.23.c of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate receipts covered by Article II.23 of the contract.

	Type of Activity											
	Research and Technological Development / Innovation (A')		Demonstration (B')		Training (C')		Management of the Consortium (D')		Other Specific Activities (E')		Total (F') = (A)'+(B')+(C')+(D')+(E')	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Total receipts											0	0

4- Declaration of interest generated by the pre-financing (in €)	
To be completed only by the coordinator.	
Did the pre-financing (advance) you received by the Commission for this period earn interest? (Yes / No)	
If yes, please indicate the amount (in €)	
5- Request of FP6 Financial Contribution (in €)	
For this period, the FP6 Community financial contribution resuested is equal to (amount in €)	43298

6- Audit certificates				
According to the contract, does this Financial Statement need an audit certificate (or several in case of Third party(ies)) delivered by independent auditor(s)? (Yes / No)				**Yes**
If Yes, does this(those) audit certificate(s) cover only this Financial Statement per Activity? (Yes / No)				**No**
If No, what are the periods covered by this(those) audit certificate(s) ?		From -to	**15.2.04-14.2.06**	
What is the total cost of this(those) audit certificate(s) (in €) per independent auditor(s) ?				

Audit certificate of the contractor (X)			
Legal name of the audit firm	Assegai Contracts Ltd	**Cost of the certificate**	€ 440
Audit certificate(s) of the third party(ies) (Ys) ***(if necessary)***			
Y1 : Legal name of the audit firm		**Cost of the certificate**	
Y2 : Legal name of the audit firm		**Cost of the certificate**	
Y3 : Legal name of the audit firm		**Cost of the certificate**	
Y4 : Legal name of the audit firm		**Cost of the certificate**	
If necessary add another Form C.		**Total (Z) = (X) + (Ys)**	
Reminders: *The cost of an audit certificate is included in the costs declared under the activity "Management of the Consortium".The required audit certificate (s) is (are) attached to this Financial Statement*			

7- Conversion rates	
Costs incurred in currencies other than EURO shall be reported in EURO.	
Please mention the conversion rate used (only one choice is possible) – Please note that the same principle applies for receipts.	
Contractor	
- Conversion rate of the date of incurred actual costs? (YES / NO)	No
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	YES

Third Party(ies) *(if necessary)*	
Third Party 1 (Y1)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 2 (Y2)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 3 (Y3)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 4 (Y4)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	

If necessary add another Form C.

8- Contractor's Certificate

We certify that:

- the costs declared above are directly related to the resources used to reach the objectives of the project ;
- the receipts declared above are directly related to the resources used to reach the objectives of the project ;
- the costs declared above fall within the definition of eligible costs specified in Articles II.19, II.20, II.21, II.22 and II.25 of the contract, and, if relevant, in Annex III and Article 9 (special clauses) of the contract ;
- the receipts declared above fall within the definition of receipts specified in Article II.23 of the contract ;
- the interest generated by the pre-financing declared above falls within the definition of Article II.27 of the contract ; incorporated in the above Statement ;
- the above information declared is complete and true ;
- there is full supporting documentation to justify the information hereby declared. It will be made available at the request of the Commission and in the event of an audit by the Commission and/or by the Court of Auditors and/or their authorised representatives.

Contractor's Stamp	Name of the Person responsible for the work	Name of the duly authorised Financial Officer
	Professor Roger Corder	**Mrs Coleen Colechin**
	Date	**Date**
	Signature	**Signature**

Form C - Model of Financial Statement per Activity for a Specific Cooperative Research for SMEs

(to be completed by each contractor)

Type of instrument	**Specific Research Project for SMEs**	**Type of Action (if necessary)**	**Cooperative Research (CRAFT)**
Project Title (or Acronym)	**PARADOX**	**Contract n°**	**COOP-CT-2003-508649**

Contractors's legal name			
Legal Type	**RTD**		
Contact Person	**Prof. Antonio Ceriello**	**Telephone**	390432559813
Telecopy		**E-mail**	ceriello@uniud.it
Cost model used (AC/FC or FCF)	**ACF**	**Indirect costs (Real or Flat Rate of 20% of Direct costs, except subcontracting)**	**20% Flat Rate**
Period from	**15.02.2005**	**TO**	**14.02.2006**

1- Resources (Third party(ies))

Are there any resources made available on the basis of a prior agreement with third parties identified in Annex I of the contract? (Yes / No)	No
If Yes, please provide the following information	

Third Party 1 (Y1) Legal name		**Cost model used**	
Third Party 2 (Y2) Legal name		**Cost model used**	
Third Party 3 (Y3) Legal name		**Cost model used**	
Third Party 4 (Y4) Legal name		**Cost model used**	

If necessary add another Form C

2- Declaration of eligible costs (in €)

Please complete only the activity covered by the relevant instrument (and type of action) indicated above and as mentioned in Article II.25 and/or in Annexes I and III of the contract.

If you are a contractor using the additional cost model (AC):

- indicate only your additional eligible costs, except for Management of the Consortium Activity for which you may indicate your full eligible costs;

do not declare eligible direct additional costs specifically covered by contributions from third parties as mentioned in Articles II.20 and II.23.a and b of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate your full eligible costs
The costs declared should distinguish between direct and indirect costs
If necessary, adjustments to previous period(s) may be included where appropriate

	Type of Activity											
	Research and Technological Development / Innovation (A)		Demonstration (B)		Training (C)		Management of the Consortium (D)		Other Specific Activities (E)		Total (F) = (A)+(B)+(C)+(D)+(E)	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Direct costs	91.311,80										91.311,80	
Of which subcontracting												
Indirect costs	18.262,36										18.262,36	
Adjustments to previous period(s)												
Total costs	109.574,16										109.574,16	

3- Declaration of receipts (in €)

If you are a contractor using the additional cost model (AC), indicate only receipts covered by Article II.23.c of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate receipts covered by Article II.23 of the contract.

	Type of Activity											
	Research and Technological Development / Innovation (A')		Demonstration (B')		Training (C')		Management of the Consortium (D')		Other Specific Activities (E')		Total (F') = (A)'+(B')+(C')+(D')+(E')	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Total receipts	0										0	

4- Declaration of interest generated by the pre-financing (in €)	
To be completed only by the coordinator.	
Did the pre-financing (advance) you received by the Commission for this period earn interest? (Yes / No)	
If yes, please indicate the amount (in €)	
5- Request of FP6 Financial Contribution (in €)	
For this period, the FP6 Community financial contribution resuested is equal to (amount in €)	109.574,00

6- Audit certificates				
According to the contract, does this Financial Statement need an audit certificate (or several in case of Third party(ies)) delivered by independent auditor(s)? (Yes / No)				**yes**
If Yes, does this(those) audit certificate(s) cover only this Financial Statement per Activity? (Yes / No)				
If No, what are the periods covered by this(those) audit certificate(s) ?		From -to		
What is the total cost of this(those) audit certificate(s) (in €) per independent auditor(s) ? € 312,00				

Audit certificate of the contractor (X)			
Legal name of the audit firm	dr. Gianluca Fantini	**Cost of the certificate**	312,00
Audit certificate(s) of the third party(ies) (Ys) ***(if necessary)***			
Y1 : Legal name of the audit firm		**Cost of the certificate**	
Y2 : Legal name of the audit firm		**Cost of the certificate**	
Y3 : Legal name of the audit firm		**Cost of the certificate**	
Y4 : Legal name of the audit firm		**Cost of the certificate**	
If necessary add another Form C.		**Total (Z) = (X) + (Ys)**	

Reminders:
The cost of an audit certificate is included in the costs declared under the activity "Management of the Consortium".The required audit certificate (s) is (are) attached to this Financial Statement

7- Conversion rates	
Costs incurred in currencies other than EURO shall be reported in EURO.	
Please mention the conversion rate used (only one choice is possible) – Please note that the same principle applies for receipts.	
Contractor	
- Conversion rate of the date of incurred actual costs? (YES / NO)	No
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	No

Third Party(ies) *(if necessary)*	
Third Party 1 (Y1)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 2 (Y2)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 3 (Y3)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 4 (Y4)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	

If necessary add another Form C.

8- Contractor's Certificate

We certify that:

- the costs declared above are directly related to the resources used to reach the objectives of the project ;
- the receipts declared above are directly related to the resources used to reach the objectives of the project ;
- the costs declared above fall within the definition of eligible costs specified in Articles II.19, II.20, II.21, II.22 and II.25 of the contract, and, if relevant, in Annex III and Article 9 (special clauses) of the contract ;
- the receipts declared above fall within the definition of receipts specified in Article II.23 of the contract ;
- the interest generated by the pre-financing declared above falls within the definition of Article II.27 of the contract ;

the above Statement ;

- the above information declared is complete and true ;
- there is full supporting documentation to justify the information hereby declared. It will be made available at the request of the Commission and in the event of an audit by the Commission and/or by the Court of Auditors and/or their authorised representatives.

Contractor's Stamp	Name of the Person responsible for the work	Name of the duly authorised Financial Officer
	Prof. Antonio Ceriello	**Prof. Leonardo Alberto Sechi**
	Date	**Date**
	21st February 2006	**21st February 2006**
	Signature	**Signature**

Form C - Model of Financial Statement per Activity for a Specific Cooperative Research for SMEs

(to be completed by each contractor)

Type of instrument	Specific Research Project for SMEs	Type of Action (if necessary)	Cooperative Research (CRAFT)
Project Title (or Acronym)	PARADOX	Contract n°	COOP-CT-2003-508649

Contractors's legal name	UNIVERSITY OF CRETE, SCHOOL OF MEDICINE, (RTD)		
Legal Type	HIGHER EDUCATION		
Contact Person	MAROUDIO KENTOURI	Telephone	0030 2810 393215
Telecopy	0030 2810 393130	E-mail	kentouri@rector.uc.gr

Cost model used (AC/FC or FCF)	AC	Indirect costs (Real or Flat Rate of 20% of Direct costs, except subcontracting)	20% Flat Rate
Period from	15.02.2005	TO	14.02.2006

1- Resources (Third party(ies))

Are there any resources made available on the basis of a prior agreement with third parties identified in Annex I of the contract? (Yes / No)	No

If Yes, please provide the following information

Third Party 1 (Y1) Legal name		Cost model used	
Third Party 2 (Y2) Legal name		Cost model used	
Third Party 3 (Y3) Legal name		Cost model used	
Third Party 4 (Y4) Legal name		Cost model used	

If necessary add another Form C

2- Declaration of eligible costs (in €)

Please complete only the activity covered by the relevant instrument (and type of action) indicated above and as mentioned in Article II.25 and/or in Annexes I and III of the contract.

If you are a contractor using the additional cost model (AC):

- indicate only your additional eligible costs, except for Management of the Consortium Activity for which you may indicate your full eligible costs;

do not declare eligible direct additional costs specifically covered by contributions from third parties as mentioned in Articles II.20 and II.23.a and b of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate your full eligible costs
The costs declared should distinguish between direct and indirect costs
If necessary, adjustments to previous period(s) may be included where appropriate

	Type of Activity											
	Research and Technological Development / Innovation (A)		Demonstration (B)		Training (C)		Management of the Consortium (D)		Other Specific Activities (E)		Total (F) = (A)+(B)+(C)+(D)+(E)	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Direct costs	117945,94						1160,00				119105,94	
Of which subcontracting												
Indirect costs	23589,19											
Adjustments to previous period(s)												
Total costs	141535,13						1160,00				142695,13	

3- Declaration of receipts (in €)

If you are a contractor using the additional cost model (AC), indicate only receipts covered by Article II.23.c of the contract.

If you are a contractor using a full cost model (FC/FCF), indicate receipts covered by Article II.23 of the contract.

	Type of Activity											
	Research and Technological Development / Innovation (A')		Demonstration (B')		Training (C')		Management of the Consortium (D')		Other Specific Activities (E')		Total (F') = (A)'+(B')+(C')+(D')+(E')	
	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)	Contractor	Third Party(ies)
Total receipts												

4- Declaration of interest generated by the pre-financing (in €)	
To be completed only by the coordinator.	
Did the pre-financing (advance) you received by the Commission for this period earn interest? (Yes / No)	
If yes, please indicate the amount (in €)	
5- Request of FP6 Financial Contribution (in €)	
For this period, the FP6 Community financial contribution resuested is equal to (amount in €)	**142695,13**

6- Audit certificates				
According to the contract, does this Financial Statement need an audit certificate (or several in case of Third party(ies)) delivered by independent auditor(s)? (Yes / No)				**Yes**
If Yes, does this(those) audit certificate(s) cover only this Financial Statement per Activity? (Yes / No)				**No**
If No, what are the periods covered by this(those) audit certificate(s) ?		From -to	**15.2.05-14.2.06**	
What is the total cost of this(those) audit certificate(s) (in €) per independent auditor(s) ?				

Audit certificate of the contractor (X)			
Legal name of the audit firm	**SOL AE** (DIKAIOSINIS 45, PC 71202 HERAKLION CRETE GREECE)	**Cost of the certificate**	15.02.2004-14.02.2005 €450 15.02.2005-14.02.2006 €710
Audit certificate(s) of the third party(ies) (Ys) ***(if necessary)***			
Y1 : Legal name of the audit firm		**Cost of the certificate**	
Y2 : Legal name of the audit firm		**Cost of the certificate**	
Y3 : Legal name of the audit firm		**Cost of the certificate**	
Y4 : Legal name of the audit firm		**Cost of the certificate**	
If necessary add another Form C.		**Total (Z) = (X) + (Ys)**	
Reminders: *The cost of an audit certificate is included in the costs declared under the activity "Management of the Consortium".The required audit certificate (s) is (are) attached to this Financial Statement*			

7- Conversion rates	
Costs incurred in currencies other than EURO shall be reported in EURO.	
Please mention the conversion rate used (only one choice is possible) – Please note that the same principle applies for receipts.	
Contractor	
- Conversion rate of the date of incurred actual costs? (YES / NO)	No
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	No

Third Party(ies) *(if necessary)*	
Third Party 1 (Y1)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 2 (Y2)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 3 (Y3)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	
Third Party 4 (Y4)	
- Conversion rate of the date of incurred actual costs? (YES / NO)	
- Conversion rate of the first day of the first month following the period covered by this Financial Statement? (YES/NO)	

If necessary add another Form C.

8- Contractor's Certificate

We certify that:

- the costs declared above are directly related to the resources used to reach the objectives of the project ;
- the receipts declared above are directly related to the resources used to reach the objectives of the project ;
- the costs declared above fall within the definition of eligible costs specified in Articles II.19, II.20, II.21, II.22 and II.25 of the contract, and, if relevant, in Annex III and Article 9 (special clauses) of the contract ;
- the receipts declared above fall within the definition of receipts specified in Article II.23 of the contract ;
- the interest generated by the pre-financing declared above falls within the definition of Article II.27 of the contract ;

in the above Statement ;

- the above information declared is complete and true ;
- there is full supporting documentation to justify the information hereby declared. It will be made available at the request of the Commission and in the event of an audit by the Commission and/or by the Court of Auditors and/or their authorised representatives.

Contractor's Stamp	Name of the Person responsible for the work	Name of the duly authorised Financial Officer
	Prof. Elias Castanas	**Prof. Maroudio Kentouri**
	Date	**Date**
	17.03.2006	**17.03.2006**
	Signature	**Signature**

Summary Financial Report

Type of Instrument	CA	Project Title (or Acronym)	PARADOX	Contract N°	COOP-CT-2003-508649
Reporting period number	2	From (dd/mm/yyyy)	15.02.2005	To (dd/mm/yyyy)	14.02.2006

Contractor n°	Organisation Short Name	Cost model used	Eligible costs (in €)	Type of activities: Research and Technological Development / Innovation (A)		Demonstration (B)		Training (C)		Management of the consortium (D)		Other Specific Activities : Coordination (E)		Total eligible costs (F)=(A)+(B)+(C)+(D)+(E)		Receipts		EC contribution	
				Contractor	Third party(ies)	Contractor	Third party(ies)	Contractor	Third party(ies)	Contractor	Third party(ies)	Contractor	Third party(ies)	Contractor	Third party(ies)	Contractor	Third party(ies)	Maximum	Requested
1	GAT	FCF	Direct eligible costs	57.175,00						18.166,00				75.341,00	0,00			49.144,00	49.144,00
			of which direct eligible costs of subcontracting											*0,00*	*0,00*				
			Indirect eligible costs	11.435,00										11.435,00	0,00				
			Adjustment on previous period(s)											0,00	0,00				
			Total eligible costs	*68.610,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*18.166,00*	*0,00*	*0,00*	*0,00*	86.776,00	0,00				
2	RODA	FCF	Direct eligible costs	7.740,00						6.230,00				13.970,00	0,00			19.622,00	10.874,00
			of which direct eligible costs of subcontracting											*0,00*	*0,00*				
			Indirect eligible costs	1.548,00										1.548,00	0,00				
			Adjustment on previous period(s)											0,00	0,00				
			Total eligible costs	*9.288,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*6.230,00*	*0,00*	*0,00*	*0,00*	15.518,00	0,00				
4	CHAPOUTIER	FCF	Direct eligible costs											0,00	0,00			14.666,00	0,00
			of which direct eligible costs of subcontracting											*0,00*	*0,00*				
			Indirect eligible costs											0,00	0,00				
			Adjustment on previous period(s)											0,00	0,00				
			Total eligible costs	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	0,00	0,00				
5	HEINRICH	FCF	Direct eligible costs	13.190,20						500,00				13.690,20	0,00			14.666,00	11.755,83
			of which direct eligible costs of subcontracting											*0,00*	*0,00*				
			Indirect eligible costs	2.638,04										2.638,04	0,00				
			Adjustment on previous period(s)	6.683,42										6.683,42	0,00				
			Total eligible costs	*22.511,66*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*500,00*	*0,00*	*0,00*	*0,00*	23.011,66	0,00				
6	TILIA	FCF	Direct eligible costs											0,00	0,00			14.666,00	0,00
			of which direct eligible costs of subcontracting											*0,00*	*0,00*				
			Indirect eligible costs											0,00	0,00				
			Adjustment on previous period(s)											0,00	0,00				
			Total eligible costs	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	0,00	0,00				
7	VINCELLER	FCF	Direct eligible costs	9.412,00						800,00				10.212,00	0,00			14.666,00	14.086,00
			of which direct eligible costs of subcontracting											*0,00*	*0,00*				
			Indirect eligible costs	1.882,00										1.882,00	0,00				
			Adjustment on previous period(s)	15.278,00										15.278,00	0,00				
			Total eligible costs	*26.572,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*800,00*	*0,00*	*0,00*	*0,00*	27.372,00	0,00				
			Direct eligible costs	4.619,00						3.000,00				7.619,00	0,00				

			of which direct eligible costs of subcontracting												0,00	0,00				
8	CINS	FCF	Indirect eligible costs	924,00											924,00	0,00			33.726,00	5.771,50
			Adjustment on previous period(s)												0,00	0,00				
			Total eligible costs	5.543,00	0,00	0,00	0,00	0,00	0,00	3.000,00	0,00	0,00	0,00	8.543,00	0,00				
			Direct eligible costs	27.549,85						2.500,00				30.049,85	0,00				
			of which direct eligible costs of subcontracting												0,00	0,00				
9	NATEX	FCF	Indirect eligible costs	5.509,97										5.509,97	0,00			38.682,00	20.926,93	
			Adjustment on previous period(s)	3.794,03										3.794,03	0,00					
			Total eligible costs	36.853,85	0,00	0,00	0,00	0,00	0,00	2.500,00	0,00	0,00	0,00	39.353,85	0,00				
			Direct eligible costs	9.973,84						1.200,00				11.173,84	0,00				
			of which direct eligible costs of subcontracting												0,00	0,00				
10	CHIROBLOCK	FCF	Indirect eligible costs	1.994,77						240,00				2.234,77	0,00			53.724,00	7.424,31	
			Adjustment on previous period(s)												0,00	0,00				
			Total eligible costs	11.968,61	0,00	0,00	0,00	0,00	0,00	1.440,00	0,00	0,00	0,00	13.408,61	0,00				

Summary Financial Report

Type of Instrument	CA	Project Title (or Acronym)	PARADOX	Contract N°	COOP-CT-2003-508649
Reporting period number	2	From (dd/mm/yyyy)	15.02.2005	To (dd/mm/yyyy)	14.02.2006

Contractor n°	Organisation Short Name	Cost model used	Eligible costs (in €)	Research and Technological Development /		Demonstration (B)		Training (C)		Management of the consortium		Other Specific Activities :		Total eligible costs (F)=(A)+(B)+(C)+(D)+(E)		Receipts		EC contribution	
				Contractor	Third party(ies)	Contractor	Third party(ies)	Contractor	Third party(ies)	Contractor	Third party(ies)	Contractor	Third party(ies)	Contractor	Third party(ies)	Contractor	Third party(ies)	Maximum	Requested
11	CAMPI	FC	Direct eligible costs	22.960,00						500,00				23.460,00	0,00			13.788,00	13.788,00
			of which direct eligible costs of subcontracting											*0,00*	*0,00*				
			Indirect eligible costs	4.592,00										4.592,00	0,00				
			Adjustment on previous period(s)											0,00	0,00				
			Total eligible costs	*27.552,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*500,00*	*0,00*	*0,00*	*0,00*	28.052,00	0,00				
12	VALMAR	FC	Direct eligible costs											0,00	0,00			7.793,00	0,00
			of which direct eligible costs of subcontracting											*0,00*	*0,00*				
			Indirect eligible costs											0,00	0,00				
			Adjustment on previous period(s)											0,00	0,00				
			Total eligible costs	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	0,00	0,00				
13	KUK	FC	Direct eligible costs	3.303,91										3.303,91	0,00			7.793,00	1.982,00
			of which direct eligible costs of subcontracting											*0,00*	*0,00*				
			Indirect eligible costs	660,79										660,79	0,00				
			Adjustment on previous period(s)											0,00	0,00				
			Total eligible costs	*3.964,70*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	3.964,70	0,00				
14	ATYS	FC	Direct eligible costs											0,00	0,00			7.793,00	0,00
			of which direct eligible costs of subcontracting											*0,00*	*0,00*				
			Indirect eligible costs											0,00	0,00				
			Adjustment on previous period(s)											0,00	0,00				
			Total eligible costs	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	0,00	0,00				
15	QMUL	ACF	Direct eligible costs	36.081,00										36.081,00	0,00			176.148,00	43.298,00
			of which direct eligible costs of subcontracting											*0,00*	*0,00*				
			Indirect eligible costs	7.217,00										7.217,00	0,00				
			Adjustment on previous period(s)											0,00	0,00				
			Total eligible costs	*43.298,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	43.298,00	0,00				
16	CERIELLO	ACF	Direct eligible costs	91.311,80										91.311,80	0,00			149.796,00	109.574,16
			of which direct eligible costs of subcontracting											*0,00*	*0,00*				
			Indirect eligible costs	18.262,36										18.262,36	0,00				
			Adjustment on previous period(s)											0,00	0,00				

			Total eligible costs	*109.574,16*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	109.574,16	0,00				
17	CASTANAS	ACF	Direct eligible costs	117.945,94						1.160,00				119.105,94	0,00			255.390,00	142.695,13
			of which direct eligible costs of subcontracting											*0,00*	*0,00*				
			Indirect eligible costs	23.589,19										23.589,19	0,00				
			Adjustment on previous period(s)											0,00	0,00				
			Total eligible costs	*141.535,13*	*0,00*	*0,00*	*0,00*	*0,00*	*0,00*	*1.160,00*	*0,00*	*0,00*	*0,00*	142.695,13	0,00				
Total eligible costs				507.271,11	0,00	0,00	0,00	0,00	0,00	34.296,00	0,00	0,00	0,00	541.567,11	0,00	0,00	0,00	872.061,00	431.319,86
				507.271,11		0,00		0,00		34.296,00		0,00		541.567,11		0,00			

Requested EC contribution for the reporting period (in €) without taking into account receipts	397.023,86	0,00	0,00	0,00	0,00	0,00	34.296,00	0,00	0,00	0,00	431.319,86
	397.023,86		0,00		0,00		34.296,00		0,00		

Amount of the financial interests generated by the prefinancing	588,00
Requested EC contribution for the reporting period (in €) taking into account the receipts	431.319,86

Report on the Distribution of the Community's contribution

Type of Instrument	Spec. Research Proj.for SMEs	Project Title (or Acronym)	PARADOX	Contract N°	COOP-CT-2003-508649

Part I — Community's prefinancing (or payment) sent to the coordinator (1)

	Reporting Period 1 (2)		Reporting Period 2 (2)		Reporting Period 3 (2)		Reporting Period 4 (2)		Reporting Period 5 (2)		Reporting Period 6 (2)		Reporting Period 7 (2)		Final payment		Total Amount (I) (3)
	From	To	From	To	From	To	From	To	From	To	From	To	From	To			
	Date	Amount (A)	Date	Amount (B)	Date	Amount (C)	Date	Amount (D)	Date	Amount (E)	Date	Amount (F)	Date	Amount (G)	Date	Amount (H)	
Total (X)																	0,00

Part II — Distribution of the Community's prefinancing (or payment) between contractors according to the consortium decision(s) (4)

Contractor n°	Organisation Short Name	Country Code	Reporting Period 1		Reporting Period 2		Reporting Period 3		Reporting Period 4		Reporting Period 5		Reporting Period 6		Reporting Period 7		Final payment		Total Amount (I') (6)
			Date(s) (5)	Amount(s) (A') (5)	Date(s) (5)	Amount(s) (B') (5)	Date(s) (5)	Amount(s) (C') (5)	Date(s) (5)	Amount(s) (D') (5)	Date(s) (5)	Amount(s) (E') (5)	Date(s) (5)	Amount(s) (F') (5)	Date(s) (5)	Amount(s) (G') (5)	Date(s) (5)	Amount(s) (H') (5)	
1	GAT	AT	5.01.2004	40.286,00	28.02.2005	24.449,00													64.735,00
					31.05.2005	40.286,00													40.286,00
																			0,00
																			0,00
			Total	40.286,00	Total	64.735,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	105.021,00
2	RODA	ES	9.06.2005	2.808,00															2.808,00
																			0,00
																			0,00
																			0,00
			Total	2.808,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	2.808,00
3																			0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	0,00
4	CHAPOUTIER	FR																	0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	0,00
5	HEINRICH	AT																	0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	0,00
6	TILIA	SLO																	0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	0,00
7	VINCELLER	HU	6.06.2005	3.911,00															3.911,00
																			0,00
																			0,00
																			0,00
			Total	3.911,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	3.911,00
8	CINS	SLO	8.02.2005	17.987,00															17.987,00
																			0,00
																			0,00
																			0,00
			Total	17.987,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	17.987,00
9	NATEX	AT	2.06.2005	18.217,00															18.217,00
																			0,00
																			0,00
																			0,00
			Total	18.217,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	18.217,00
10	CHIROBLOCK	DE	2.06.2005	8.836,00															8.836,00
																			0,00
																			0,00
																			0,00
			Total	8.836,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	8.836,00

Report on the Distribution of the Community's contribution

Type of Instrument	Spec. Research Proj.for SMEs	Project Title (or Acronym)	PARADOX	Contract N°	COOP-CT-2003-508649

Part II — Distribution of the Community's prefinancing (or payment) between contractors according to the consortium decision(s) (4)

Contractor n°	Organisation Short Name	Country Code	Reporting Period 1		Reporting Period 2		Reporting Period 3		Reporting Period 4		Reporting Period 5		Reporting Period 6		Reporting Period 7		Final payment		Total Amount (I') (6)
			Date(s) (5)	Amount(s) (A') (5)	Date(s) (5)	Amount(s) (B') (5)	Date(s) (5)	Amount(s) (C') (5)	Date(s) (5)	Amount(s) (D') (5)	Date(s) (5)	Amount(s) (E') (5)	Date(s) (5)	Amount(s) (F') (5)	Date(s) (5)	Amount(s) (G') (5)	Date(s) (5)	Amount(s) (H') (5)	
11	CAMPI	ES																	0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	**0,00**
12	VALMAR	FR																	0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	**0,00**
13	KUK	AT	2.06.2005	752,00															752,00
																			0,00
																			0,00
																			0,00
			Total	752,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	**752,00**
14	ATYS	AT																	0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	**0,00**
15	QMUL	UK			8.02.2005	46.972,86													46.972,86
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	**46.972,86**	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	**46.972,86**
16	CERIELLO	IT			9.12.2005	39.945,60													39.945,60
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	**39.945,60**	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	**39.945,60**
17	CASTANAS	GR			22.12.2004	862,53													862,53
					20.01.2005	68.104,00													68.104,00
					3.08.2005	68.104,00													68.104,00
					29.2.2006	34.052,00													34.052,00
			Total	0,00	Total	**171.122,53**	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	**171.122,53**
18																			0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	**0,00**
19																			0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	**0,00**
20																			0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	**0,00**
21																			0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	**0,00**
																			0,00

22																		0,00
																		0,00
																		0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	0,00

Report on the Distribution of the Community's contribution

Type of Instrument	Spec. Research Proj.for SMEs	Project Title (or Acronym)	PARADOX	Contract N°	COOP-CT-2003-508649

Part II — Distribution of the Community's prefinancing (or payment) between contractors according to the consortium decision(s) *(4)*

Contractor n°	Organisation Short Name	Country Code	Reporting Period 1		Reporting Period 2		Reporting Period 3		Reporting Period 4		Reporting Period 5		Reporting Period 6		Reporting Period 7		Final payment		Total Amount (I') *(6)*
			Date(s) *(5)*	Amount(s) (A') *(5)*	Date(s) *(5)*	Amount(s) (B') *(5)*	Date(s) *(5)*	Amount(s) (C') *(5)*	Date(s) *(5)*	Amount(s) (D') *(5)*	Date(s) *(5)*	Amount(s) (E') *(5)*	Date(s) *(5)*	Amount(s) (F') *(5)*	Date(s) *(5)*	Amount(s) (G') *(5)*	Date(s) *(5)*	Amount(s) (H') *(5)*	
23																			0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	0,00
24																			0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	0,00
25																			0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	0,00
26																			0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	0,00
27																			0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	0,00
28																			0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	0,00
29																			0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	0,00
30																			0,00
																			0,00
																			0,00
																			0,00
			Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	0,00
Total (Y)			Total	92.797,00	Total	322.775,99	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	Total	0,00	415.572,99

Part III — Difference between Community's prefinancing (or payment) sent to the coordinator and Total Distribution of the Community's prefinancing (or payment) between contractors according to the consortium decision(s) *(4)*

	Reporting Period 1	Reporting Period 2	Reporting Period 3	Reporting Period 4	Reporting Period 5	Reporting Period 6	Reporting Period 7	Final payment	Total Amount
Community's prefinancing (or payment) not yet distributed between contractors (Z) *(7)*	-92797,00	-322775,99	0,00	0,00	0,00	0,00	0,00	0,00	-415.572,99

I certify that the information set out in this(these) form(s) is accurate and correct and agreed by all contractors.

Name *(8)*	Surname *(8)*	Date (dd/mm/yyyy)	Signature of the administrative official authorised to commit the organisation of the coordinator *(8)*

GIMENO	BARBARA	31.03.2006	

Explanatory notes

(1): To be filled in only by the Commission services.

(2): Established in conformity with articles 4.2 and 6 of the contract.

(3): (I) = (A) + (B) + (C) + (D) + (E) + (F) + (G) + (H)

(4): To be filled in only by the coordinator.

(5): Insert the dates (dd/mm/yyyy) and the amounts (x,xxx.xx €) transferred to a contractor (including the coordinator) for a reporting period. If there are more than one transfer to a contractor during a reporting period, identify each date and each relating transferred amount.

(6): (I') = (A') + (B') + (C') + (D') + (E') + (F') + (G') + (H')

(7): (Z) = (X) - (Y)

(8): One the following persons : authorised contact person or first or second administrative official authorised to sign the contract, as mentioned in your Contract Preparation Form (Form A2b)