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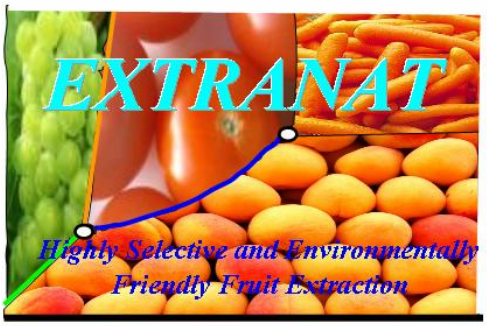

Contract Nº: COOP-CT-2004-512550

EXTRANAT

Highly Selective and Environmentally Friendly Fruit Extraction using Supercritical Fluids Technology

Final Activity Report

Due date of deliverable: September 2007
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Project co-funded 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

Abbreviations

Fundación CARTIF ..... CARTIF
Cooperativa Agrícola Industrial de Cosecheros Exportadores de San Nicolás de Tolentino, S. Coop. ..... COPAISAN
Bodega Matarromera, S.A. ..... MATARROMERA
Enviplan Ingenieurgesellschaft mbH ..... ENVIPLAN
Gradiens Termékfejlesztö, Kft. ..... GRADIENS
Aldivia, S.A. ..... ALDIVIA
EXXENTIA, S.A.. ..... EXXENTIA
Dulces y Conservas Helios, S.A. ..... HELIOS
Universitá di Pavia ..... UNIPV

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Publishable Executive Summary

In the last decades, the food science and technology have developed new products in a safer way, improving the quality and the availability of the final products, and providing thus a higher added value for the consumers. At the same time, the consumers have greatly increased the demand for natural obtained products.

On the other hand the environmental protection and sustainable development have reached the highest possible levels of interest among citizens, not only throughout Europe but also all around the world.

Right now, the food and drink industry is a very diverse sector with role players ranging from big industries to SME´s. This industry is of prime importance to the economy of the European Union; it is among the largest industry sectors in the Union:

- With more than **282.000 companies** across the EU-25.
- employing some **4.1 million people** (3rd industrial employer),
- an annual turnover **of 815 billion euro (14% of total turnover EU-25)**.
- **279.000 SME´s** (98.9%) employing **61%** of the workers, and producing **49%** of the turnover.[1]

The addition of these two interest points together is from where the Extranat project was born. It brings together the necessity of the vegetable processed producers to find a way to reuse their wastes and the will of the food additives, cosmetic and pharmaceutical producers to obtain natural and healthy products from a cheaper source.

Waste represents an unwanted cost for Food and Drink industries and therefore, any reduction in waste would be good in terms of competitiveness. Reduction programmes and recycling efforts have already led to a decrease in the volume of waste, but much remains to be done.

For SME's processing vegetables, a reduced competitiveness caused by waste management, and a more restrictive legal frame, creates an important problem that may put in question even their survival. Extranat tried to tackle this problem by obtaining a **high added value product** from vegetable wastes.

Most of the food and agricultural industry organic residues are currently disposed or used on a low technological and economical level. Two of the most popular reutilisations of these wastes are the production of compost or biomass fuels. However these two possibilities do not yield economic benefits for the vegetable producers, they only try to solve the environmental problem of wastes disposals.

In order to generate the desired economic advantages it is necessary to look closely to the composition of the wastes to be revalorised. These subproducts and wastes are generally very rich in bioactive, mainly antioxidant and anticarcinogenic substances, which are considered to be responsible for the health promoting properties of the dietary habits in which fruit and vegetables are relevant ingredients[2].

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[1] Data and Trends 2005*; Confederation of the food and drink industries of the EU, CIAA*

[2] *Technology Transfer Day. Biotech Andalucia 2005. Technology Catalogue*

Nowadays the most usual way to recover these substances is the organic solvent extraction that consists first in steaming the product to be extracted in a rotating drum and then rinsed for several hours using dichloromethane. The solvent is evaporated when the extracted product is steamed for a second time and then vacuum dried. Unfortunately some solvent residue remains.

This traditional functional compound extraction uses organic solvents and presents the following limitations:

- First of all, **environment** wise, it is impossible to completely eliminate the organic solvent.
- Secondly, the treatment employed to intent removing most of the organic solvents causes a rapid degradation of the obtained final products.
- On the **production** side, organic solvents do not show selective activity in the extraction process, limiting the use of the products obtained
- **Health** wise, the presence of organic solvents radically hinder any use of the final products for human consumption such as nutraceutical complements or pharmaceutical pills even thought their contents in natural fibre would have high value in the food industry.
- Finally, since the final product, as the organic dissolvent contenting compound, has no other use than food additive or complement, it is clear that it has no added value as a sub product.

Supercritical Fluid Extraction may be regarded as a healthy alternative to traditional extraction methods. It is one of the most important new applications of high-pressure processes[3]. It has become a successful technique for the separation of compounds from natural and synthetic sources and widely accepted for extraction, purification, re-crystallization, and Fractionation operations in many industries[4]. Other possible applications are precision cleaning of semiconductors and other electronic equipment, textile dyeing and dry cleaning, impregnation (wood, polymers, and catalysts), coatings and varnishing, etc

Furthermore, supercritical fluid extraction is far more efficient than traditional solvent separation methods. Supercritical fluids are indeed selective, thus providing the high purity and product concentrations[5]. Extraction is efficient at modest operating temperatures, for example, at less than 50°C, thus ensuring maximum product stability and quality.

These characteristics have made SFE an optimal technique to be used in the food industry. Like other successful processes of the last 50 years (deep freezing, freeze drying, vacuum techniques, membrane separation) SFE preserves product quality by applying moderate temperature[6,7,8,9]. The process has as well achieved success in

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[3] *Patent number: US5866004, Patent title: Automated Supercritical Fluid Extraction Method And Apparatus.*

[4] *http://www.phasex4scf.com/nutra.htm*

[5] *Supercritical Fluid Extraction: Principles and Practice, Second ed. McHugh, M.A., V.J. Krukonis. Butterworth-Heinemann, Boston (1994).*

[6] *"Supercritical Fluid Extraction in Food Engineering", Dunford Nurthan, USDA.*

[7] *"Supercritical Fluid Processing of Food and Biometerials", Edited by S.S.H. Rizvi.*

cleaning products for human consumption where it is becoming synonymous with the highest purity and quality.

Extranat aimed to develop and implemented a highly selective and environmentally friendly method to extract compounds with anti-oxidant activity from fruit and vegetable waste, based upon the Supercritical Fluid Extraction (SFE) technique.

This project arose from the ideas and needs of several European SME´s and Research Centres. The Consortium was formed by the following partners:

-CARTIF (Coordinator). Research centre located in northern Spain with great expertise in SFE and Food Technology.

-COPAISAN. (SME). Vegetable producer from the Canary Islands (Spain). It produces Tomatoes, (several varieties) and Papaya.

-ALDIVIA. (SME): Commercialises natural products for their utilisation in sectors ranging from cosmetics to industrial hygiene.

-GRADIENS (SME): Develops new natural products from medicinal herbs to be used in cosmetic and nutritional industries.

-Bodega MATARROMERA (SME): This Spanish winery produces on of the most awarded wines from the Ribera del Duero.

-ENVIPLAN (SME): Engineering Company from Germany. It designs, constructs and build machinery and equipment mostly in the food processing industry.

-Universitá di Pavia. The Universitá di Pavia is one of oldest European Universities. Department of Hematology and Department of Experimental Medicine are involved in the project.

-EXXENTIA (SME): is a manufacturer of dry extracts from selected medicinal plants, fulfilling the needs of costumers in the dietetic, pharmaceutical, nutritional and cosmetic markets

-Helios (IND): Centres its activities in the production of canned fruits and vegetables based in their more than a century expertise and quality processes.

This project was approved and financed by the European Commission under the 6th Framework Programme and it started in April 2005. Some of the results achieved:

First Year Results:

The main objectives of the project for this first year were:

- To define functional components and their availability in vegetable wastes.
- To study Supercritical Fluid Extraction applied to the food industry.
- To select the kind of waste to be used during the project
- To design the pilot plant in which the method will be tested.

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[8] *Krukonis, V.J., Gallagher-Wetmore, P.M., Coffey, M.P.: Food Processing with Supercritical Fluids: Facts and Fiction, Ch. 14, in Science for the Food Industry of the 21st Century, M. Yalpani, ed., ATL press, Science Publishers, Mt. Prospect, IL (1993).*

[9] *Krukonis, V.J., Supercritical Fluid Extraction in Flavor Applications, 154, in Characterization and Measurement of Flavor Compounds, Bells, D.B., Mussian, C.V., eds, ACS Symp. Series 289, ACS, Washington (1985)*

Second Year Results:

The main objectives of the project second year were:

- To end the construction of the pilot plant, specifying as much as possible the design, in order to minimize the future actions of final tuning.
- To run laboratory tests to assure characterise a much as possible the extracts.
- To continue with the dissemination and publicity activities.

Since the main target of the Project is the isolation and production of an extract from vegetal wastes with functional or nutraceutical properties, the initial action to proceed in the selection of the wastes to be used in the Project was the definition of a criteria for this choice:

- The expected behaviour of the raw material against SFE.
- The expected activity and possible use of the compounds of the materials (antioxidant, colorant, etc)
- The content of the compound of interest in the raw material
- The aspect of the material (roots, stem, grape, pomace…)

The following species were considered in the definition of the set of raw materials, all of them related to some of the partners activity:

Strawberry (Fragaria Vesca)

Apricot (Prunus Armeniaca)

Grape (Vitis Vinifera)

Pepper (Capsicum SPP)

Tomato (Licopersicon escolentum)

Carrot (Daucus Carrota)

In the definition of the criteria, the properties of active compounds contained in the selected vegetal wastes were considered. These properties should be useful from an industrial point of view. The considered active compounds, which could be extracted, were:

Terpenes and tocopherols: These compounds are usually present in green and coloured vegetables. The main useful activity in the industry is their high antioxidant activity. On the other hand, they could act as colorants, but they could be neither competitive nor profitable.

Flavonoids and polyphenols: The main activity related with flavonoids and polyphenols is the antioxidant activity. Their tendency to act as chelating agents could be used as a functional property and it is suspected them to have anticarcinogenic activity.

Among the mentioned compounds, the most interesting property from an industrial point of view, it is the antioxidant capacity. It could be considered as a secondary property the colouring ability of some of these compounds.

Finally, it should be considered if the selected compounds or family of compounds are representative or not. So, the results obtained in the project could be used to estimate the application of the process to other vegetable species.

In order to evaluate the efficiency and the quality of the extraction, several methods to measure antioxidant capabilities were studied. Most of these assays are quite well-known such as the oxygen radical absorbance capacity assay (ORAC), the Folin-Cicolteau method (F-C) the Trolox equivalent antioxidant capacity assay (TEAC), the TRAP Assay, Total Oxidant Scavening Capacity (TOSC), the chemiluminescence (CL), Low-Density Lipoprotein Oxidation (LDL), Croton or β-Carotene Bleaching by LOO, Ferric Reducing Antioxidant Power (FRAP), Copper Reduction Assy (CUPRC), DPPH…Once these methods were confronted with the samples chosen and their characteristics, three of them were chosen to be used during the project: Folin-Cicalteau, DPPH and TEAC.

Through the experiments carried out in a Supercritical fluid extraction plant at laboratory level, the consortium was able to decide the flow chart of the final process, including pre-treatments and the optimum parameters (temperature, flow rate, pressure…) to be used during the extraction. With this design, the pilot plant has been constructed in CARTIF's facilities and after its finalisation, it has been finally transported and mounted in the Matarromera's installations and the process finally optimised.

For More Information: www.idetra.com/extranat
linosanchez@idetra.com

Co-ordinator contact Details: Fundación Cartif
Parque Tecnológico Boecillo
47151 Boecillo (SPAIN)
www.cartif.es

Section 1.- Project Objectives and major achievements during the project.

1.- General overview

Following the workplan designed for this project, the main R&D efforts were devoted to the design and construction of the pilot plant after deciding, through a bibliographic and a laboratory study on the samples target of the project.

In order to expose the achievements of the project in a clear way, the information will be distributed among the workpackages. This will only be a summary, please refer to section 2 for a much deeper report.

Work package 1.- Specifications and needs

Work package 1 had, as a primary objective, to define the "healthy" components found in vegetables and the suitability to be extracted using the Supercritical Fluid Extraction. The workpackage included two different tasks, first a study on the characterisation of functional components found in vegetable wastes and then a study of the application of Supercritical Fuid Extraction applied to the food industry.

The first task was clearly focused on providing data to make the right choice of samples to perform the subsequent studies to optimize the extraction method. Therefore before this study was carried out, a survey was run among the partners to determine the raw materials produced inside the consortium. Once these were identified, the bibliographic study was carried out on the following products:

- Strawberry (Fragaria Vesca)
- Apricot (Prunas Armeniaca)
- Grape (Vitis Vinifera)
- Pepper (Capsicum spp)
- Tomato (Licospersicon esculentum)
- Carrot (Daucus Carrota)
- Papaya (Asimila triloba or Carica papaya)

The facts gathered during this study were used in workpackage 2 to make a decision on which samples were more suitable to perform the project.

The detailed and updated bibliographic revision of the SFE technique was produced in order to start the laboratory assays in WP2 with a deeper knowledge of the technique applied to the food industry. The results from this study were also applied to make a better choice of samples to be used on the project.

As an outcome of this first work package two bibliographic studies can be reported. Both of them showed that the Supercritical Fluid Extraction is an adequate technique to obtain compounds with anti-oxidant capacity that can be found in several vegetables.

Many interesting compounds were found in several of the samples available, although, as will be described in workpackage 2, many seemed not feasible for extraction due to their low concentration or to the difficulty of the process.

Regarding the technique revision, it was found that the SFE is being widely applied in the industry, particularly in the food industry, for different processes such as the

extraction of carotene from tomatoes. The most used fluid and the most feasible for this project was found to be CO_2.

In this Workpackage, four SMEs and both of the RTD made contributions, although, as the WP leader the biggest effort was done by CARTIF. The SMEs involved were Enviplan, Gradiens, Aldivia and Pavese, being the contribution from the first two focused on the study of functional compounds from vegetable wastes, and those from the last three to the technique revision. UNIVPV contributed also in the evaluation of the functional compounds from a health point of view.

This Workpackage was completed without any remarkable problem and in due time.

Work package 2.- Selection of samples and laboratory tests

The main objective of this workpackage was to determine at laboratory level the extraction parameters and define as precisely as possible the procedure to extract a certain compound from a certain sample, so the design of the pilot plant could be made in a more efficient manner.

In order to define this procedure, the first steps that should be taken were those focused on determining which compounds and which samples were the most interesting. According to the results obtained on WP1, a selection of raw materials was made. The criterion to select the best samples was firstly established:

- the behaviour of the raw material in supercritical fluid extraction (SFE),
- the expected activity an possible use of the compounds of the materials (antioxidant, colorant, etc),
- the content of the compound of interest in the raw material,

It was also considered if the selected compounds or family of compounds were representative or not, so the results obtained in the project could be used to estimate the application of the process to other vegetable species.

According with the mentioned criteria two vegetable species were chosen to continue with the process:

- Grape pomace. (flavonoids and polyphenols)
- Carrots. (terpenes)

These two species were found representative, since although they both showed antioxidant capacity, they presented different chemical behaviour. While the extracts obtained from grapes will be composed mainly by polar compounds, those from carrots will be non polar, therefore it should be easy to translate the results obtained to a wide range of other samples and interest compounds.

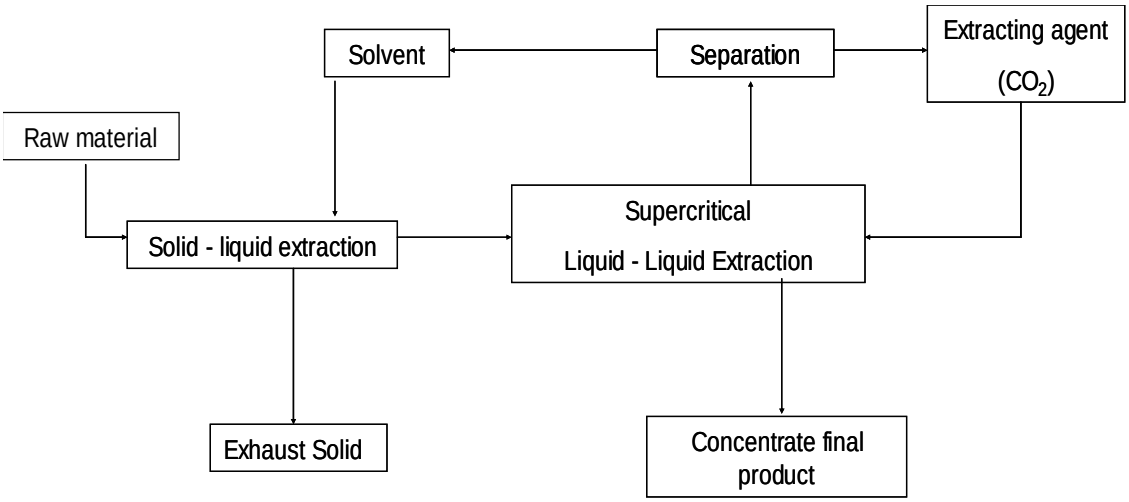
The quantities in which the compounds of interest are found in the samples are high enough so a feasible amount of raw material will be used to obtain enough extract.

This theoretical decision was supported also with some extractions performed at laboratory scale on the SFE plant sited on the coordinator facilities.

One of the facts that clearly arose when applying directly the SFE to the samples, was that it yielded poor results. This was caused by the low solubility of the interest compounds in CO_2, even when co-solvents are used and by the low concentration of these compounds in the raw materials. In order to solve this problem a change on the strategy was implemented.

It was found that CO_2 had a high tendency to retire solvents such as Ethanol or Ethyl Acetate leaving behind compounds with different polar characteristics. To take advantage of this effect it was thought to apply a solid liquid-extraction with this kind of solvents previous to the SFE. Then the solvent could be removed by the CO_2 and a precipitation of the compounds will occur. The solvent can be separated by a rapid expansion in another vessel, and later, it can be reused in the extraction of the raw material. This way the SFE would be acted as a concentrator.

The following diagram shows the main stages of the process:



This idea was presented and accepted by the partners during the six month meeting. After this acceptance, the new diagram and idea, since it would mean some changes to the overall project, was also consulted with the project officer. He agreed with the new layout of the process and therefore the idea was accepted as the line to follow for the future procedure.

Besides the process, some other laboratory assays had to be done to establish a method to evaluate the efficiency and quality of the extraction. The antioxidant capacity (AOC) was chosen as the best parameter to evaluate the applicability of the extract. A big amount of methods to measure this capacity can be found in the bibliography ranging including: oxygen radical absorbance capacity assay (ORAC), the Folin-Ciocalteu method (F-C), the Trolox equivalent antioxidant capacity assay (TEAC), the TRAP Assay, Total Oxidant Scavenging Capacity (TOSC), the Chemiluminescence (CL), Low-Density Lipoprotein Oxidation (LDL), Croton or β-Carotene Bleaching by LOO*, Ferric Reducing Antioxidant Power (FRAP), Copper Reduction Assay (CUPRAC, AOP-90), 2,2-Diphenyl-1-picrylhydrazyl Assay (DPPH), etc…

In order to choose the most adequate AOC methods, a selection criteria was defined:

- The raw materials chosen.
- Future possible standardisation
- Recommended assays by expert panels.
- The adequacy of these assays to industry and companies requirements,
- Use and know-how.
- Availability of facilities and lab apparatus.
- Simplicity in procedures, shortness of the required time and costs.

The choice of this method was agreed by the partners during the six month meeting and the final decision was to use the Folin-Cicolteau method and DPPH for grapes extracts, and TEAC for carrot extracts. It was also pointed that ORAC is growing a big acceptance in the cosmetic market, so it was taken in consideration to subcontract this measurements (none of the partners had the possibility to perform this measurement).

The laboratory assays were supposed to end by month 7, but the changes on the process previously described enlarged the duration of this task and therefore the workpackage. It was necessary to determine also the optimal parameters for the solid-

liquid extraction to accomplish an efficient design of the pilot plant, and this also had some influence on the supercritical fluid extraction.

These changes on the process also affected the design of the pilot plant. This design started at the same time as the laboratory assays that pointed out the necessity of the improvement of the process, so some of the first design calculations had to be discarded. This caused a delay on the design of the pilot plant. In order not to create a pause in the development line and to try to balance the delay by shortening future actions, and being the design of the pilot plant very similar to the existing pilot plant at CARTIF, it was agreed to carry on more assays at laboratory scale trying to better describe the extracts and the extraction conditions.

These assays were carried out and the results evaluated by the two RTDs, CARTIF and UNIPV.

The inclusion of the solid-liquid extraction in the general process, also influenced another task envisaged to take place under this Workpackage, the study on the effect of different pre-treatments in different raw materials in order to be able to apply SFE. This task was readdressed to the study of the extraction were the main parameters to be defined were, moisture, storage conditions, particle size and extraction conditions.

Workpackage 3: Design of the Pilot Plant.

The clear objective of this Workpackage was to design and construct the pilot plant in which the extracting procedures will be implemented. As programmed, these tasks started with the information gathered in the first workpackage and developed simultaneously with work package 2. With the technical changes (introduction of solid-liquid extraction) yielded from the first tasks of WP2 in hand, it was made clear that the design of the plant was delayed.

The first Task had as objective to fix the necessary pre-treatments to prepare the raw materials before entering the pilot plant including the determination of how the samples should be presented and conserved. But when the solid-liquid extraction prior to the SFE was noted as mandatory for the process, this task was also devoted to characterise this first step of the pilot plant.

Task 3.2 comprises the efforts to design the Supercritical fluid Extraction pilot plant, including the Piping and Instrumentation (P&I) diagram of the process. This design was done taking into account all the choices and decisions agreed in previous tasks, so the future pilot plant would be able to fulfil all the requirements and to carry out the extraction with the defined parameters with enough flexibility to allow slight changes in the optimum extraction conditions.

The design achieved in this task reflects high innovative characteristics, like the combination of two extractions in the same plant, the recovery of the solvents and a new heat recovery device. This design is currently beyond the state-of the art of the extraction processes being used in the food industry and it means the achievement of a big objective of the project.

The third task of this workpackage is intended to gather the construction and the start-up of the pilot plant. In order to build it, the first thing to do was to choose among the existing suppliers the best offers on those parts defined in the design. The delivery time of certain parts, caused a big delay of this task and made impossible to start-up the plant by the time programmed in Task 3.4.

The technical objective of Task 3.4 was to adjust the working parameters of the control system and to characterise the dynamic properties of the process. The prototype had to be adjusted to operate in a different range of conditions, which fulfils the required conditions of the “Extranat Process”.
It was only possible to develop this task once the plant was totally assembled, and once it was ensured that all was working properly. According to the selected control strategy during design stage, the control of the prototype was decided to be based in a set of a distributed PID controller.

The development of this workpackage has differed in several ways from what was firstly programmed caused mainly by two factors:

-Technical changes explained in WP2 made the workpackage to start 3 months later than programmed.

-The high delivery time of several parts of the future pilot plant caused the construction of the pilot plant to be carried out several months after what was programmed.

In order not to stop the technical development of the project, it was agreed to continue with the characterisation of the extraction, including both the process and the extracts. The extraction tests were carried out using the Supercritical fluid extraction

plant located at Cartif facilities. The results achieved in this plant were found succesfull, and so it was decided to design the Extranat pilot plant taking as a starting point the design of the existing plant. This facilitated the assumption that the extracts obtained from the future plant will be very similar to those yielded by the Cartif's plant.

The analysis made over the samples of extracts were made thinking on those that are programmed to be done on the extracts from the pilot plant in Workpackage 6. This way the delay on the construction of the pilot plant was absorved by working in advance in tasks programmed after this construction.

The partners involved in this workpackage are:

- Copaisan and Matarromera: only involved in the first task, devoted to the selection of methods to be used on raw materials prior to the extraction process.
- Enviplan: leader of the Workpackage and involved in every task.
- Gradiens: This SME is involved in the first tasks of the Workpackage, from the selection of the pre-treatment to the construction of the plant.
- Cartif: As RTD and responsible of Workpackage 2, from where this one starts, has an important role to play.

Workpackage 4: Manufacturing process establishment at laboratory scale

This workpackage is meant to comprise the tasks envisaged to reproduce the results of the extractions carried out in the WP2 with the prototype and to identify possible failures and problems when applying the final selected raw materials.

To carry out these studies, was mandatory to have the pilot plant constructed and running. As was explained before, in workpackage 3, this construction was delayed in time and therefore, workpackage 4 started after the plant was available.

This workpackage was based on the Extraction of the selected fruits with the prototype, the estimation of the conditions to obtain the best yield for the required product, the study of alternatives conditions for the same product and the Final adaptations of the prototype to perform the process.

The objective ot the first task, Supercritical fluid process running, was to perform the extraction of selected materials according to their different properties and thermo dynamical conditions. Several extraction series on the selected fruits (about seven species) were performed.

The obtained result of the extraction series of the previous task were analysed to identify the main parameters affecting the performance of the process (Task 4.2). After this, some correctios to to deal with every species of fruits to define the standard procedure were applied (task 4.3)

The partners involved in this workpackage were: Enviplan, Gradiens and Cartif.

Workpackage 5: SME Implementation. In Situ Measurements and Final Validation

The purpose of the workpackage 5 was to implement the prototype in one of the SME's facilities and to reproduce the results obtained along the project. Due to the modification happened during the projects as well as the delays in the execution of previous workpackage it was decided to **modify the order of the tasks** to be more efficient in the development of the tasks. Thus it was decided to perform the training in CARTIF facilities (Task 5.4) and then to proceed with the rest of the tasks. This allowed to be more effective in the definition of requirements of the facilities in Matarromera.

The objective of the training on the operational performance of the pilot plant (Task 5.4) was to provide the technicians of MATARROMERA with the necessary skills to operate the pilot plant effectively and safely.

The transport and installation of the system at the SME facilities (Task 5.1) was devoted to relocate the SFE pilot plant from the facilities of CARTIF to those of MATARROMERA and carry out the adequate installations in the latter.

The objective of the Performance of measurements during real processes (task 5.2) was to get the prototype fully operational on the SME facilities and to study the performance of the measurement at industrial level.

The Analysis / Validation of the measurements and final adjustments (Task 5.3) was focused on the adequate adjustments for the system to be fully usable in the company's area. An analysis and a validation of the measurements was necessary before the system was fully usable.

The partners involved in this workpackage are:

- Matarromera: leader of the Workpackage and involved in all tasks.
- Enviplan, Gradiesn, Aldivia and Exxentia: participated mainly on the validation of measurements.
- Cartif: suported a highly workload for all tasks.

Workpackage 6: Production Test

The aim of this work package was to make all appropriate analyses for the final product to be used as additive in the food industry or as a complement in the cosmetic /nutraceutical / pharmaceutical sectors.

First of all, an assessment of the ideal conservation and treatment of the final product before being supplied to final users as Exxentia (food industry, cosmetic,pharmaceutical and nutraceutical partners) was done. The optimum concentration of functional agents was defined by the RTDs competences.
A characterisation of the final product composition was realised to assess the overall quality and degradation profile of the functional compounds.

The anti-oxidant activity was tested in vitro by employing the bleaching of the stable 1,1-diphenyl- 2-picrylhydrazyl radical (DPPH test). In order to give information about the real radical scavenging activity towards a radical species being generated in organs, cells and tissue of living systems, an assay will be used, based on the electron spin resonance spectrometric detection of a DMPO or a DEPMPO radical adduct.

The organoleptic properties of the final products were analysed according to standard methods. At the same time, colouring effect were also evaluated.

Finaly, the Product quality parameters and composition were evaluated to assess the quantity of functional components in cosmetic stuff, capable of maintaining anti-oxidative properties, without toxic effects, at optimum concentration.

The trials on final products was done by the RTD performers, participating also the SMEs (COPAISAN, MATARROMERA, ALDIVIA, EXXENTIA, CARTIF, HELIOS, UNIPV).

Workpackage 7: Exploitation and dissemination

The main objectives of this workpackage were to achieve a successful relationship among partners and with the outside of the project in two main issues: how to exploit the results and how to disseminate the project achievements. During the development of the project one of the main aims was related to the exploitation of the results. As a first approximation to the exploitation a study of the user needs and socio-economic aspects of the potential market and marketing analysis was planned, during the first period of the project. In order to make this analysis as closed to reality as possible, was mandatory to have the real costs of the production of the final products from an estimation done with the extrapolation of the costs of the pilot plant.

Also, comprised this exploitation efforts, a plan for future actions was foreseen. This plan cannot be initially fully established before reaching the results and studying their commercial feasibility, but a draft for foreseen exploitation actions could be agreed. After the complete development of all the tasks planned in the technical annex, the definitive version of the Plan for Exploitation of Knowledge has been included in this report as an Annex.

The actions agreed during the project development included to write a publishable project summary, to design a project logo, to send these two tools to publications and to start up a **website** that can be used as an information forum for the partners and as a dissemination tool, and the publication of **CD-ROMs** with useful information about the project. The **project summary** along with the logo, was sent to electronic and paper publications in English and in the languages of the project participants. This action had a noticeable impact: it was published in more than 10 websites and their paper versions, and through the website many information request from different parts of the food sector were received.

During the second period of the project, the main objective of this action was to execute different dissemination actions about the progress of the project. In this sense, several actions were taken.

The Technical delay, mainly in the construction of the pilot plant, caused to enlarge task 7.1, until the production cost analysis could be done. The main objective of this task was to collect and extract information from target sectors. Since the technology should be used by the SME's involved in the Consortium, main research was devoted on to the perception of public acceptance of extracts enriched on antioxidants refereed as functional products. The main targets of the research were focused to gain knowledge on perception of the population on these products, the reasons to acquire these products or to refuse them, the knowledge about the uses.

For Task 7.3 a subcontract was foreseen to help in this tasks, a minor part of the innovation activities aimed to the dissemination beyond the consortium and study of the standardisation and regulatory aspects that influence the exploitation of the results. These are Innovation activities for the project as a whole but it was considered that the most suitable approach was to allocate the budget for this potential sub-contracting (18000 €) to a given partner rather than sharing its costs among the whole consortium. In order to have this dissemination strongly coordinated with the exploitation of the results and ensure that use of the pilot plant as demonstrator is adequate it has been envisaged to allocate the costs of the contract to ACOMSA, afterwards substituted by MATARROMERA (where it is intended to implement the plant).

Section 2.- Workpackage progress of the period.

In this section the workpackages developed during the project development will be presented separately and in a deeper way.

1.- Workpackage 1: Specifications and needs.

The partners involved in this work package were: Enviplan, Gradiens, Aldivia, Pavese, Cartif and the University of Pavia. Its main objective was to gather all the information required to start efficiently the practical work. This workpackage was divided in two tasks: 1.1 Bibliographic revision and characterisation of functional components and 1.2 Bibliographic revision of Supercritical Fluid Extraction technique applied to the food industry.

The main objective of this Workpackage is to gather all information required to start efficiently the practical work. Its starting point is the same as that for the project, and therefore, future developments will be based on these bibliographic studies. The technical development of the Workpackage is presented below:

Task 1.1 Bibliographic revision and characterisation of functional components

This task involved several partners that identified, through a bibliographic study, the "healthy" components found in fruit and vegetables.

The first element to be solved along this task was the definition of a set of raw materials related with the interest of the partners involved in the Project. After that, a selection was made among them in order to select the more suitable for the success of the Project. The selection criteria of the raw materials were based in:

- the expected behaviour of the raw material against the supercritical fluid extraction (SFE),
- the expected activity an possible use of the compounds of the materials (antioxidant, colorant, etc),
- the content of the compound of interest in the raw material,
- the aspect of the material (roots, stem, grape, pomace, etc).

The following species were considered in the definition of the set of raw materials: Strawberry (Fragaria Vesca), Apricot (Prunus Armeniaca), Grape (Vitis vinifera), Pepper (Capsicum spp), Tomato (Licopersicon esculentum), Carrot (Daucus Carrota). All of them were related with some of the partners' activity (producer and/or end user)

A description of every raw material is given now as a part of the task.

Tomato (Licopersicum esculentum):

Nutritional composition of 100 g. of tomato

Compound	Content	Unity
Water	94,00	%
Carbohydrates	4,0	g
Proteins	0,80	g
Lipids	Traces	g
Calcium	7,30	mg
Phosphor	22,76	mg
Iron	0,50	mg
Potassium	183,00	mg
Sodium	8,00	mg
Vitamin A (value)	1130,00	UI
Thiamine	0,06	mg
Riboflavin	0,05	mg
Niacin	0,56	mg
Ascorbic Acid	18,00	mg
Energetic Value l	20,32	cal

It is a very good source of Vitamin A (833 IU/100g), specially licopene (2573 mcg/100g), β-carotene (449 mcg/100g), lα-carotene (101 mcg/100g), and lutein+ zeaxanthin (125 mcg/100g). Also vitamin C (12,7 mg/100g) and K (7,9 mcg/100g) are quite important. It is quite rich in amino acids, especially in isoleucine (20 mg/100g) and methionine + cystine (18 mg/100g). Also, it has some minerals such as potassium (237 mg/100g) and manganese (0,1 mg/100g) in remarkable quantities.

An important remark concerns to very different compositions depending on different tomatoes varieties: Italian, cherry, large (3" day), medium (2-3/5" day) or small whole (2-2/5" day) and/ or the different types of processing. For example:

Compounds of interest depending on size and type of tomatoes**.**

TYPE	COMPOUNDS					
	LICOPENE	β-CAROTENE	α-CAROTENE	LUTEIN+ ZEAXANTHIN	VITAMIN C	VITAMIN K
Italian/ Plum	1595 mcg/62 g	278 mcg/62 g	62,6 mcg/62 g	76,3 mcg/62 g	7,9 mg/62 g	4,9 mcg/62 g
Cherry	437mcg/17 g	76,3 mcg/17 g	17,2 mcg/17 g	20,9 mcg/17 g	2,2 mg/17 g	1,3 mcg/17 g
Large whole	4683 mcg/ 182 g	817 mcg/182 g	184 mcg/182 g	224 mcg/182 g	23,1 mg/182 g	14,4 mcg/182 g
Medium whole	3165 mcg/ 123 g	552 mcg/ 123 g	124 mcg/ 123 g	151 mcg/ 123 g	15,6 mg/123 g	9,7 mcg/ 123 g
Small whole	2342 mcg/ 91 g	409 mcg/ 91 g	91,9 mcg/ 91 g	112 mcg/ 91 g	11,6 mg/91 g	7,2 mcg/ 91 g

In raw tomatoes, qualities and nutrient concentration not only depend on genetic variations but also upon different other factors such as: the amount of exposure to the sun, fertilizer, soil, cultivation, handling, maturity, water used and seasonal variation

Compounds of interest in tomato:

The compounds of interest in tomato are mainly carotenoids: lycopene, ß-carotene, α-carotene, Lutein or ("xanthophyll" / (3R,3'R,6'R)-beta, epsilon -carotene-3,3'-diol), zeaxantin and Vitamin K or Phytonadione. Carotenoids are important because they are considered anti-carcinogenic agents, preventing cardiovascular diseases and regulating the immune system. At the same time, they present antioxidant activity and they act as a vitamin A precursor. The molecular structures are showed in figures 1 to 5. The main idea to remark about those compounds is that all of them are highly lipophilic substances, and so, it is expected to obtain a high solubility with the CO_2.

Lycopene, represents more than 85% of total carotenoids in tomato. It is found in the water insoluble fraction and in the skin. The concentration of lycopene varies between 30 to 200 mg/kg in the fresh fruit, and from 430 to 2950 ppm on a dry basis.

β-carotene and α-carotene are found as a fat-soluble pigment not only in tomato but in many higher plants of dark green leafy and yellow vegetables such as carrots, collards, turnips, sweet potatoes, and squash, and in yellow fruits such as apricots, oranges, peaches, and cantaloupes.

Lutein and zeaxhantin (3,30-dihydroxy-a-carotene) isomers are xanthophylls, carotenoids with two hydroxyl groups in the conjugated polyene chain. Epidemiological studies provide evidence to suggest that lutein may protect against Age-related Macular Degeneration (AMD), a leading cause of blindness in people over 65.

Strawbery (Fragaria vesca):

Strawberries have vitamins, proteins, sugars and salts. In leaves and rhizomes there are condensated tannins (in roots these are pirogallic and catechinics), flavones, flavonoids, fraganol and leucoanthocyanidins. They are used as astringents and red colourings. In roots procyanidins B1, B2 and B5 were found together with catechins (extracted by fermentation of some tannin extracts from Fragaria).

The content on ellagic acid was 630 μg/g dry wt. In strawberries, 95.7% of the ellagic acid was found in the pulp while 4.3% was contained in the seeds. Its juice contained only negligible amounts of ellagic acid.

Strawberry antioxydants may act to extend shelf-life and enhance quality preservation by delaying senescence created by oxidative degradation. Antioxydants include enzymes, ascorbic acid-glutathione cycle and phenolic compounds, such as proanthocyanidins, which may also contribute to quality preservation acting both as antifungal compound and as antioxydant molecules. It has been detected also anthocyanins in strawberry. Total DMHF quantities of 26.2 mg/kg (ratio free/bound = 2/13) and 60.2mg/kg (ratio free/bound = 3/2) were determined in Spanish and Italian strawberries.

An average content of 60 mg/100 g is high enough to consider strawberry as one of the richest sources of Ascorbic acid among fruits. Ascorbic acid is the predominant form of vitamin C present in fruits, and the primary oxidation product. L-Dehydroascorbic acid (DHA) is also important because it also has biological activity. Since the oxidized form is more prone to decomposition, leading to the loss of biological activity, the changes in Ascorbic acid forms are important in both, technological and nutritional terms.

Concerning flavonols, the total flavonol content ranged from 5.2 to 8.2 mg/100 g FW (fresh weight). Only quercetin and derivatives are present and, in general, the amount of

quercetin derivatives is more than double that of kaempferol derivatives. Kaempferol was the main flavonol in strawberries and its concentration varied between 0.2 and 0.9 mg/100 g fresh weight. Quercetin concentration varied from 0.3 to 0.5 mg/100 g.

Total phenolics content is of 300mg/100g FW (fresh weight), total flavonols (7.5 mg/100 g FW) and free ellagic acid (2.25 mg/100g FW) represents only a small percentage of total phenolics, mainly constituted of p-coumaric acids, ellagitannins and glycosylated derivatives of ellagic acid, and anthocyanins. (See table below).

Apricot (Prunus armeniaca):

Apricots are one of the richest fruit in carotene contents with pro-vitamin A activity, specially β- carotenes. However there are also minor portions of other carotenes such as α and γ carotenes and cryptoxantin. The β- carotene, on top of turning into vitamin A in our body, has been related to carcinogenic, cardiovascular illnesses, cataracts and macular senile degeneration prevention.

They also have little contents of flavonoids, such as quercetin (a flavone with bioactive activity) to which it has been attributed antioxidant and antitrombogenic activity. It is thought to play an important role on cardiovascular diseases prevention. Moreover some studies have indicated that quercetin can inhibit certain tumoural growings.

The apricot has in its composition organic acids like malic and citric acids (which is able to enhance vitamin C action, favour the calcium intestinal absorption and facilitate toxic residues elimination from our body, like the uric acid). It is necessary to remark that the maturation process makes these acids quantities to decrease.

The next table shows the average composition of apricots:

Basic composition of apricots

	Quantity per 100 g per edible portion	Recommended intake
Water (g)	87.6	-
Energy (kcal)	39	3000 - 2300
Proteins (g)	0.8	54 - 41
Hydrates of carbon (g)	9.5	450 - 350 (a)
Lipids(g)	Traces	90 - 80 (a)

Functional compounds composition of apricots

	Quantity per 100 g per edible portion	Recommended intake		Quantity per 100 g per edible portion	Recommended intake
Vitamins			**Carotenes without activity provitaminic A**		
Vitamin A (Equivalents. Retinol) (µg)[3]	280	1000 - 800	Lutein (µg)[2]	Traces	-
Total Carotenes (µg)[3]	1800	-	Zeaxantin (µg)[2]	Traces	-
Alfa-carotene (µg)[3]	37	-	**Special Bioactive Compounds**		
Beta-carotene (µg)[3]	1600	-	Kaempferol (mg)[3]	0.2	-

Crytoxantin (µg)[3]	106	-	Quercetin (mg)[3]	3.2	-
Vitamin B1 (mg)[1]	0.05	1.2 - 1.1	Organic Acids		
Vitamin B2 (mg)[1]	0.07	1.3 - 1.2	Citric Acid (mg)[3]	400	-
Niacin (mg)[1]	0.6	16 - 15	Chlorogenic Acid (mg)[3]	7.5	-
Vitamin B6 (mg)[1]	0.07	1.5 - 1.3	Malic Acid (mg)[3]	1000	-
Folates (µg)[1]	5	400	**Fibre**		
Vitamin C (mg)[1]	7	60	Total fibre (g)[3]	1.54	> 30 (a)
			Total fibre (g)[4]	0.71	> 30 (a)
			Soluble (g)	0.83	12 (a)

Grape (Vitis vinifera):

From long time ago is generally known that grapes consumption has given numerous benefits to human health like the diminution of coronary diseases, degenerative illnesses, etc. This is mainly caused by grapes composition and by their content on phenolic compounds.

The quantity and variety of these phenolic compounds depend on several factors such as the variety of grape; the maturity grade, the climatic conditions, the kind of storage…. Phenolic compounds have a basic functional structure, a phenolic ring at least. Basically, 4 types of phenolic compounds can be found within the grapes:

- Phenolic acids.
- Hydroxycinnamic acids.
- Flavonoids
- Tannins

These effects are based mainly on the antioxidant properties of these compounds, which put out and capture the free radicals (especially hydroxyl and peroxyl radicals) through the constitution of stable phenolic radicals due to resonance properties.

The grape pomance is composed of pressed skins, disrupted cells from grape pulp, seeds and stems. It could reach up to 20% of total weight of grapes. The next table shows the standard composition of the grape pomance:

Composition of grape pomance in compounds of interest

	Origin		
Component	Warm press	Cold press	Fermented pomance
Seed (1%)	15 -30	22 -36	18 – 37
Total soluble solids (%)	13 -10	12 - 9	15 –4
Tannins (mg/100g)	391 -444	254 -120	178 – 120
Total antociannins (phenolic comp.)	460 -900	200 - 79	360 - 211

Most of the phenolic compounds in grapes are flavonoids. The flavonoids are polyphenolic compounds with 15 carbon atoms; two benzene rings joined by a linear three carbon chain. The basic structure of flavonoids is showed in the next figure. This skeleton can be represented as the C6-C3-C6 system. Favonoids can vary in their basic structures, type number and position of sustituyents. Most of them are joined to a sugar.

Anticarcinogenic activity of phenolic compounds has been reported and related to the inhibition of some types of cancer such as: lung, liver and breast cancer. Some of these compounds are: resveratrol, quercitin, cafeic acid, elagicg acid, flavan-3-ols. Flavonoids and polyphenols inhibits the origin of cancer by a modification of the metabolic activity.

Flavonoids are potent antioxidants, free radical scavengers and metal chelators; they inhibit lipid peroxidation and exhibit various physiological activities including antiinflammatory, antiallergic, anticarcinogenic, antihypertensive and antiarthritic activities. These compounds represent an important source of antioxidants in human diet. Resveratrol has been correlated with serum lipid reduction and inhibition of platelet aggregation and its cancer chemopreventive activity has been recently reported. Phenolic compounds may be synthesized by plants for the defence against

microorganisms or strong UV radiation. For instance, t-resveratrol concentrations have been correlated with resistance to fungal infections.

1

Pepper (Capsicum spp):

The composition of pepper shows that water is its major component, followed by carbon hydrates, which makes it a low caloric vegetable. It is a good source of fiber that improves the intestinal transit. Like the rest of vegetables, its protein content is very low and it has hardly fat. Regarding their vitamin content, peppers are an excellent vitamin source reaching two times the amount of vitamin C than in fruits as orange or strawberries, and the same vitamin A amount that 1/3 of carrot.

Peppers, in general, are low in Saturated Fat, and very low in Cholesterol and Sodium. They are also a good source of Thiamin, Riboflavin, Niacin, Folate, Pantothenic Acid and Magnesium, and a very good source of Dietary Fiber, Vitamin A, Vitamin C, Vitamin E, Vitamin K, Vitamin B6, Potassium and Manganese.

Capsicum annuum L. is an excellent source of ascorbic acid and a fair source of provitamin A carotenoids (Haytowitz and Matthews, 1984). In addition, peppers are rich in flavonoids (Lee et al., 1995) and other phytochemicals (Duke, 1992).

Because of their antioxidant properties, ascorbic acid, carotenoids, and vitamin E are currently the object of much attention due to possible links to the prevention of certain types of cancer (Olson, 1989; Ziegler, 1989; Krinsky, 1989), cardiovascular diseases (Kritchevsky, 1992; Machlin and Bendich, 1987), atherosclerosis (Mezetti et al., 1995), and delay of the aging process (Packer, 1996). Ascorbate acts in oxidation/reduction reactions with metal ions associated with metalo-enzymes and as a free radical scavenger in animal and plant tissues (Foyer, 1993). Carotenoids with nine or more conjugated double bonds are quenchers of reactive oxygen species and act as antioxidants at low oxygen pressure (Bendich, 1989). Also, they may protect tissues against free radical damage and peroxidation (Machlin and Bendich, 1987).

There is a worldwide growing interest in the application of natural colorants for food products, and red pepper (Capsicum annuum L.) or paprika has been used since ancient times as a colorant to enhance or change food colour. Carotenoids are one of the most important groups of natural colorants, and paprika oleoresin is an important natural source of these pigments (Nienaber,& Schwartz, 1999). The main constituents of the carotenoid fraction are capsanthin and capsorubin, which are almost exclusive to the genus Capsicum and are responsible for the final red colour.

The oleoresin is the liquid extract of pepper, obtained in oil form, characterised by its intermediate viscosity and its intense red colour and typical aroma of paprika. This oleoresins are constituted of a mixture of light (e.g., fatty oils) and heavy components (e.g., pigments). It contains all the components of paprika that are extracted selectively by a suitable fatty solvent, from the fruits of different varieties of the species "Capsicum annuum L." dehydrated and milled.

The more important carotenoids in the pepper resin present the following functional groups:

Functional groups of the most important capsicum carotenoids.

	HYDROXYL GRUPS	*CARBONYL GRUPS*	*DOUBLE CONJUGATED BOUNDS*	*EPOXI*
Capsantene	2	1	10	0
Capsorrubene	2	2	9	0
Beta-carotene	0	0	11	0
Zeaxantene	2	0	11	0
Cryptosantene	1	0	11	0
Violaxantene	2	0	9	2
Cryptocapsene	1	1	10	0

Carrot (Daucus Carota):

Carotenoids in carrot

Carrots contain α-carotene (35.0 mg/g) and β-carotene (61.5 mg/g) as principal carotenoids and lutein (5.1 mg/g) as a minor component.

Lutein is the carotenoid related with the risk reduction of cataract and macular degeneration (Moeller et al. 2000; Seddon et al, 1994; Snodderly, 1995). It has been related as a preventive agent against colon cancer. The typical lutein contents values are: 5.6 μg/g for medium size carrots and 3.6 μg/g for larges sizes (Müller 1997).

Anthocyanins are widely distributed in nature in various plant species. They are mainly found in flowers and fruits. They have high potential as food colorants because of their low toxicity (Markakis, 1982). Anthocyanins have been researched from a “black carrot" variety. It was reported to have six anthocyanins with only cyanidin as an aglycone (Glabgen, Wray, Strack, Metzger & Seitz, 1992). In another study, malvidin and/or peonidin glucosides have also been reported (Harborne, 1967).

Two anthocyanin pigments were isolated from cell cultures of Carrot, Nentes scarlet-104 local variety. Chemical hydrolysis, column and paper chromatography, HPLC, proton and 13C NMR and mass spectroscopic studies indicated the presence of cyanidin-3-lathyroside [cyanidin-3-0{β-D -xylopyranosyl (1→2) β-D-galacto pyranoside}] (90%) and cyanidin-3-β-D -glucopyranoside (10%) in the callus cultures, whereas only cyanidin-3-lathyroside (0.05%) was found in the carrot. The significant difference was that there was no acylated anthocyanin present as reported in other varieties of carrot.

Flavonoids (quercetin) content of violet carrot juice
83.7 ± 0.5 μg/l

The quercetin, whose systematic name is 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4- one, and its molecular formula is $C_{15}H_{10}O_7$, is widely distributed in the plant kingdom, especially in rinds and barks, clover blossoms, and ragweed pollen.

Papaya (Asimina triloba or Carica papaya); PawPaw

Papaya tree and fruit.

Papaya, also called pawpaw is used for human consumption as fruit (yellow) or as vegetable (green). Nowadays, Papaya is considered one of the most economically important and nutritious fruits, being a rich source of antioxidant nutrients such as carotenes, vitamin C and flavonoids; the B vitamins folate and pantothenic acid; the minerals potassium and magnesium; and fiber. In addition, papaya is the source of the digestive enzyme papain, which is an industrial ingredient used in brewing, meat tenderising, pharmaceuticals, beauty products and cosmetics.

Papain

It is a water-soluble globular protein (EC 3.4.22.2). It is a thiol enzyme (sulfhydryl proteases) obtained from the latex and unripe fruit of Carica papaya (tropical melon or papaw). Papain is a carbohydrate free, basic, single chain protein with a molecular weight of 23,350 Da and consists of 212 amino acid residues (methionine absent; IP 8.75) with four disulfide bridges and catalytically important cysteine (position 25) and histidine residues (position 158). (Ghosh)

The interesting feature of this molecule is its molecular structure divided essentially in the form of two distinct lobes separated by a cleft. The structural stability of each lobe is at least partly due to the hydrophobic core to which many side chains contribute. It is suggested that the folding of each lobe is independent of the other and this feature of the papain may indicate its binuclear nature.

Papain is a chemical (proteolytic enzyme), which can be found in their leaves and in the green fruit of the pawpaw tree.

Uses of papain: (FAO database1)

- To make meat or octopus tender, as commercialised meat tenderisers.
- To treat insect stings, to lessen the pain by rubbing with the juice of papaya.
- To kill intestinal worms (juice, mixed with honey and hot water)
- As a treatment for indigestion

- To wash clothes and remove stains (young crushed pawpaw leaves instead of soap)

Task 1.2 Bibliographic revision on Supercritical Fluid Extraction (SFE) applied to the food industry.

Extraction procedures using supercritical fluids (SCFs) rather than organic solvents have been growing in popularity. A fluid whose temperature and pressure are simultaneously higher than its critical temperature and pressure is supercritical. Actual solubility of non-volatile solutes in SCFs may be as much as 106 times higher than would be calculated assuming ideal gas behaviour at the same temperature and pressure.

The most common SCF, carbon dioxide (CO_2, Tc= 304. 1 K, Pc= 73. 8 bar), is a gas at ambient conditions. In a supercritical state, it is essentially a compressed, high density fluid at mild temperature. It is relatively innocuous, inexpensive and non-reactive under most operating conditions. Other SCFs may have higher Tc and Pc and may not be innocuous. Contrary to liquids, the density, solvent power or selectivity of a SCF can be easily altered with relatively small changes in pressure or by addition of small amounts of an organic solvent. The change in CO_2 density (with pressure at 35 ºC determined using an equation of state developed specifically for CO_2) does not increase linearly with increasing pressure. Small changes in pressure can produce large changes in density when operating closed to the critical point, for instance at 83 bar where the compressibility of CO_2 is high. Relatively large changes in pressure may result in relatively small changes in density when operating at higher pressures, for instance at 700 bar where CO_2 compressibility is low.

Because of its gaseous nature, a SCF is also characterized by a higher diffusivity and lower interfacial tension than liquids, and has the ability to freely penetrate a matrix such as pores in a catalyst with no phase change. A SCF such as CO_2, can also be vented out of an extractor, leaving no residue and no need for drying.

Numerous gases other than CO_2 may be converted to SCFs at temperatures and pressures commonly employed in industry, including, without limitation, hydrocarbons (e. g. methane, ethane, propane, butane, pentane, hexane, ethylene and propylene), halogenated hydrocarbons, and inorganic compounds (e. g., ammonia, carbon dioxide, sulfur hexafluoride, hydrogen chloride, hydrogen sulfide, nitrous oxide and sulfur dioxide). SCFs have been used to extract numerous compounds including aliphatic and aromatic hydrocarbons, organic esters of inorganic acids, organosilicons and organometallics.

The general rules of extraction with carbon dioxide can be summarised as follows:

- Lipophilic compounds such as hydrocarbons, ethers, esters, ketones and aldehydes could be extracted.
- Polar substances such sugars, polysaccharides, amino acids, proteins, phosphatides, glycosides and inorganic salts are not soluble.
- Fractionation is possible when the substances display differences in volatility, molecular weight or vapour pressure.

The main drawback encountered is the low dissolving power of CO_2 for many of the interesting compounds present in natural products, particularly when these have polar characteristics or very long chain lengths. In many cases CO_2 can be regarded as a typical non-polar solvent.

Industrial applications of supercritical carbon dioxide.

Besides the application focused on the separation of compounds, there are more industrial applications where carbon dioxide could be used:

- Reactions:
  - Hydrogenation.
  - Hydroformylation
  - Oxidation.
  - Enzymatic chemistry.
  - Diels-Alder chemistry.
  - Lewis acid catalysis / Friedel Craft chemistry.
- Polymerization and polymer processing.
- Formation of fine particles using carbon dioxide..

Latest advances in supercritical fluid extraction.

Supercritical fluid and ultrasound extraction. There are some investigations related to the use of supercritical fluid extraction plus ultrasounds in order to improve the extraction method.

- *Advances in sample preparation.*

Meng Zhong, in pantent number B01D11/02 (C11B9/00, A23L1/064, A23L2/02 and CN 200410010653) called Extraction method of wild rose under supercritical condition, proposes the supercritical CO_2 extraction of obtaining wild rose extract. The difference with other extraction methods is the sample preparation procedure. The process includes freeze drying of wild rose at 0 deg.c to -30 deg.c, crushing and pulping, extracting in an extracting reactor with CO_2 at flow rate of 40-80 kg/hr at 30-50 deg.c and 10-20 MPa for 3.5-5 hr. This is the first separation to collect coarse extractive. The second separation takes place in a separating reactor at 30-50 deg.c and 10-16 MPa for 3.5-5 hr.

Patent number CN1253951 describes a new technological process for extracting and concentrating natural vitamin E by using supercritical fluid extraction. It uses the difference of solubility of natural, vitamin E and other components in supercritical fluid to make separation and concentration.

Wu Jiasen proposes, in patent CN1258511, the supercritical multiple bee glue extracting process. It features that bee glue is first soaked with solvent and then extracted in extractor through supercritical multiple extracting process to obtain ultimate products polycyclic fats as well as polysaccharides, flavones and terpenes separately.

- *Use of entrainers.*

Cosolvents or entrainers are commonly used in supercritical fluid extraction. The solvatation power may be altered by the presence of a co-solvent. It should be considered that the extract is diluted or mixed in the separation stage when a cosolvent is used, and so, a further separation stage could be required. Ethanol is the cosolvent most used but there are new process where using anothers.

Patent AU2003232854 propose a method for the fractionated extraction of natural source carotenoids with a high lycopene content, using supercritical fluids. They use a conventional supercritical fluid extraction using water as entrainer.

- *New equipment.*

Allington Robert W. et al, from Isco inc, propose in their patent US6294088 a system valid as well for supercritical extraction as for supercritical chromatography. This system provides a novel technique for reducing the loss of sample and for reducing the recovery time. The invention consists in a variable-orifice fluid restrictor for use with a supercritical extractor or chromatograph.

In CN1297782, the technological process of supercritical CO_2 extraction includes CO_2 extraction, CO_2 separation and CO_2 recovery, and features the CO_2 recovery course, during which residual CO_2 in extractor is first made to pass through flow limiting orifice plate to CO_2 gasifier without passing through two-stage reducer. The technological process needs less equipment, simple control, less investment and low consumption, and is favorable to continuous operation, programmed control and automation.

Legal restrictions to extraction solvents used in food stuffs.

A brief description of the present regulation on the use of solvents in extraction is given. It should be noted that when CO_2 is used with an entrainer, the legislation will affect to the final extract, since it will occur some type persistence of the cosolvent.

The council directive 88/344/EEC of 13 June 1988 applies to extraction solvents used in the production of foodstuffs or food ingredients including those imported into the European Community. They do not apply to extraction solvents used for the production of additives, vitamins and other nutritional additives not listed in the annex to the Directive nor to extraction solvents exported from the Community. Member States must, however, ensure that the use of these substances does not result in dangerous levels of extraction solvent residue in foodstuffs.

Member States shall authorize the use of extraction solvents listed in the annex to these Directives. They shall not authorize any others.

Deviations from the project workprogramme

This first workpackage was carried out without any remarkable problem. Its start was slightly delayed, since the starting date of the project (April 1st) was communicated 10 days late, which caused the celebration of the kick-off meeting later that month. The work was started before that meeting by Cartif, and since that meeting the rest of the partners involved in this workpackage, Enviplan, Gradiens, Aldivia, Pavese and Univpv contributed to the completition of the tasks.

Enviplan and Gradiens helped mainly in the bibliography study about the latest improvements on the Super critical fluid extracion techniques, and its application in the food industry. Gradiens, Aldivia, Pavese and Univpv helped identify the interst compounds present on the raw materials that will be used in the project. Copaisan, Helios and, by that time Acomsa (it will later on leave the consortium), defined the possible fruits and vegetables wastes that would be available from them.

List of Deliverables and Milestones

No Milestones nor deliverables were assigned to the development of this Workpackage.

2.- Workpackage 2: Selection of samples and laboratory tests.

The partners involved in this work package were: Copaisan, Matarromera and in its first stages Acomsa, Helios, Cartif and the University of Pavia. Most of the work being very technical, was assumed by the RTD performers.

The SMEs involved and Helios, as a big company, centred their contribution in the first task, selection of samples, were they expressed their preferences and provided the needed samples to carry out several tryouts. This provision of samples was also used for the second task.

The objective of this work package is to analyse at laboratory level the optimal parameters to extract fruits by SFE and to decide the best option to implement in the future pilot plant.

The information gathered in the first workpackage was used as the starting point for this one. It was needed in order to take a good decision in the selection of sample as well as for the definition of the overall process. The technical progress will be showed sorted by tasks.

Task 2.1 Selection of samples from various types and origins

Since the main target of the Project is the isolation and production of an extract from vegetal wastes with functional or nutraceutical properties, the initial action to proceed in the selection of the wastes to be used in the Project was the definition of a criteria for this choice. Therefore, this criterion was used to select among the species mentioned in WP1.

In the definition of the criteria, the properties of active compounds contained in the selected vegetal wastes were considered. These properties should be useful from an industrial point of view. The considered active compounds, which could be extracted, were:

- Terpenes and tocopherols: These compounds are usually present in green and coloured vegetables. The main useful activity in the industry is their high antioxidant activity. On the other hand, they could act as colorants, but they could be neither competitive nor profitable.
- Flavonoids and polyphenols: The main activity related with flavonoids and polyphenols is the antioxidant activity. Their tendency to act as chelating agents could be used as a functional property and it is suspected them to have anticarcinogenic activity.
- Phytosterols: They are usually present in most of green and yellow vegetables. They have the ability to block the cholesterol absorption.
- Non digestible oligosaccharides.

Among the mentioned compounds, the most interesting property from an industrial point of view, it is the antioxidant capacity. It could be considered as a secondary property the colouring ability of some of these compounds.

On the other hand, and considering the selected extraction technique in this project, additional problems can be found like the chemical form of the compounds to be extracted or the content and distribution of these compounds in the waste.

So, the following situations can be found:

- The compound shows a very poor solubility in the supercritical fluid, even if it is used high quantity of entrainer.
- The content of the compound of interest is so low, that it is needed to process a big quantity of waste to obtain a representative sample of the final product.

Finally, it should be considered if the selected compounds or family of compounds are representative or not. So, the results obtained in the project could be used to estimate the application of the process to other vegetable species.

Attending to the former information, the following species have been selected to be used in the process:

- Grape pomace. (flavonoids and polyphenols)
- Carrots. (terpenes)

The two selected species are considered as representative ones. Both of them will act as antioxidants. On the other hand, the compounds contained in both wastes show a different chemical behaviour. The extracts obtained from grape pomace will be composed by polar compounds mainly, and the extracts obtained from carrots will be composed by non polar compounds.

The amount of compounds of interest contained in the selected wastes is high enough. Then, it is possible to obtain representative samples processing an acceptable quantity of raw material.

The selected species will be representative of the set of wastes mentioned in the Project. So, the results obtained from carrots could be applied to pepper and tomato. The result obtained from grape pomace could be applied to strawberry and apricot.

Finally it will be pointed out that the methods of analysis that will be used during the project will be focussed to estimate the antioxidant activity and to quantify key components of the extract.

Task 2.2 Laboratory assays

One of the studies carried out to evaluate the efficiency and quality of the extraction, was focused on the choice of the method to measure the antioxidant capacity.

There is a big amount of different methods or assays to measure antioxidant activity (AOC) of a compound. Most of these assays are quite well-known such as the oxygen radical absorbance capacity assay (ORAC), the Folin-Ciocalteu method (F-C), the Trolox equivalent antioxidant capacity assay (TEAC), the TRAP Assay, Total Oxidant Scavenging Capacity (TOSC), the Chemiluminescence (CL), Low-Density Lipoprotein Oxidation (LDL), Croton or β-Carotene Bleaching by LOO*, Ferric Reducing Antioxidant Power (FRAP), Copper Reduction Assay (CUPRAC, AOP-90), 2,2-Diphenyl-1-picrylhydrazyl Assay (DPPH), etc. Currently there is not a standardisation for AOC methods and there is little consistency in methods that are being reported, thus comparison of activities and reported results is nearly impossible. The number of papers published and sent to journals in this topic area has increased dramatically due to the actual relevance of antioxidants on the present industry products, so the scientific community is doing urgently a great effort to try to standardise at least some of these methods (Prior et al.).

Therefore, a study on the different methods to measure antioxidant activity was carried out, in order to select the most appropriate ones according to our raw materials, different scientific opinions, their future possible standardisation as recommended assays by expert panels, the adequacy of these assays to industry and companies requirements, use and know-how and our available facilities and lab apparatus.

In addition, simplicity in procedures, shortness of the required time and costs of their application were taken into account.

Concerning the raw materials chosen (grapes and carrots) and the antioxidants contained on them:

Grapes Phenolic compounds will be the compounds mainly extracted and for this reason we have chosen the Folin-Ciocalteau assay. Also and besides other considerations, as these are hydrophilic compounds, we have selected too the DPPH assay.

Carrots DPPH method can not be used to analyse carotenoids, since both DPPH and β-carotene absorb at the same range on the spectrophotometer (around 515-520 nm). Carotenoids are lipophilic compounds, therefore F-C methods cannot be used either. These are the reasons (among others) why the TEAC assay was selected.

Considering their future possible standardisation as AOC Assays by expert panels:

Attending quotations made at the First International Congress on Antioxidant Methods: “From evaluation of data presented at the First International Congress on Antioxidant Methods in 2004 and in the literature, as well as consideration of potential end uses of antioxidants, it is proposed that procedures and applications for three assays be considered for standardization: the oxygen radical absorbance capacity (ORAC) assay, the Folin-Ciocalteu method, and possibly the Trolox equivalent antioxidant capacity (TEAC) assay.” (Expert Panel during the first Inter. Cong .on AOM). (Finley).

Taking into account other research groups expertise on some of the methods and the possibility of comparing results among different R&D performers in the project:

The third selected method, DPPH assay was chosen in order to be able to compare the AOC results between Pavia University and CARTIF, at least for one kind of samples (skin grapes), since they were going to use this assay and they have been already working with it.

Finally, it has to be remarked that none of the proposed methods is perfect. Each one of them has their own advantages and disadvantages, considerations on their applicability and feasibility that must be taken into account when making trials, accurate procedures in order to properly obtain results and mechanisms and kinetics of reaction of their own. Therefore, it is essential to know quite well each one of the proposed methods and their limitations in order to apply them accurately. (Huang et al.)

General Description of the process

As it was referred in previous reports, the direct use of supercritical CO_2 on solid material (in solid to liquid extraction stage) yields poor results even if an entrainer such ethanol is used. The main reason to maintain such asseveration is based on the initials results obtained in previous work packages. These reasons can be summarised as follows:

- The solubility of the compounds of interest is very low in CO_2 due to their polar characteristics. This situation can be observed even if cosolvents are used (Those allowed by the present normative).
- The total content of the natural product with commercial interest is very low in the raw material. This situation will force to use a high volume extraction vessel to obtain profitable results. And, as it is refereed in some bibliographic studies, intense extraction condition are required to perform the extraction. It should be considered the large quantities of industry by-products to be treated and the low content in compounds of interest (for instance antioxidant).

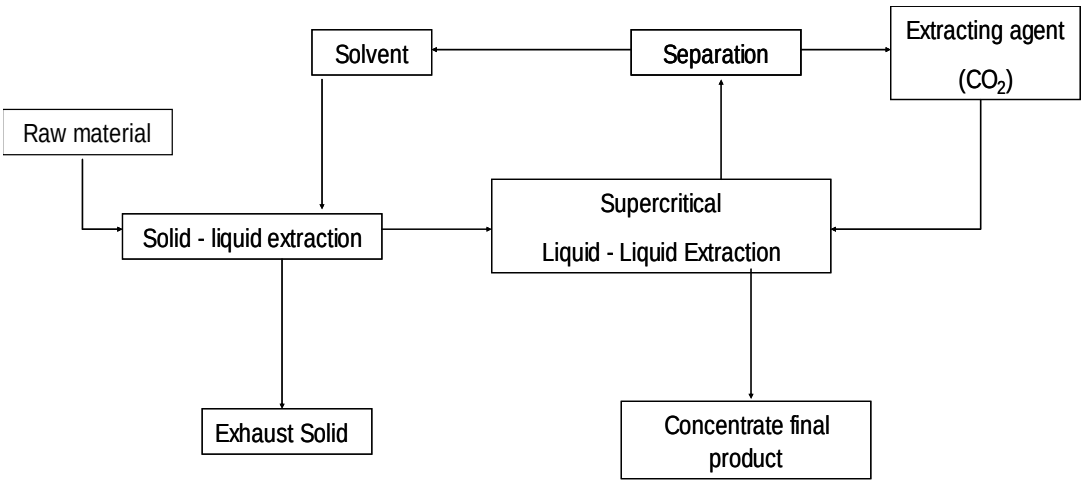
In order to obtain results which could be applied by the enterprises of the consortium, a change on the strategy of the process was required. The points of interest which could improve the performance of the process are:

- To increase by some way the quantity of antioxidant to be treated with CO_2.
- To focus efforts on the extraction of the surrounding material (solvent). Use the supercritical fluid as a purification stage.

It can be noted that the solubility of the natural compounds in the cosolvents selected for the project (Ethanol and Ethyl Acetate) is high. In fact, they are used at the present in the isolation of some of the compounds. On the other hand, the solubility of the entrainer in the supercritical fluid is very high (they can be considered as completely miscible with the CO_2). This situation will permit to use the supercritical fluid as an extracting agent for the cosolvent.

So, if the raw material is extracted by the mentioned solvents, two solutions are obtained with some content in the natural compound. Then the solvent could be removed by the CO_2 and a precipitation of the compounds will occur. The solvent can be separated by a rapid expansion in another vessel, and later, it can be reused in the extraction of the raw material. In this way, the CO_2 extraction unit would be acting as a concentrator.

The two refereed points are satisfied by this method. A block diagram of the process is presented in the figure 1:



At this moment, it is possible to think about different questions to consider:

- It could be noted that the main reasons of using supercritical fluids is that the use of organic solvents is avoided, and no persistence in the extract is observed. It seems that the advantage of using this technology is loosed.
- Which could be the advantages of the process vs traditional processes such as vacuum evaporation?

The first question should be considered from a unique point of view. Thinking on the raw materials selected for the project, as far the direct extraction with supercritical CO_2 will produce a single extract composed by waxes and non polar substances with a low commercial value, the use of a cosolvent is compulsory for the process. If a cosolvent is used, a solution composed by the cosolvent and extract is obtained in the separator. The following points should be noted when applying the traditional process:

- The content of cosolvent needed to promote the entrainer effect ranges between 3% and 15% of the supercriticial phase.
- The solubility of natural products will be very low for the selected compounds.
- The initial quantity of compounds in the raw material is very low.

So, the final product obtained in the separator will be a dilute solution of the required compound in the cosolvent and, a further purification stage would be required. The advantages observed if the process is applied in the standard way are not so clear when if a cosolvent is used (which is required if it is required a sort of solubility). It should be considered also the cost of the extraction vessel of the standard process several times higher than the vessel needed in the proposed process. Then, from this point of view, the extract obtained by the proposed would be better than the solution of cosolvent.

The second question should be considered from two points of view: the economic aspect of the process, and the technical aspect.

From a technical point of view, the new process present the possibility of adjust the solubility. The supercritical fluid allows to select the solvatation power if the operation conditions (pressure and temperature) are varied. This situation could permit to modify in some aspect part of the composition of the final product. It is expected that some aromas or other volatile compounds would be removed by this method.

From an economical point of view, further studies are required but, it can be noted that the equipment needed to process large quantities of product is more simple and smaller than the needed in solid to liquid supercritical extraction. On the other hand, a continuous operation can be obtained, which will allow reducing operating cost.

It is proposed to recycle the CO_2 and the solvent. The recover of both solvents occurs as a part of the process, and this is an important difference against the vacuum evaporation, where a refrigeration of the vapours is required.

The size of the equipment should be comparable to vacuum evaporation but the technology required should be more simple than the traditional process. The energy requirements should be quite similar.

In conclusion, the configuration of the proposed process could be a reliable way of using supercritical fluids to obtain the selected products. In addition to that, the further applications which could be derived to that technology could offer new commercial

benefits for the Consortium. For example, if the feed solution is treated to vary the molecular structure of the compound of interest to improve the solubility (for instance, use of surfactants, pH variation, selective hydrolysis, etc), a short of selection in extraction could be achieved.

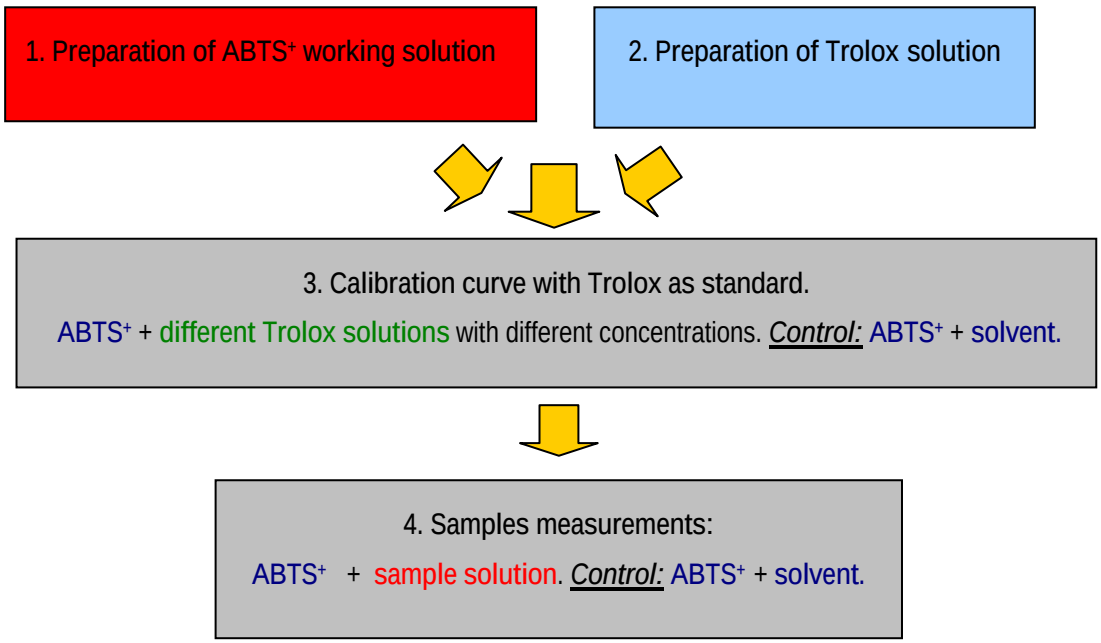
The selected method to measure the antioxidant activity of the extracts obtained by SFE and raw material samples taken from carrots was TEAC, according to the main compound (β- carotene). DPPH was eliminated as option, since DPPH and β- carotene absorb at the same wavelength range in the spectrophotometer (around 515-520 nm).

TEAC ASSAY

It is a decolourisation method applicable to both lipophilic and hydrophilic antioxidants. Therefore, we wanted to apply it, in order to know the antioxidant capacity of the beta-carotene (lipophilic antioxidant).

The addition of antioxidants reduces cation radical $ABTS^{+}$ into its colourless form. We measure this loss of absorption (colour) at 734 nm. The higher the absorbance reduction (loss of colour), the bigger the antioxidant capacity of the antioxidant analysed.

The extent of decolourisation as percentage of inhibition of $ABTS^{+}$ is determined as a function of concentration and calculated relative to the reactivity of Trolox.



- Results were reported as *mg equ. of Trolox /L solvent.*

Procedures and lab work:

First, we did a Calibration Curve with TROLOX as standard using Isopropanol or 2-propanol as solvent, which was the one used previously when we try with the sunflower oil solvent (see previous Commission report). As we saw before, TROLOX and Isopropanol were soluble. In new the calibration curve, the R2 was 0,9953 and equation: y =– 0,0005 x + 0,8566.

Problems encountered. Causes and changes proposed:

- *Problem:*

The sunflower oil showed a high antioxidant activity and the effect of both antioxidants (β-carotene and sunflower oil) were overlapping, therefore added up. It was impossible to have an accurate measure of the beta-carotene antioxidant activity. A blank with sunflower oil was used to take off the effect of the antioxidant capacity of the oil. Although, there were different unknown concentrations of oil in each sample, so an accurate measure was not feasible.

This was the reason why paraffin was used instead the old SFE co-solvent.

- *Solution:*

It was decide to use paraffin as substitute for the sunflower oil to act as catcher in the SFE process. SFE process as presented in this Project needs an oily base in order to avoid the extraction along the ethyl acetate, and at the same time, this base had to be a neutral oily base (without any antioxidant activity).

It was needed to carry out different tests to check the sample solubility (this time solved with paraffin) to try different solvents in order to apply antioxidant assay.

The solvents used were:1- Butanol, Isopropanol, EtOH , MeOH and Acetone.

Less soluble acetone, then MEOH, after EtOH, finally Isopropanol and 1- Butanol which was soluble.

The sample was only soluble in 1-butanol with the others, it was not soluble, emulsions and vesicles floating in the cuvettes were obtained. The reason was the co-solvent used in SFE: paraffin, which needs organic solvents of long chain to be solubilized).

As paraffin was used as new co-solvent and sample was only soluble with 1- Butanol, $ABTS^{+}$ solubility in 1- Butanol was tested. It was soluble in $ABTS^{+}$.

- *Problem:*

Finally, we did a calibration curve with TROLOX and 1- Butanol. The R2 were 0,9744 and 0,9810, so very bad coefficient of linear regression were obtained.

- *Cause:*

The reason was that microemulsions were formed (1-butanol and TROLOX were not completely soluble), therefore, cloudiness was produced and the spectrophotometer could not read properly).

TROLOX is a heavy and complex molecule (MW= 250,29 and C14 H18 O4) and only was soluble in solvents of small size such as MeOH, EtOH, etc., due to its size.

However, these solvents were not soluble with paraffin.

- *Results:*

TEAC method means TROLOX Equivalent Antioxidant Capacity. Therefore, this method cannot be applied because the solvents soluble with the samples were not soluble with the TROLOX.

- *Solution:*

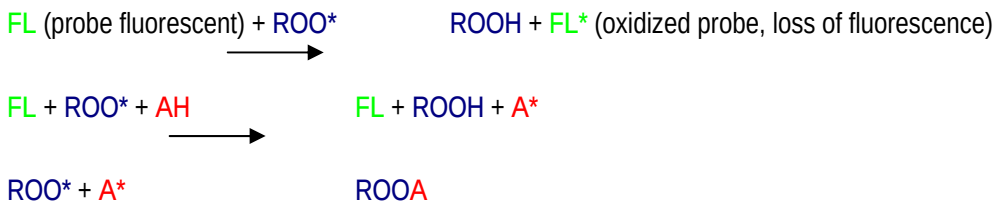
To apply other method to measure beta-carotene antioxidant activity. The ORAC method, which is widely spread with wastes of food industry and by-products of food industry and that can be used for both hydrophilic and hydrophobic antioxidants is a good candidate.

ORAC ASSAY

Introduction and definition of ORAC method:

A peroxyl radical (which induces oxidations) reacts with a fluorescent probe to form a non-fluorescent product, which can be quantified easily by fluorescence.

ORAC measures antioxidant inhibition of peroxyl radical.



Calculation of protective effects of an antioxidant (AO) is obtained from the net integrated areas under the fluorescence decay curves, [AUC AO – AUC no AO].

ORAC values are usually reported as TROLOX equivalents.

Materials and equipment:

- *Fluorescein* (3', 6'- Dihydroxy-spiro (isobenzofuran-1 (3H), 9' (9H)-xanthen)-3-one).

- *AAPH* (2, 2'- Azinobis (2-aminopropano) dihidrochloride)

- *Phosphate buffer* (pH = 7,4, 75 mM)

- *TROLOX* (as standard)

- *Spectrofluorometer*.

Advantages:

– Normally, it is used for hydrophilic antioxidants, but it can be adapted to detect both antioxidant capacity of hydrophilic and lipophilic antioxidants (Prior et al., 2003), by altering the radical source and solvent. For lipophilic antioxidants , the sample solution will be appropriately diluted with 7% of RMCD solvent (w/v) made in a 50% acetone-water mixture (v/v) and shaken for 1 h at room temperature on an orbital shaker at 400 rpm. The sample solution will be ready for analysis after further dilution with 7% RMCD solvent.

- It can be used for both food and physiological systems.

Disadvantages:

- ORAC reaction is temperature sensitive, close temperature control is needed (so incubation of the reaction buffer at 37 °C prior AAPH being dissolved is required).
- The long analysis time (1 hour more or less) has also been a major criticism.
- It is needed at least 2 mL of sample.
- It is necessary to have expensive equipment: spectrofluorometer.
- Procedures of the method are complicated.
- Fluorescein is pH sensitive, therefore fluorescence decays a lot under pH = 7. Buffer is always required.
- The protection of fluorescein by the antioxidants (AO) camouflage the actions that other biological substrates would do, so it can be some imprecision.

Protocol:

- *Sample preparation*

For liquid samples, a 20-mL aliquot of sample is centrifuged for 15 min, and the supernatant was ready for analysis after appropriate dilution with phosphate buffer (ph= 7,4). (Ou et al, 2001, Huang et al., 2002).

- *Procedures*

The ORAC-fluorescein (ORAC-FL) assay will be based on that proposed by Ou et al., 2001 and further modified by Dávalos et al., 2004, Avello and Pastene, 2005, etc.

Briefly, the reaction will be performed in 75 mM phosphate buffer (pH=7,4) and the final assay mixture (200 μL) will contain: (Ou et al., 2002)

- Fluorescein (120 μL, 70 nM final concentration) as oxidizable substrate,

- AAPH (60 μL, 12 mM final concentration) as oxygen radical generator,

- Antioxidant (20 μL, either trolox [1– 8 μM, final concentration] or sample [at different concentrations]).

The reaction will be performed at 37 ºC and fluorescence will be recorded every minute for 80 min.

A blank (control) using phosphate buffer instead of the antioxidant will be carried out in each experiment.

The fluorescein stock solution (1,17 mM) will be made in 75 mM phosphate buffer (pH= 7,4) and stored under dark conditions at 4 ºC up to 4 weeks maximum, when necessary.

AAPH and TROLOX solutions in 75 mM phosphate buffer (pH= 7,4) will be prepared daily.

When spectrofluorometer will be used the required wave lengths will be: 530 ± 20 nm for emission and 485 ± 20 nm for excitation.

All reaction mixtures will be prepared in duplicate and at least three independent runs will be performed for each sample.

Fluorescent measurements will be normalized to the curve of the blank (no antioxidant).

Data interpretation

The final ORAC values are calculated by using a regression equation between the Trolox concentration and the net area under the FL decay curve and were expressed as Trolox equivalents as micromole per litre or per gram. The area under curve (AUC) was calculated as:

$$AUC = 1 + f_1/f_0 + f_2/f_0 + f_3/f_0 + f_4/f_0 + \ldots + f_{34}/f_0 + f_{35}/f_0$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i.

The net AUC was obtained by subtracting the AUC of the blank from that of the sample. The relative ORAC value (Trolox equivalents) was calculated as:

Relative ORAC value = $[(AUC_{Sample} - AUC_{Blank})/(AUC_{Trolox} - AUC_{Blank})]$ X (Molarity of Trolox/ Molarity of Sample).

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CONCLUSIONS

1. It was not possible to measure antioxidant activity with TEAC method due to the co-solvent required to extract the carrots waste with CO2 supercritical.
2. As it is impossible to separate β-carotene from the co-solvent, a new method to measure antioxidant activity was required (ORAC method was proposed) or a way to allow the solubility of the extract in compatible solvents with TEAC method (micelle encapsulation was proposed).

Task 2.3 Definition of isolation parameters for pure functional products

As a result of the previous task 2.1, some changes in the development of the project have been proposed. Selection of two representative wastes containing polyphenols and carotene was done and the results will be extrapolated to rest of wastes. The extraction with supercritical CO_2 will be carried out on the liquid extract obtained by direct extraction of wastes with traditional GRAS solvents (Ethanol and Ethyl acetate).

So on, the task 2.3 (*definition of isolation parameter for functional products)* of the technical annex, which technical objective was to study the effect of the pre-treatment in the raw materials, will refereed to the characterisation of the extraction. The solid to liquid extraction is considered as a pretreatment stage. The selection of the extraction conditions with supercritical fluid was selected to allow the higher quantity of organic solvent under mild conditions. A new parameter was defined to characterise the extraction: the solid to liquid equilibrium relation.

The main parameters were defined, as well as the methods to be used in the determination.

Moisture

The wastes were obtained as fresh. Although it was expected to work with some dry samples, the wastes are not processed to improve the conservation due to the cost. On the other hand, the fresh samples present high water content, which could affect to the average yield of the extraction operation.

The moisture was measured for all the selected wastes using a laboratory oven at 105º C. The samples were hold in the oven at 105º C until no loss on the weigh was observed. The total moisture was obtained by the difference between weights before and after the oven.

Storage conditions

The storage of samples was highly conditioned by the moisture. If normal conditions are used for storage (20º C without light), degradation occurs. For the grape pomance, the presence of mold colonies and strong odour (due to fermentation process) appears in a few days. Carrots can be storage for a couple of weeks, but changes in the aspect of the root can be easily observed.

If the storage is done in a refrigeration chamber (4º - 8º C) the conservation time can be improved up to three weeks, but a decrease in the content of antioxidant is observed. This situation shows that if this form of conservation is considered, the raw materials should be processed during the first week.

If the storage is done with the freeze samples, long time of conservation can be obtained without important degradation in the selected compounds. The conservation time can be maintained up to six month.

Particle size

The wastes require a reduction in the average particle size to increase the contact area within the solvent. This will allow to reduce the extraction rate since the resistance to mass transfer is reduced. The operation of particle size reduction is affected by the storage conditions. The size reduction operation was different depending on the selected waste.

So, when working with grape pomance, milling with a grinder was the selected procedure. The selection of the particle size with sieves with defined pore size was done (1 mm and 0,5 mm). It was only possible to carry out the operation with frozen samples, since with fresh samples the grinder blades removed the mass only, quite similar to type of dough.

Especial attention should be taken when the milled samples get in contact with the air, since rapid degradation is observed due to oxidation. It is highly recommended to performance the extraction of samples immediately after the size reduction operation.

When it was used carrot as raw material, the initial size reduction operation was focused to obtain sliced pieces from the root. This procedure was not accepted due to the formation of paste when the grinder was used on fresh carrots. The paste was hard to handle and it was needed a final filtration stage which plugged easily. As far it was observed, since the extraction of carrots slices is low, it was decided to grind the carrots when frozen as in the case of the grape pomance.

Extraction conditions

The selection of the extraction conditions was fixed by the nature of the compounds. Since the most of them are thermolabile compounds, the higher recommended temperature is 40º C. The idea of using lower values of temperature will imply the increase of total extraction time.

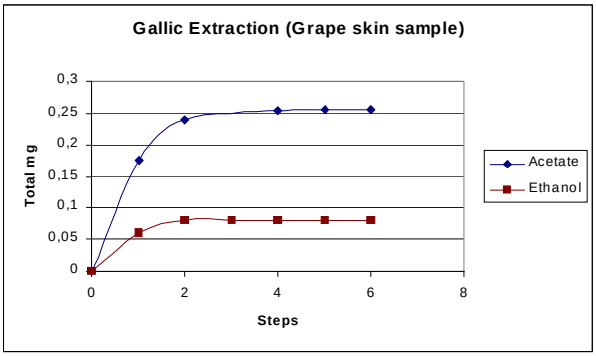
The variables to consider in the characterisation of the extraction were the effect of the particle size, the solvent and the time mainly. The characterisation of the extraction was based on the determination of the total time required to reach the equilibrium per every extraction stage and the equilibrium line between solid and liquid during the extraction.

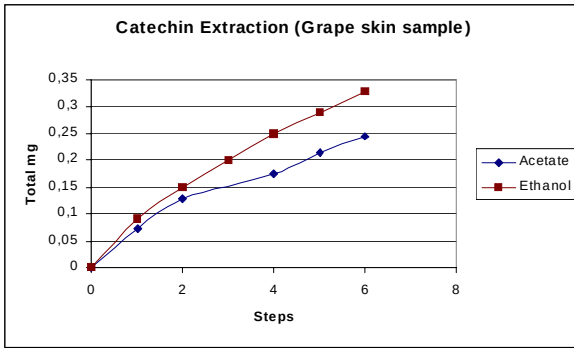
For the determination of the required time to reach equilibrium a fixed quantity of waste material was selected as solid sample. A set of flask filled with the raw material, were filled up to different values of volume of organic solvent. So, the system was left under controlled conditions (Stirring, without light and at constant temperature). At different times the content of the compounds was analysed. Once was observed that the value of concentration maintained constant along a period of time, it was accepted that the equilibrium was obtained.

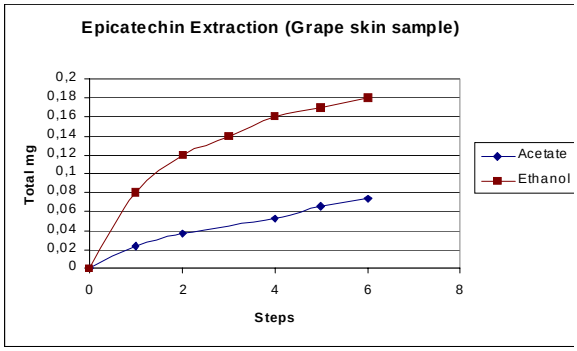
Grape pomance

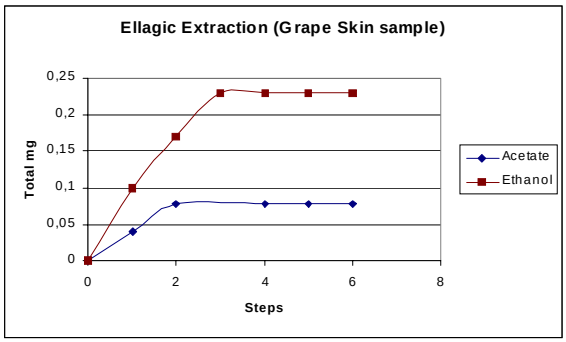
For the characterisation of the grape pomance, the extraction temperature was fixed at 40º C. The solvents used in the characterisation was ethyl acetate and ethanol. The samples were extracted with and without grinding. Different relations of sample and solvent were used at the extraction different times.

Final results showed that the total extraction of compounds of interest depends strongly on the solvent used. Next figure shows the effect of the solvent in the composition of the extract:



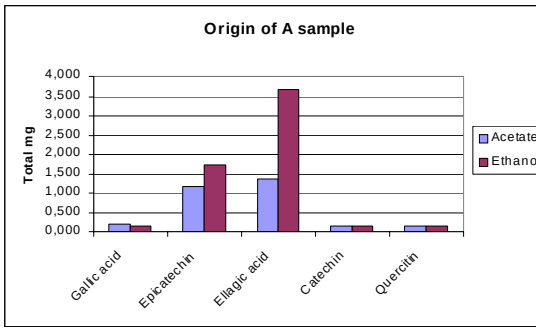


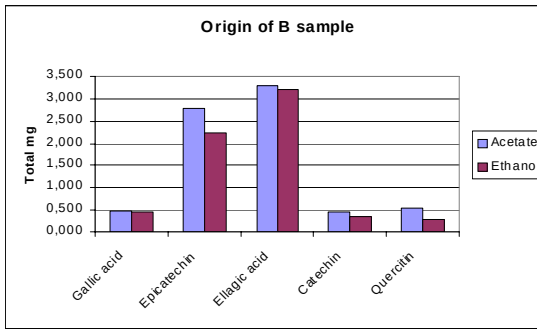




Evolution of the composition of extracts in time. Effect of the solvent.

Another effect observed is the variability in extracted compounds among different samples origins in higher when ethyl acetate is used.





Effect of the variation of origin of sample and solvent.

Finally it was concluded that the best extraction conditions to obtain a solution of grape skin pomance were:

Extraction time: 6 hours.

Required temperature: 40º C.

Required solvent: Ethanol (96%).

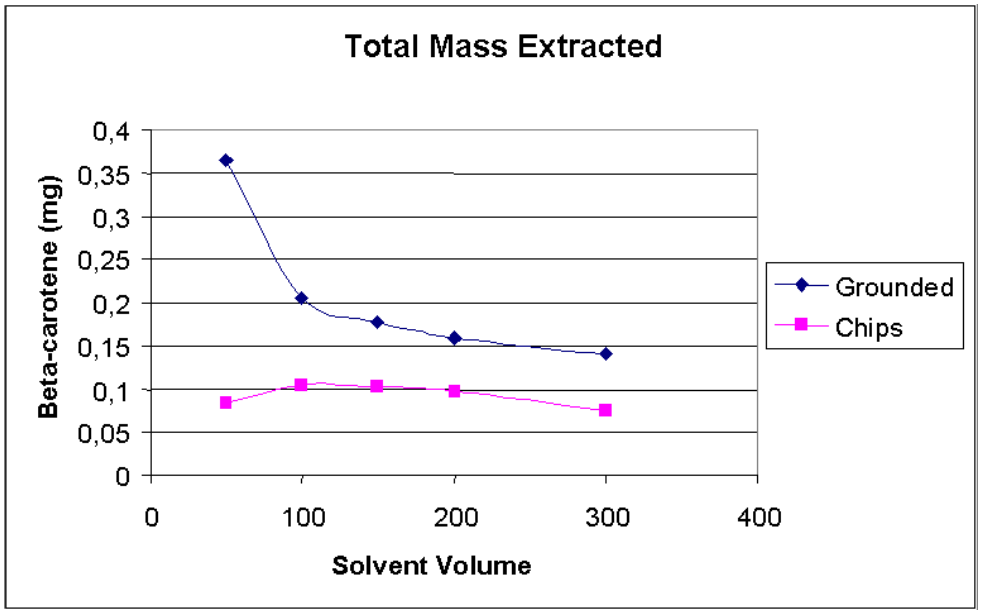
Sample/solvent load ratio: 0.15

Size reduction required.

Carrots

For the characterisation of the carrots, the extraction temperature was fixed at 40º C. The solvents used in the characterisation were restricted to ethyl acetate since the solubility on ethanol of carotenes is very low. The samples were extracted ground and sliced. Different relations of sample and solvent were used at the extraction different times.

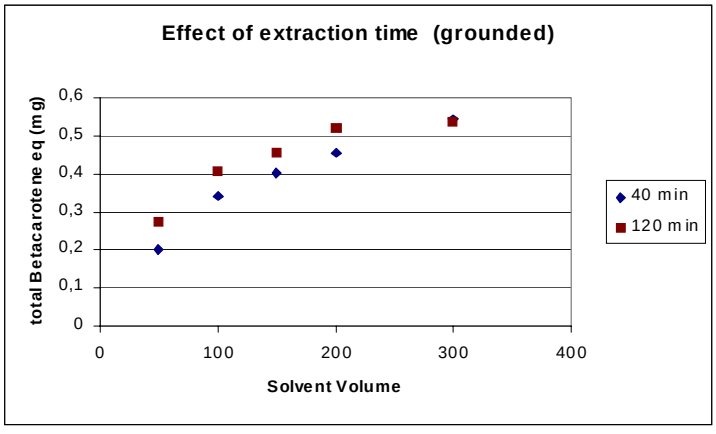
The next figure shows the effect of the shape and particle size on the extraction efficiency



Effect of the size and solvent volume in the extraction of carotene.

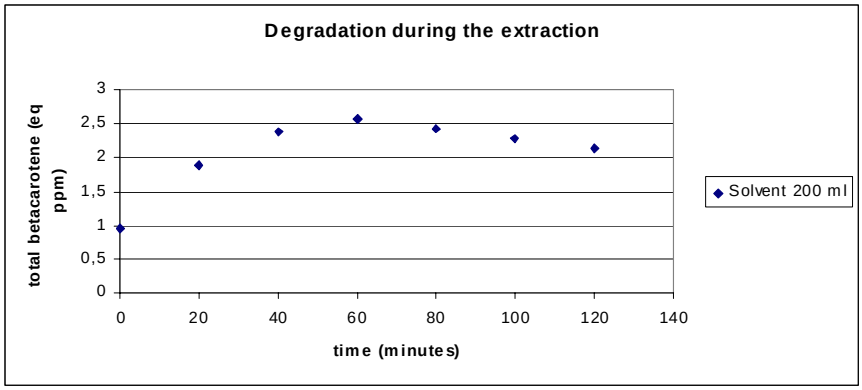
As it can be observed, the best results are obtained when carrots are grounded. As far the volume of solvent is increased the less is the effect of shaking on the extraction. These values can be very important if a low solvent to solid ratio is used.

It was determinate the effect of time on the extraction. So the folowwing figure shows the effect:



Effect of the extraction time of carotene.

As it can be appreciated in the previous figure there is a poor increment when the extraction time is increased three times. Far of optimise the extraction, the risk in the degradation of carotenes when extraction time is increased. This can be observed in next figure:



Degradation of carotene during solvent extraction.

As far the extraction time is increased, total content of extracted β-carotene decreases.

Finally it was concluded that the best extraction conditions to obtain a solution of carotene compounds were:

Extraction time: 40 minutes.

Maximum required temperature: 40º C.

Required solvent: Ethyl acetate

Sample/solvent load ratio: 0.15

Size reduction required.

Estimation of the solubility of the organic solvent in supercritical CO_2

Although the solubility of the solvents can be estimated, the real value is strongly dependent on the configuration of the system to be used. So, smaller values than the theoretical maximum can be expected. On the other hand, the presence of water (specially with ethanol) will affect the performance of the operation.

Different determinations were made to determinate the performance of the system using different ratios of CO_2 and solvent. The final obtained values were used in the definition of the design requirements of pilot plant.

It was required to modify the configuration of the supercritical fluid plant of CARTIF to reproduce the selected process. The selected configuration for the pilot plant was done in open loop.

The final selected disposition of the plant performed the following operations: pumping the CO_2 in liquid state up to required pressure conditions, then, using another pump, the solvent was pumped to the system. The total quantity of organic solvent was measured as a percentage of the value reported by the CO_2 mass flow meter. Once the streams were mixed, the system was heated up to 40º C. The stream reached the operation conditions and in this state was introduced in the extractor vessel. From the

extractor vessel it was throttled to the separator and it was vented from the separator to the atmosphere.

The system was worked in this mode during different periods of time. At the end, when the system was stopped, it was measured the following parameters: total CO_2 used, total solvent used, solvent collected in first extractor and solvent collected in the separator.

Once it was possible to performance the process, the total mass flow for the CO_2 was 6 kg/h. The total volume of organic solvent processed with the CO_2 stream was 3 % for the ethyl acetate and 2 % from the ethanol. Higher values promoted dragging of organic solution to the separator. These values are representative when working at 90 or 120 bar in the extractor.

Extraction conditions

Once it could be concluded the total quantity of organic solution to be processed by the supercritical fluid plant, different trials were done on the organic solutions to determinate the optimal supercritical extraction conditions and, finally, to use these values in the design of the new plant.

The effect of the extraction on the grape pomance solution was determined. So, samples was extracted for the conditions selected in previous point. The main conclusions obtained are related now.

When the solution is extracted, a final solution is obtained in the bottom of the extractor. Since the grape pomance was extracted with ethanol, part of the water content was mixed with solvent. Finally, the total alcoholic grade was lowered. Since the solubility of water in the CO_2 is extremely low, it was depleted with the antioxidants. In this sense, it acted as a natural liquid trap for the compounds.

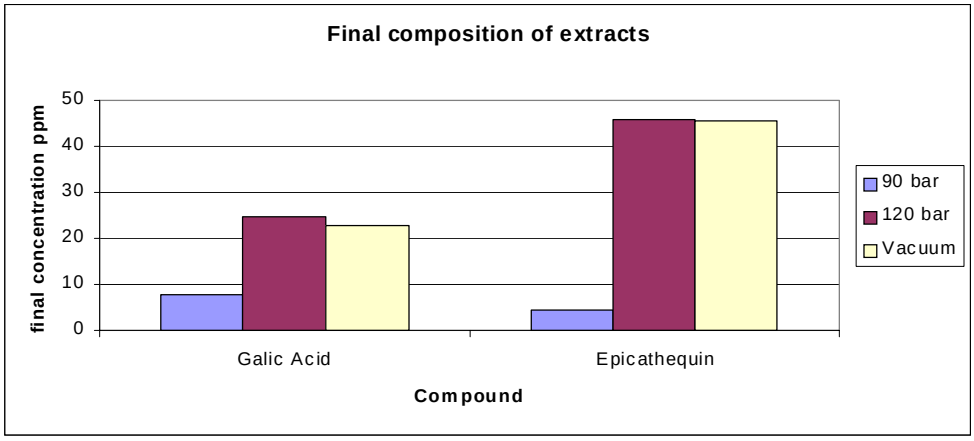
In the following table can be appreciated the effect of the variation of the extraction pressure.

Pressure	Initial	extract	sep
90	3140	6.307	N.D.
120	3140	13.561	N.D.
Vacuum	3140	10.268	N.D.

Effect of pressure on the extract

The values are expressed as antioxidant capacity of extracts, and it was measured by Folin Assay and expressed as meq of Galic acid per litre. It can be appreciated that the antioxidant value of supercritical extract is higher than the initial. The differences in final values are due to the presence of ethanol in samples for a fixed extraction time. It can be appreciated that when the extraction is carried out at 120 bar, the solution present higher antioxidant capacity than vacuum concentrated extract.

The following figure shows the obtained concentration for two key compounds in the three situations.



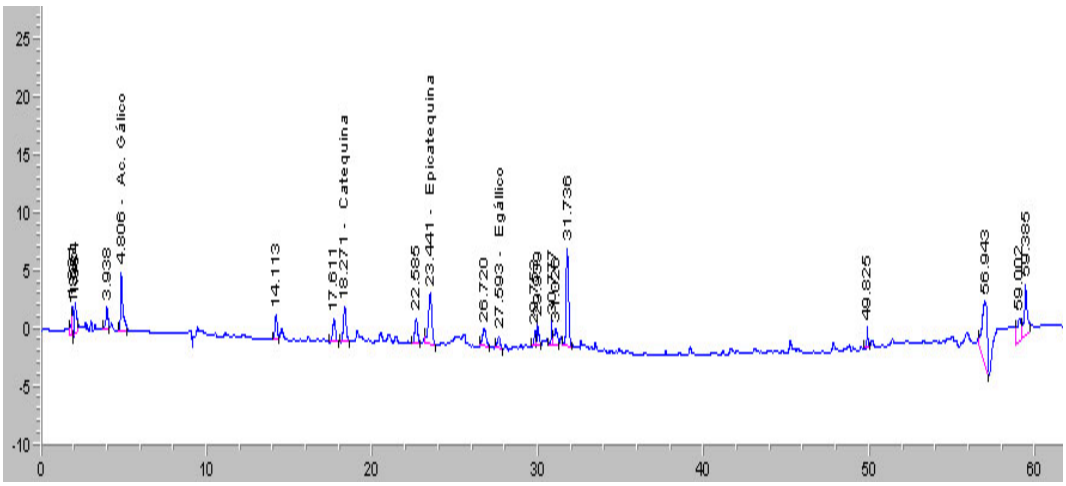
Effect of the extraction pressure on composition.

One important observed effect during the extractions was that it was impossible to recover all the solvent used. So part of the organic solvent flowed away from the separator. This situation will affect to the performance of the process when a close loop will be used since the total organic solution allowed per cycle will lower than the applied in the CARTIF plant. Next table shows this effect:

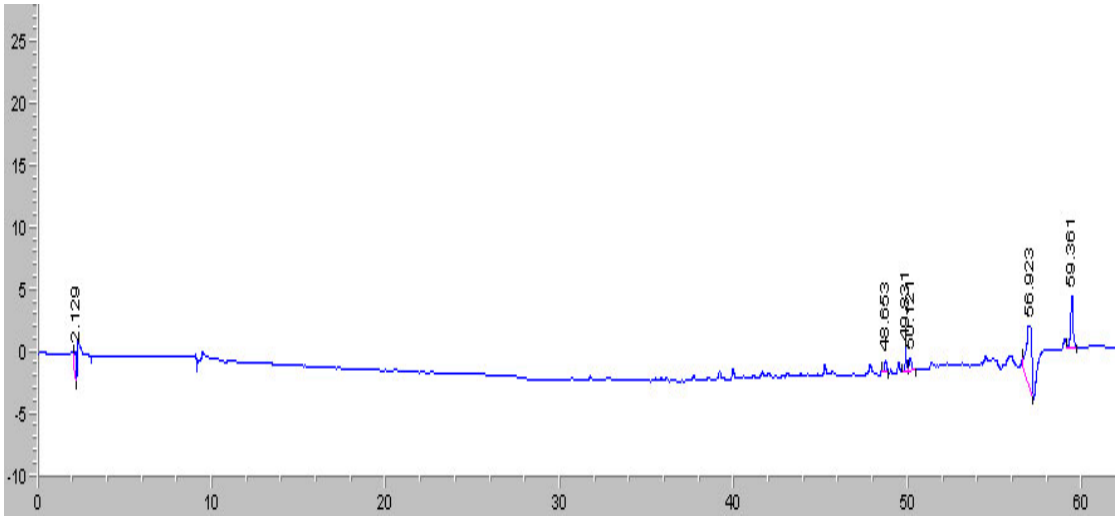
Conditions (Bar)	Solubility (ml/kg of CO2)	Recovered Solvent	Conc. Factor
90	27	60,00%	7,89
120	25	73,33%	20,69

Effect of pressure on the extract

Finally, it will be showed that once the process was adjusted to work properly, there was no presence of compounds of interest in the separator. Next images shows the chromatograms of samples from the extractor and from separator.



Chromatogram of final extracted sample by supercritical fluid.



Chromatogram of final sample of ethanol in the separator after extraction.

Finally it was concluded that the best isolation pressure conditions when working with grape pomance extract was 120 bar since the more ethanol is removed for the same time.

Concerning to the antioxidant capacity of obtained samples it could be noted that the removal of the ethanol by the CO_2 did not affect to the antioxidant composition of the sample since the EC50 value obtained by DPPH was constant before and after extraction. Total antioxidant capacity was increased since a concentration was done.

The effect of the extraction on the carrots solution was also determined. So, solutions of ethyl acetate samples were obtained for the conditions selected in previous point. The solutions were used to determinate the optimal conditions. Since temperatures higher than 40º C could promote degradation, it was fixed to this value to ensure to be in supercritical state. Temperature values under 40º C could suppose that the CO_2 would be in liquid state instead.

The first extractions showed some operational problems. The final extract was a solid and the collection from the vessel in order to quantify was very difficult. On the other

hand the green aspect of samples pointed to a degradation due to the process. Following figure shows this situation:

(A)

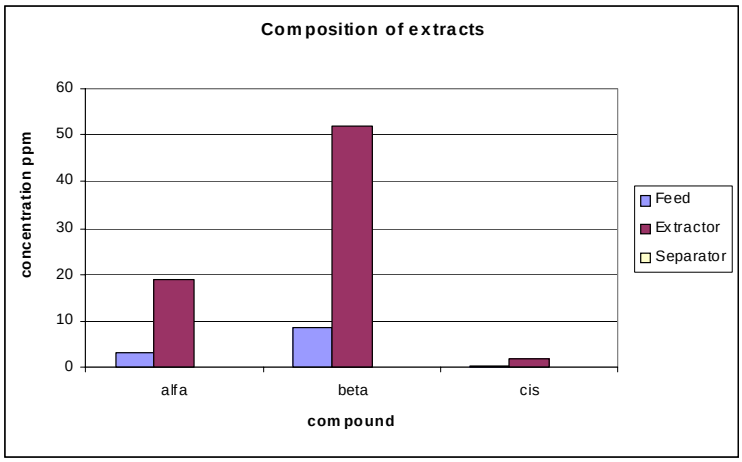
(B)

(A) carotene depleted in solid state after removal of the ethyl acetate by supercritical fluids. (B) Degradation of sample due excessive heating of system. Green aspect of the solid collected a re-suspended in ethyl acetate

This moved us to introduce some variation from the process applied for grape pomance. First, a more precise tuning of the heater was required to performance the process. On the other hand, and in order to make easier the collection of carotene, it was added sun flower oil to the organic solution before to the supercritical extraction. For the proposed extraction conditions the sunflower oil is not soluble in supercritical CO_2. So it obtained and the bottom of extractor, and due the lipophilic behaviour of carotenes, they are solved in the oil and handled in liquid state. This allowed to measure the effectiveness of the process with better precision.

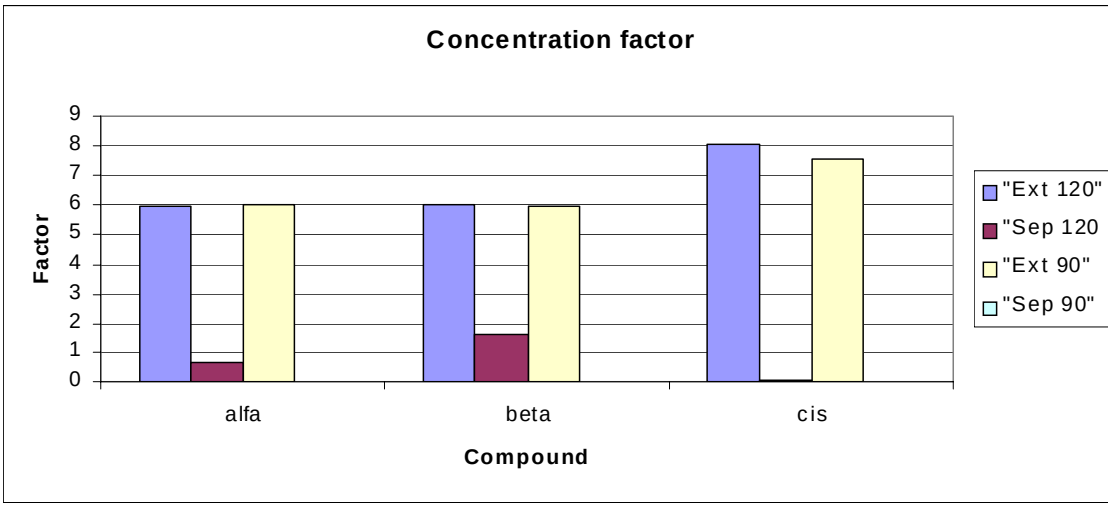
Finally the obtained results showed the following conclusions:

The ethyl acetate is selective removed by the CO_2 and no presence of carotene can be found in the separator. Next figure shows this situation:



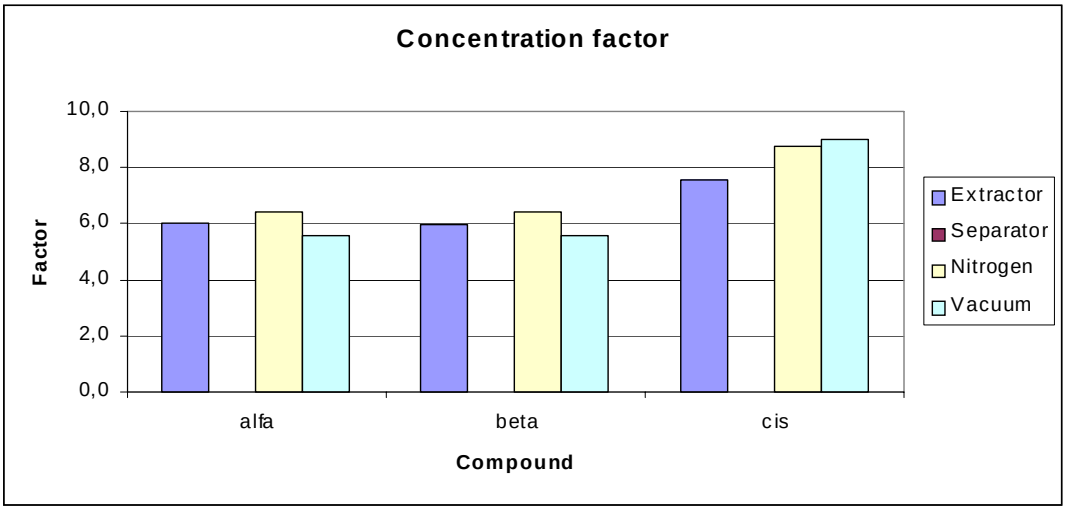
Composition of extracts obtained in SFE process

When the extraction is carried out at 120 bar, some of the carotenes can be found in the separator:



Alpha, beta and cis carotene content in the extractor and separator for extraction carried out at 90 and 120 bar.

The effect of the supercritical extraction was compared with vacuum concentration and with nitrogen evaporation, the result showed that no degradation (estimated through the cis-carotene content increase) was lower when SFE is applied:

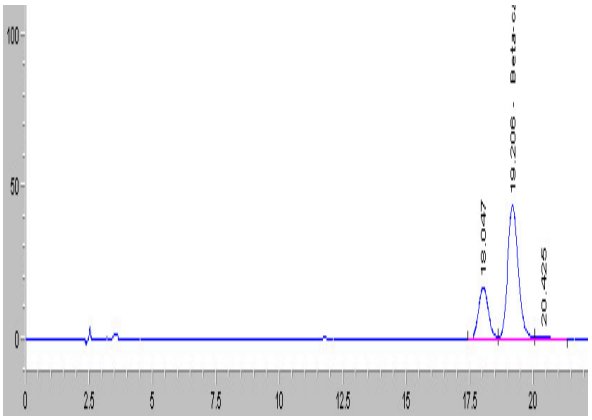


Comparative values for different operation techniques.

Due to the presence of the sun flower oil, it was not possible to apply the proposed antioxidant assays. This was due to the antioxidant capacity of sunflower oil which overlapped the effect of carotenes and to the low solubility of oil in the assay solvents. It was decided to make a variation in the TAEC assay by using isopropanol instead of ethanol to improve the solubility of samples during the assay. As far the results can not be considered, due to the variation promoted by the oil in the reference, the tendency in all the situation was that the SFE extract presented better antioxidant capacity.

It is expected to solve this problem in the new plant since it will not be used oil as a fixing agent. A solid it is expected to be obtained at the end of the process.

By the moment it was measured the quantity of carotene by HPLC. Next figure shows the aspect of one of the chromatograms obtained for SFE extracts:



HPLC of ethyl acetate solution extracted by CO_2. The observed peaks corresponds to alpha-carotene and beta-carotene

The final supercritical extraction conditions should be 90 bar and 40 degrees according to the results of the trials.

It can be concluded that the definition of the isolation parameters of the task 2.3 mainly done.

Actual activities are focused to the characterisation of the stability of the samples obtained by supercritical methods. The first results shows, as it was expected that:

Direct exposition to the light should be avoided.

Direct exposition to the air will degrade the compound in time.

Temperatures higher than 40º C degrades very fast the compounds when they are in solution, and freeze of samples is strongly recommended.

The water can be used as a carrier of the polyphenols. A dewatering operation will produce a solid which could be more stable in time, but this operation could suppose a degradation during the evaporation.

On the other hand, the obtained samples holds their activity fairly well if they are storage in a cold and dark ambient. These trials will be carried out properly on the obtained extract of the pilot plant.

Toxic and Antioxidant Evaluation of the Extracts

As a continuation of this task, and given the delays registered in Workpackage 3 and 4, a task firstly assigned to workpackage 6, Analysis of anti-oxidant activity and Toxicity, was started in this wp2.

Cytotoxicity and antioxidant activity both *in vitro* and *in vivo***,** of the following extracts obtained in the coordinator facilities were analyzed:

1) Ext CO_2-SFE Ethanol obtained from Grape waste soaked in ethanol and extracted with supercritical fluid plant (GSE = Grape Supercritical Extract).
2) Ext Co_2 SFE obtained from Carrots, additioned with 75ml of sunflower oil and finally extracted with the supercritical fluid plant (CAR).

Materials and methods

Cell culture

Rat hepatoma cell line (MH1C1) was used. It was obtained by the Istituto Zooprofilattico (Brescia, Italy) and grown in nutrient mixture Ham's F-10 medium supplemented with 20 % foetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin.

Cytotoxicity assay

In order to find the concentration of grape and carrots extracts not able to induce cytotoxicity, cell viability was determined by the MTT colorimetric assay [1], measuring the mitochondrial function using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT). Briefly, cells were incubated in 24-well tissue culture plates for 24 h with grape extracts at a concentration of 100, 50, 10, 5, 1, 0,5 ml/l and then 0.4 mg/ml (0.9 mM) of MTT dissolved in PBS was added to the medium in each well, and incubated for 2 h at 37°C. The medium was then removed, the formed blue formazan dissolved in 1 ml of DMSO, and quantified by absorption measurements at 570 nm using a microtiter plate reader (Biorad-Mod. 550). Cell viability was calculated measuring the difference in optical density of treated samples with respect to control cells.

Moreover, cytotoxicity was assessed also with the trypan blue exclusion test that evaluates cell membrane damage.

Antioxidant effect

The antioxidant activity of grape extracts was evaluated *in vitro* by the DPPH method [2] and the citronellal thermo-oxidation inhibition test. In addition, antioxidant activity was assessed in cells by measuring lipid peroxidation inhibition, redox state alterations and cellular ROS level.

The cells were incubated for 2 h with the grape extract at the concentration of 10 ml/l or 1 ml/l and then treated for 1 h with 200 μM/1 mM Fe^{2+}/ascorbate to induce oxidative damage.

DPPH reduction method

Grape extract (50 μl) was added to 2950 μl of a 0.1 mM DPPH solution in methanol. The exact initial DPPH concentration in the reaction medium was calculated from a calibration curve. The decrease in absorbance was determined at 515 nm at 0 min, at 30 sec, every 1 min for 15 min, and every 2 min until the reaction reached a plateau (about 30 min). Antiradical activity was expressed as the EC_{50}, *i.e.* the antioxidant concentration necessary to decrease the initial amount of DPPH by 50 %.

Lipid peroxidation assay

After treatment, lipid peroxidation was measured by a fluorometric method for the determination of hexanal using 1,3-cyclohexanedione reagent and HPLC (HP1090 Hewelett-Packard Walbronn, Germany) according to Yoshino *et al.* and Cabré *et al.*

Cellular redox state determination (GSH/GSSG)

The activity of grape extracts in altering the cellular redox state was measured calculating the reduced glutathione to oxidized glutathione ratio (GSH/GSSG).

The cellular contents of GSH and GSSG were measured using the Cayman Chemicals (Ann Arbor, USA) enzymatic recycling/spectrophotometric assay kit. Spectrophotometric readings were carried out in a Biorad Mod. 550 microplate reader. The dermination of GSSG was obtained derivatizing GSH with 2-vinylpyridine.

Cellular redox status 2'7'-dichlorofluoroscein (DCF) assay

Cellular ROS level was quantified by the 2,7-dichlorofluorescein (DCF) assay. After the treatment, cells were incubated for 10 min at 37°C with 50 μM dichlorofluorescein-diacetate, then washed, harvested with a policeman and resuspended in cold physiological solution. Cells were analysed with a Coulter Epics XL (Coulter) flow cytometer.

Antiproliferative effect

Clonogenic assay

Cells were treated for 24 h with the grape extract at the concentration of 10 ml/l or 1 ml/l, then washed twice with PBS and fresh medium was added. After 10-15 days, the colonies were stained with crystal violet and counted. The clonogenic efficiency was calculated as the mean percentage with respect to control cells.

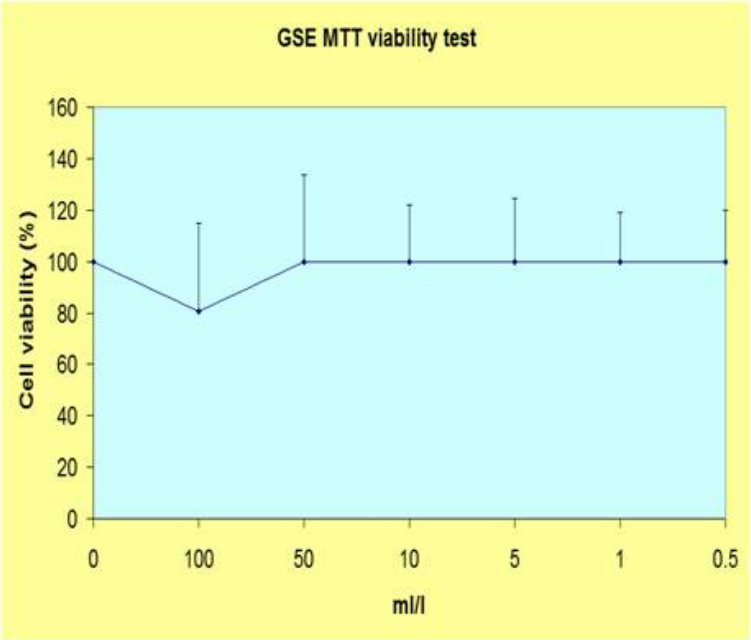
RESULTS

Grape waste supercritical fluid extract (GSE)

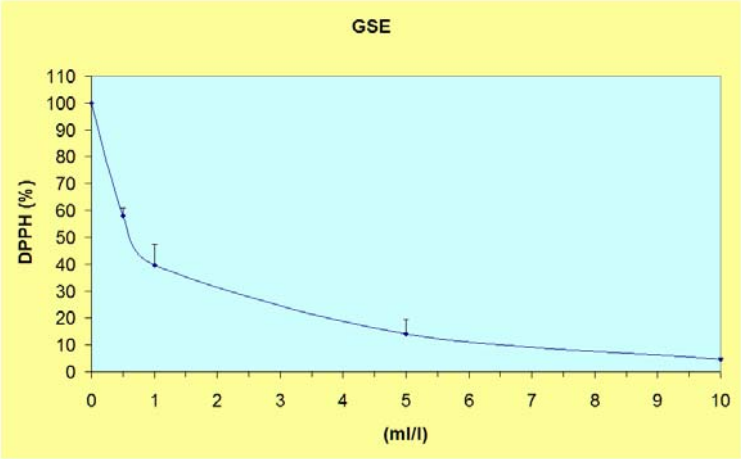
Effect of grape extract on cell viability

Preliminary experiments were carried out to determine the cytotoxic effect of the grape extract on MH1C1 cells by the MTT test. The grape extract induced a weak cytotoxicity only at the highest concentration (100 ml/l) with a cellular survival of 80 % (Fig. 1).

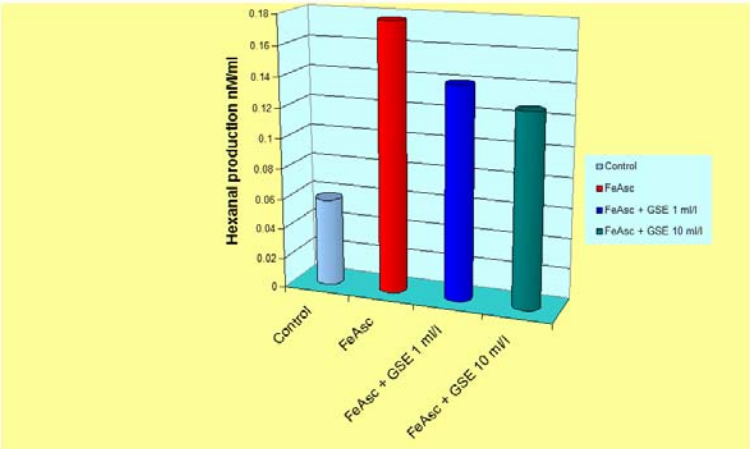
Superimposible results were obtainded with the trypan blue exclusion assay (data not shown).



Antioxidant activity of the grape extract

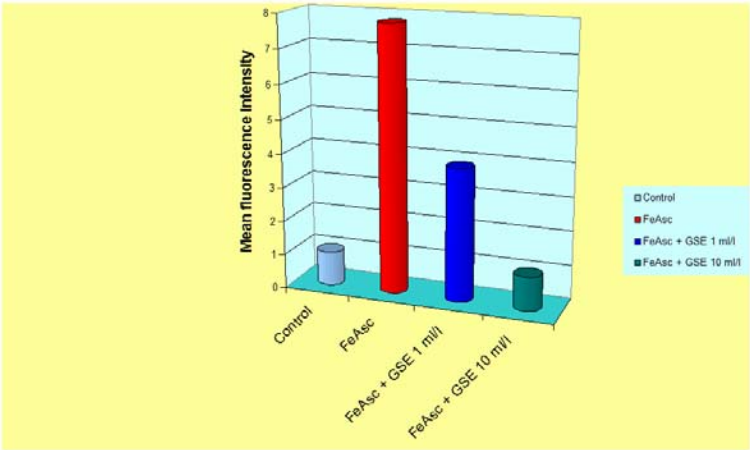


Reports the dose-dependent anti-radical activity of GSE. Antiradical activity, expressed as the antioxidant concentration necessary to decrease the initial amount of DPPH by 50% (EC50.) is 0.65 ml/l.



Previous figure shows the effects of grape extract on hexanal production induced by Fe^{2+}/ascorbate in MH1C1. Incubation of the cells for 1 h in the presence of 200 μM/1mM Fe^{2+}/ascorbate significantly increased membrane lipid peroxidation, raising the hexanal production to 0.177 nM/ml from the level of 0.058 nM/ml measured in untreated control samples. Grape extract inhibited the hexanal production in a dose-dependent manner by about 20% and 30% at 1 and 10 ml/l concentration respectively.

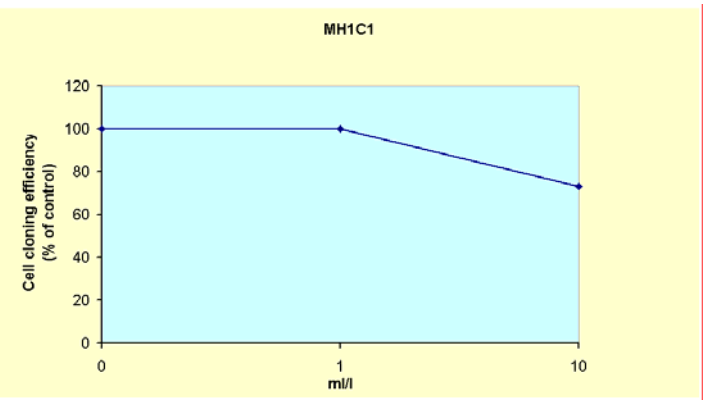
The grape extract reduced Fe^{2+}/ascorbate-induced ROS production in a dose-dependent manner (Fig. 5). In particular the highest concentration (10 ml/l) reported the ROS to the level of control cells.



Since the oxidative damage generally involves the cellular GSH system, we measured GSH/GSSG cell content after redox challenge with FeAsc, ± presence of GSE. However, preliminary results using the MH1C1–Fe_2-ascorbate model system do not indicate a significant activity of grape extract on thiols cellular redox state. Further experiments are foreseen with a more adequate model system to evaluate extract effects on the GSH/GSSG ratio.

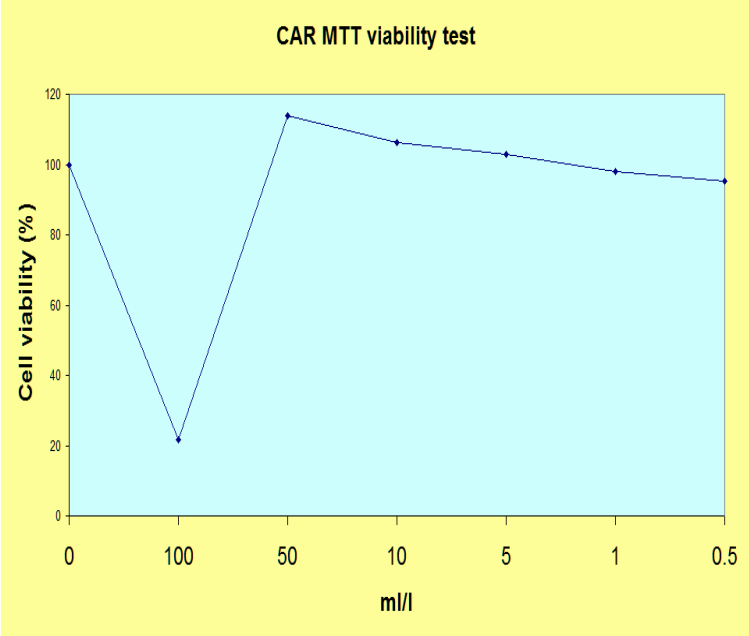
Effect of grape extract on clonogenic efficiency

Figure 21 shows that grape extract induced a reduction in clonogenic efficiency. At the highest dosage this reduction was about 30%. This indicates that GSE shows also an antiproliferative effect.



Carrot supercritical fluid extract (CAR)

As far as carrot extract is concerned, it is to point out that the first sample received for Partner 9 were dissolved in sunflower oil after SFE, due to technical reasons. This was not an issue for toxicity assessment.



CAR extract seems to be non-toxic at concentrations below 50 ml/l). Nevertheless, sunflower oil presence impaired measurement of anti lipid peroxidation and anti radical activities since it is by itself a powerful antioxidant containing relevant amounts of vitamin E.

CONCLUSION

On the whole our results demonstrate that **GRAPE WASTE SUPERCRITICAL FLUID EXTRACT** *(*GSE) is non-toxic and shows an antioxidant effect both *in vitro*

and in a cellular system, in contrast an improvement of the extraction procedure is necessary to evaluate the antioxidant activity of **CARROT SUPERCRITICAL FLUID EXTRACT (CAR).**

Task 2.4 Characterisation of extraction properties and stability

As it was told before, the selected compounds of the project could be very useful in the food, cosmetic and pharmaceutical industries as they can be used as substitutes for synthetic colorants and antioxidants. One of the possibilities is the use those compound as a colorant. Natural plant colorants strongly demanded by the food industry to replace synthetic dyes. However, replacing synthetic dyes is a challenging task because they tend to show greater stability with respect to light, oxygen, temperature and pH, among other factors.

Although selected compounds have a high potential for use as natural additive or colorants due to their colours and innocuousness, they could present stability problems.

The colour and stability of products should be dependent on several factors, such as:

- Time
- Temperature storage
- PH storage.

and other factors such as the presence of copigments, metallic ions, enzymes, etc. From the point of view of the project, the previous points are the most relevant. When using the proposed extraction method, the extraction procedure is some how selective and a number of other plant constituents should not be co-extracted along with the desired compounds. So, the expected degradation due to those products (for instance sugars) will not occur.

Any way, and due to the nature of the compounds, a type of degradation could be obtained. Based on that, it was necessary to study the characteristics, stability and antioxidant capacity of extracts. The next points show the developed test to be applied on the products obtained by the pilot plant. These tests have been defined and are still under development since the pilot plant has not been constructed, but they have been carried out using samples obtained in the existing pilot plant at the coordinator facilities.

The results of the trials will afford enough information to estimate the stability of the products. Some very positive results has been obtained using the samples obtained at laboratory scales but they are not reported since further confirmation is required with new samples.

The previously named parameters were the ones used during this stage. (Time, Temperature storage and PH storage)

Other effects such as water content were not considered since the final extract consists mainly in a solution of water and polyphenols. In the case of β-carotene extracts, it is required to define a new antioxidant measurement method. The lipophilic behaviour of extracts result in a bad mixing of different reagents used in TEAC and DPPH. In this sense, the stability of these samples will be checked by a new method (ORAC) which is at the present under development by the Laboratory.

The stability test has been developed on grape extracts obtained by the new process proposed along this Project.

Definition of trials

The first approximation to define the stability test was bibliographic revision on other stability test carried out on similar materials. The idea was to detect any parameter to be used to follow the evolution of the product with the time. It was found that the main parameter to follow the evolution for this kind of extract was the colour.

The colour of extract will depend mainly on the extracted polyphenols, any change in it can be related with a type of degradation of the sample.

In order to get an estimation of this evolution it was decided to measure the absorption of samples with a spectrophotometer in the visible range. The measurements were carried out during different days. The values of the maximum absorption peaks of the spectrum were used as the main parameter.

In order to consider the effect of ambient in sample, It was decided to use five different pH values and two different temperatures. The idea of using different pH values is to consider the possible effect if the extract is used in different mediums or formulations.

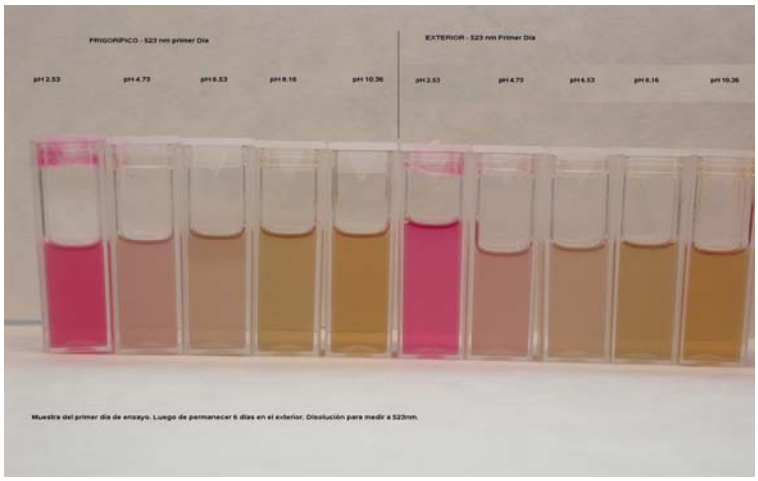
So the selected conditions values for the grape extract were:

- Storage temperature: ambient and 4º C.
- pH condition: 2.5; 4.5; 6.5; 8; 10.

The samples of extract were stored in separated flask and direct light contact was avoided. Different aliquots were taken at any selected period of time to make the proper dilutions to measure the absorption.

Results :

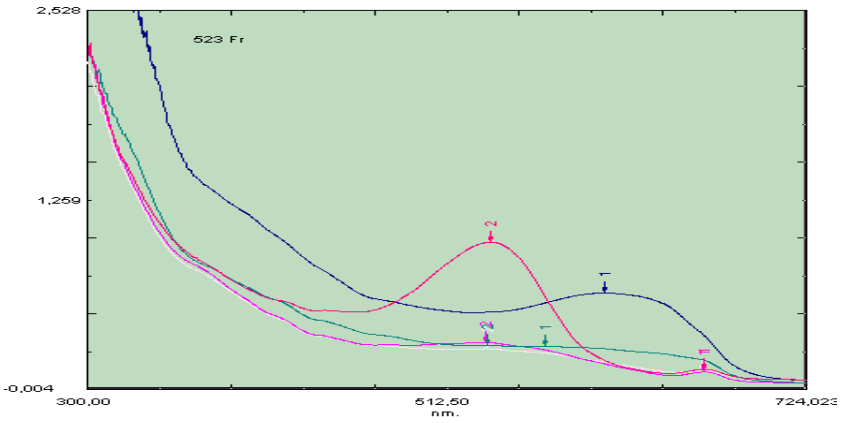
The first effect observed is that the product (grape extract) changes the colour when the pH is varied in the proposed range. The next figure shows this effect.



Effect of pH on solution.

It is clear that this effect should be considered when the extract is applied as a colorant for different products. From the point of view of stability it can be reported the effect of pH on the structure of polyphenols.

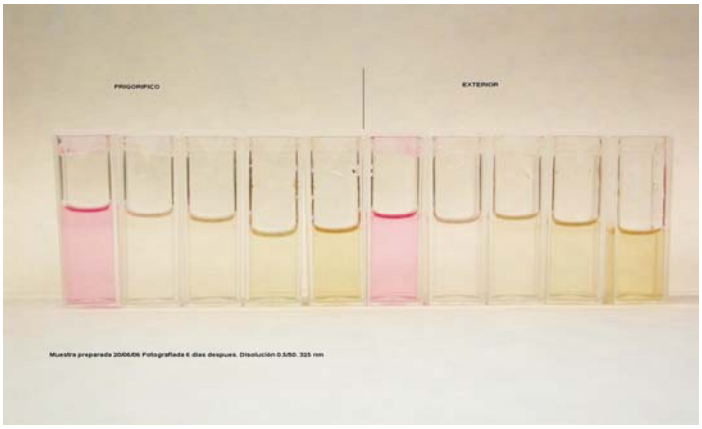
This effect can be observed in the spectrophotometer. The next figure shows the different visible spectrum for each solution:



Effect of pH on solution (Visible spectrum). Red line corresponds to pH 2.47; magenta line corresponds to pH 4.43; grey line corresponds to pH 6.45; Green line corresponds to pH 8.47; Blue line corresponds to pH 10.

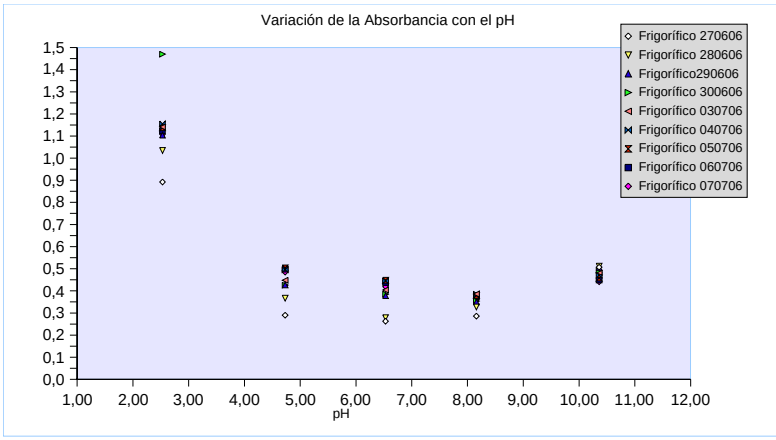
The observation of the visible spectrum shows that there is displacement of peak as far the pH is increased. This will be used as a parameter to estimate the evolution of the samples. It can be observed also that there is an increment in the absorption band between 300 and 400 nm as far the pH is increased.

In all samples it was observed the presence of a solid precipitation with the increase of time. This situation is related with the ability of polyphenols to form aggregates, which present a low solubility in aqueous solution. This situation promotes a decrease in the content of free polyphenols with the time. The following figure shows the same solutions that were used in the previously after the trials. The loss in the intensity of the colour can be appreciated.

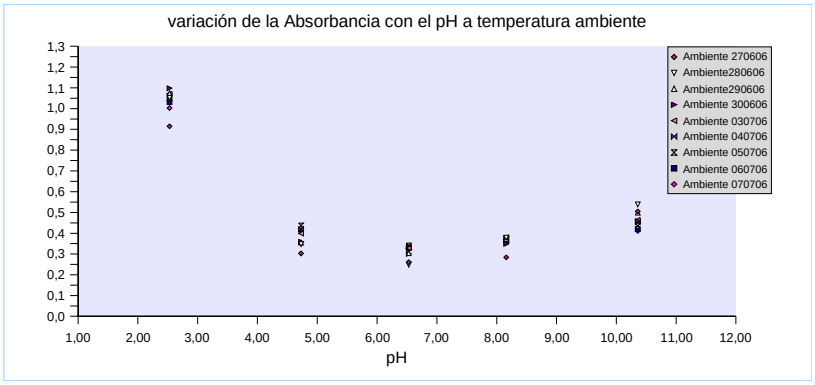


Effect of pH on solution after 15 days (Visible spectrum). Samples present a loss in the intensity of the colour.

The effect of the time and temperature can be derived from the following graphs. It is shown the evolution of absorbance at different pH with the time. The measurement was taken at two different wavelengths: 325 and 523 nm

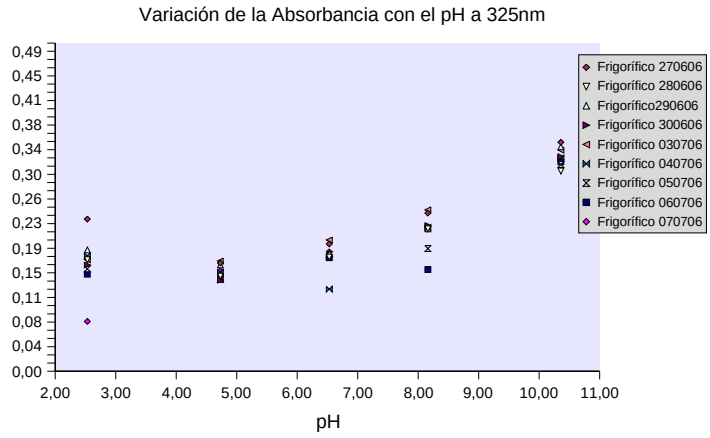


(a)

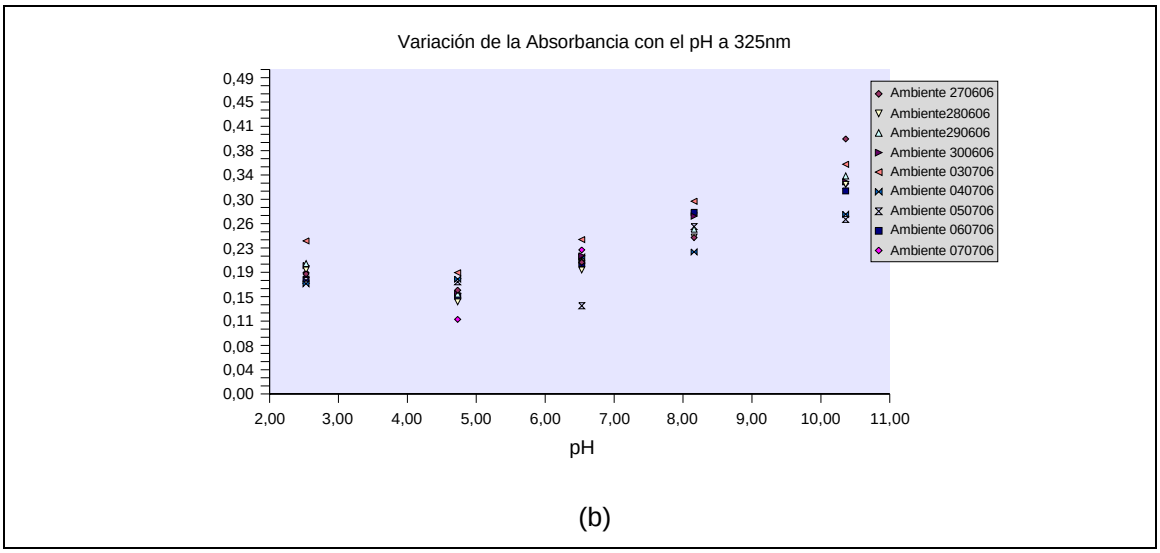


(b)

Evolution of absorbance at 523 nm with time at different pH values and at different temperature storage conditions



(a)



(b)

Evolution of absorbance at 325 nm with time at different pH values and at different temperature storage conditions

As a conclusion it can be reported that a type of instability was observed in the grape extract. Slight differences can be noted when the storage is performed at low temperatures (4º C) derived from the loss in the solubility. Changes in the colour can be obtained adjusting the pH.

Deviations from the project workprogramme

As explained before, this second workpackage has suffered several changes from what was envisaged in the workprogramme. It was firstly developed as stated in Annex I, but while the work was being carried out, it seemed clear that some technical changes were mandatory (see General Description of the process), and that these changes would affect the workplan.

The first Task was developed as stated in the original workplan and its conclusions, after crossing the choice of the interest compounds present in the raw materials and the possibilities offered by the Supercritical Fluid Extraction, pointed to the necessity to use a previous solid-liquid extraction with an appropiate organic solvent.

These findings caused that Task 2.2 had to be elongated in time to make sure that the new process was feasible technically, and to gather enough knowledge to design the pilot plant efficiently.

In order to include this new stage prior to the SFE, the tasks devoted to the design of the pilot plant were delayed several months. The action agreed to compensate this delay was to enlarge the laboratory tests in the SFE pilot plant at the coordinator facilities, before the avilability of the pilot plant. With this action, it was intended to shorten the tasks programmed after the workpackage devoted to the design and the construction of the pilot plant, achieving thus the final objectives at the same time as initially programmed.

The studies over toxicity and anti-oxidant capacity of the extracts from Cartif's plant were easily extrapolated to those obtained through the Extranat´s pilot plant, where the studies were firstly assigned.

All these changes only affected the RTD performers, Cartif and Univpv, since they were the respoinsable for WP2 and WP6.

Another effect of the technical changes affected greatly also to the content of Task 2.3, since the pretreatments became a part of the process, instead of as a previous stage.

List of Deliverables and Milestones

Only Milestone 1: Characterisation of Fruit Extraction Parameters was assigned to the development of this Workpackage.

Milestone no.	Milestone Name	WP no.	Date due	Actual delivery date	Lead contractor
1	Characterisation of Fruit Extraction Parameters	2	Month 9 (Dec'05)	Month 12 (March'05)	CARTIF

No Deliverable was programmed for this Workpackage.

3.- Workpackage 3: Design of the pilot plant.

This workpackage had a clear objective, to set up all design features and legal requirements for the manufacturing process to be implemented at pilot scale.

The partners involved in this workpackage are:

- Copaisan and Matarromera: only involved in the first task, devoted to the selection of methods to be used on raw materials prior to the extraction process.
- Enviplan: leader of the Workpackage and involved in every task.
- Gradiens: This SME is involved in the first tasks of the Workpackage, from the selection of the pre-treatment to the construction of the plant.
- Cartif: As RTD and responsible of Workpackage 2, from where this one starts, has an important role to play.

The workplan establishes that the tasks of design, and construction of the pilot plant has to be carried out at the same time as those comprised in the previous workpackage about the laboratory tests and the characterisation of the process. This workplan did not foresee the necessity to improve the SFE technique in order to achieve a successful process, but, as reported previously, a solid-liquid extraction prior to the SFE and a new technical point of view were found as the best choice to extract the interest compounds.

Thus the starting point of this workpackage was foreseen to be directly the information gathered through WP1, but finally this starting point consisted in the general layout of the process defined in Task 2.2: Laboratory assays.

The technical progress will be showed sorted by the active subtasks.

Task 3.1: Selection of pre-treatment methods and definition of the conditions of raw materials

The technical objective of this task was to define the pre-treatment operations to carry out on the raw material in order to optimise the performance of the operation. Since the overall definition of the process has changed due to the low solubility of compounds in supercritical CO_2, a liquid to liquid extraction with GRAS solvents is proposed as pre-treatment method.

As it was refereed in previous reports, the direct use of supercritical CO_2 on solid material (in solid to liquid extraction stage) yields poor results even if an entrainer such as ethanol is used.

So the pre-treatment selected to proceed with the project had to extract the raw material with two organic solvents depending on the selected compounds to be extracted. To achieve the best results it was needed to grind the raw material to offer a higher surface contact, so the extraction times were reduced.

The method to obtain the solutions to be introduced in the SFE plant are described below for both selected raw materials: grape pomance and carrots.

Grape pomance extraction.

As it was determined in the development of the task 2.3, the extraction parameters for the grape pomance can be summarised in the following points:

Extraction time: 6 hours.

Required temperature: 40º C.

Required solvent: Ethanol (96%).

Sample/solvent load ratio: 0.15

Size reduction required.

The procedure to performance the solid to liquid extraction was defined in the following steps:

Storage of original sample in frozen state at -18º C or lower.

Grinding in frozen state. Final average particle size: 1 mm diameter or lower.

Total mass weight per load: 1 kg.

Total Ethanol: 1.40 kg

Number of ethanol cycles: 8

Total extraction time: 6 hours

The extraction is carried out in an separate vessel. The proposed extraction method is in direct contact between phases. Ethanol is pumped in the system at the ratio required by the CO_2 stream. Recovered ethanol on the separator is reused on the solid to liquid extraction.

Carrots extraction.

As it was determined in the development of the task 2.3, the extraction parameters for the carrots can be carried out taking into account the next parameters:

Extraction time: 40 minutes.

Maximum required temperature: 40º C.

Required solvent: Ethanol (96%).

Sample/solvent load ratio: 0.15

Size reduction required.

The procedure to performance the solid to liquid extraction was defined in the following steps:

Storage of original sample in frozen state at -18º C or lower.

Grinding in frozen state. Final average particle size: 1 mm diameter or lower.

Total mass weight per load: 1 kg.

Total Ethanol: 1.40 kg

Number of ethanol cycles: 8

Total extraction time: 6 hours

The extraction is carried out in an separate vessel and the ethanol is pumped in the system.

Task 3.2: Supercritical fluid plant design

The technical objective of the task was to develop the piping and instrumentation (P&I) diagram of the supercritical extraction process as well as the selection of the equipment and control strategy of the pilot plant. The main design features of the pilot plant and its expected uses during the project are described.

As it was reported in the previous workpackage, the direct use of supercritical CO_2 in the solid-liquid extraction of natural products of interest from vegetables/ fruit raw materials (e.g polyphenols), provide low yields due to the solubility limitations of the polyphenols in $scCO_2$. The use of a co-solvent such ethanol or ethyl acetate is required to enhance the solubility of the antioxidant compounds, this complicates the separation and solvent recovery steps as well as involving an considerable increment of facilities costs.

An alternative approach for the supercritical fluid process was proposed, which consists basically in performing a selective extraction of a solution obtained from the extraction of raw material with a GRAS solvent. The CO_2 removes the solvent and some non polar substances and a concentrate active principle is obtained. Under certain temperature-pressure conditions (above the critical point of the mixture), the solvent is totally soluble with the CO_2; nevertheless, by regulating the pressure-temperature conditions, the system can split into a binary mixture in equilibrium. On one side, is obtained a liquid phase highly enriched with the compounds of interest, and in the other, a supercritical phase composed of CO_2 with the solvent which is removed.

In order to improve the performance of the process when working with an acetate feed, a low volatile solvent can be added along with (for instance a vegetable or paraffin oil). This will promote a ‘trap’ effect which will hold-up the antioxidant compounds in the liquid phase.

Therefore, the process of separation of extracted compounds can be achieved by means of two separation steps:

1.- The extract solution (oil/ethyl acetate or ethanol/water) is pumped and compressed to the operating pressure and subsequently mixed with the $scCO_2$ stream before entering the extractor. Then, the compounds of interest (mainly antioxidants) are majority retained in the extractor (liquid phase), at the same time the $scCO_2$ and the solvent flow out from the vessel and goes into the separator vessel prior a controlled expansion stage.

2.- The second separation step involves a controlled expansion of the mixture CO_2-solvent (ethanol or acetate) that permits the separation of the co-solvent from the CO_2. Two phases are obtained, a liquid phase (along with the soluble part of antioxidants or off-flavours) and the CO_2 in the vapour phase, ready for the process feedback.

3.- The CO_2 is recycled to the system again to perform a new extraction cycle. The solvent is re-used to extract more raw material.

In addition, the separation process will be accomplished in two steps for a better control of the P-T conditions so that the expansion process can be approximated an iso-enthalpic process.

Moreover, the expansion system is often a critical/limiting stage of the continuous operation in pilot plant. The physical behaviour of the system forces a limit in the use of the expansion valve. Thus, when the pressure in the outlet of a valve decreases to half of the inlet pressure, the gas leaves the orifice at the speed of sound. The gas cannot exceed the velocity of sound and, therefore, maximum flow rate is limited by this condition. This situation is known as choked flow. Any further decrease in outlet pressure does not increase flow, even if the outlet pressure is reduced to zero.

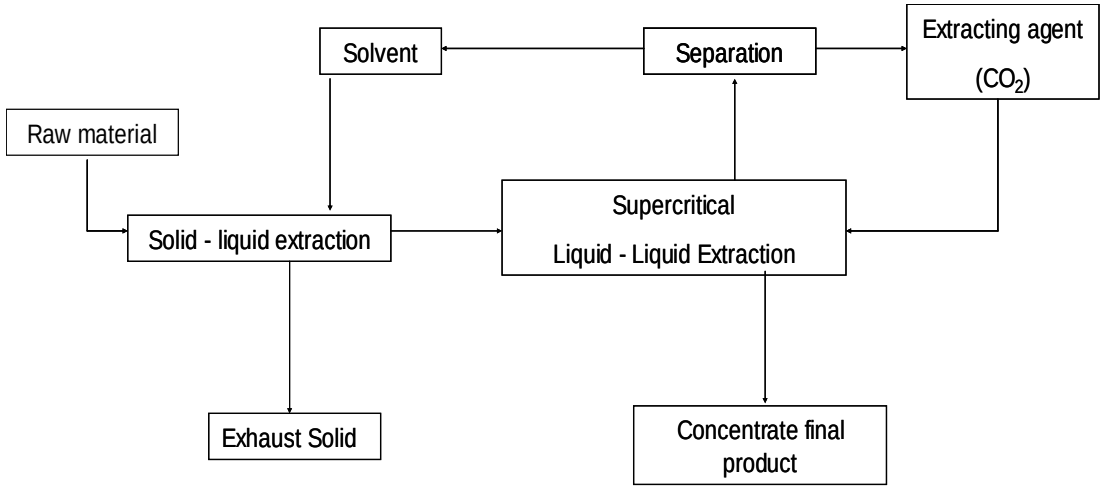
To avoid this situation two serial valves are considered in the design. By this way, the control of the process can be ensured in a wider range than using a single valve. Thus, if the extractor were operating at 20 MPa, a two stage-expansion would be needed to recirculate the CO_2 gas at 5 MPa.

On the other hand, a pump membrane pump will be used to compress the CO_2 up to supercritical pressures. Then, from a technical point of view, there is a minimum limit for the separation pressure of the system. This limit is imposed by the NPSH (net positive suction head) of the CO_2 pump, below which, the pump would start to cavitate. Then, the pressure in the separator should be slightly higher.

The process could be performed at higher pressure with the same valves but it should be necessary to rearrange the configuration of the PID controllers and the pump flow for such situation will be affected.

It should be noted that, when a gas expands, it requires energy from the environment. If there is no enough energy available, the temperature of the gas will decrease. This is known as the Joule-Thomson effect. It is always present when working with supercritical fluids and especially important when CO_2 is employed, so that the expansion valves need to heated for a good control the process.

A scheme of the block diagram of the process is presented in figure 1. As it was mentioned in previous reports, the philosophy of the process has changed substantially. The process is characterised by two steps: 1) A conventional solid-liquid extraction performed with a GRAS solvent (ethanol, ethyl acetate); and 2) a $scCO_2$-assisted process for the concentration of the natural products of interest extracted in the previous stage and selective removal of the solvent.



Description of the pilot plant

The aim of this task was to accomplish the technical design, assembly and set up of the pilot plant. The piping and instrumentation diagram (P&I) was developed in order to

make a full description of the operating parameters of the process, equipment and control strategy.

P&I diagram

Feed and pre-treatment

The P&I diagram describes the piping and instrumentation design of the plant as well as all the process units and control loops involved in process.

The pilot plant comprises two feed lines: one for the co-solvent line (F-100 tank) another one for the CO_2 line. The main feature of the continuous plant operation is that the CO_2 stream is separated from the organic liquid phase and then fed back to the process, along with the CO_2 make-up line. The CO_2 flow rate is set at 10 Kg/h, with a maximum value up to 20 Kg/h.

Prior the compression step, the CO_2 stream is cooled down to 0-4 º C to ensure that, at 50 bar approx., it is totally liquefied and sub-cooled, so cavitation problems in the pump are avoided. The chiller, (E-210) is a double-tube heat exchanger designed to provide a duty of 1.6 KW employing ethylene glycol (30 wt.%) as refrigerant. The plant is equipped with a refrigeration system (E-500) to provide ethylene glycol (30. wt%) as cooling fluid by means of a basic refrigeration cycle (10 KW). The liquefied CO_2 is subsequently pumped to the desired flow rate 10-20 Kg/h and compressed to the extraction pressure (10-20 MPa). The CO_2 mass flow rate is measured by a coriolis flowmeter (K-223) and controlled by regulating the speed of the motor of the pump. The CO_2 pump, a high pressure dosing pump, provides a maximum pressure of 20 MPa and maximum flow rate of 20 Kg/h. The compressed CO_2 is mixed with the cosolvent stream (M-240) and then is pre-heated before entering the extractor vessel (D-300) at the desired pressure-temperature conditions.

Extraction/ concentration unit: (D-300)

The concentration/extraction stage takes place in the extractor unit D-300. As explained in the previous section (fundamentals of the process), the concentration of the antioxidants is performed by removing the organic cosolvent (ethanol or ethyl acetate) from the heavy liquid phase. The CO_2 stream dissolves and drags part of the solvent. Under supercritical conditions the CO_2-cosolvent-antioxidant system splits into two phases: vapour (composed mainly by CO_2 and ethanol / ethyl acetate) and liquid (CO_2+ ethanol/ ethyl acetate + antioxidant compounds). Thus, part of the cosolvent is removed from the antioxidant solution, and, although the CO_2 is partially dissolved in this step, it can be easily removed by rapid expansion later on.

When the feed solution is composed by a non polar compound (e.g. Betacarotene), the CO_2-cosolvent system becomes one homogeneous phase. The process can not be achieved then and a fixer agent is required.

An alternative solution to facilitate the concentration of the antioxidant compounds, consists in using a non-volatile liquid that retains the compounds of interest, for instance vegetable or paraffin oil, which, at the same time is not soluble with CO_2. In this sense, the oil would be acting as a catcher. The liquid phase obtained from this initial stage is basically oil with a high concentration of antioxidant compounds and a small concentration of organic cosolvent. The gas phase is composed of CO_2 and the cosolvent removed from organic feed.

Control and instrumentation

The pressure of the extractor vessel is regulated downstream by a controlled automated valve (V-320). The temperature of the extractor is measured internally in the bulk of the fluid and externally on the wall of the vessel. The temperature of the wall permits to fix an upper-limit at which the oven stops heating as a kind of alarm or on/off variable. This way, the situations of over-temperature in the extractor unit are avoided, since the thermal inertia of the vessel is quite high, and therefore, over-temperature can be easily reached if the temperature of operation is tried to achieve rapidly.

A rupture disc and a safety valve have been included as passive safety elements in case of over-pressure, so that the system is rapidly depressurised by the release of fluid to safe place.

The solvent is obtained from the separator and it is possible to use it in the extraction stage with out further modifications.

Separation of cosolvent and CO_2 recovery: expansion system and CO_2 Separator (D-400)

The separation of the CO_2 stream from the cosolvent is performed by simple depressurisation in a controlled multi-stage expansion. The CO_2-ethanol (or ethyl acetate) system is depressurised in two steps as reported in section 2, (Fundamentals). For a certain pressure-temperature conditions (60-50 bar and 30 ºC), the CO_2-cosolvent system splits into a vapour phase, mainly composed by CO_2, (0,97-0,99 molar fraction), and a liquid phase, (cosolvent, saturated with CO_2). The vapour CO_2 phase is recirculated subsequently and mixed with the CO_2 make-up in order to keep constant the flow required in the process. A CO_2 purge is also required to control the CO_2 flow and improved the stability of the process since there is not an intermediate process tank for the CO_2 feedback.

Process units

The pilot plant comprises the following units:

- CO_2 pump (P-220)

The CO_2 stream is compressed with a dosing pump, (DOSAPRO MILTON ROYAL, model, Milroyal B, Diaphragm-type). The pump supplies up to 29 l/h at the maximum working pressure (20 MPa).

- Co-solvent pump (P-110)

The co-solvent is pressurised by means of a dosing pump, (DOSAPRO MILTON ROYAL, model, Milroyal D, Diaphragm-type). The pump supplies up to 2.2 l/h at the maximum working pressure (20 MPa).

- Flowmeter (K-223)

A coriolis mass flowmeter (Rheonik RHM 015 GNT, 1/4 inch) was employed to measure the CO_2 mass flow,(max. allowable pressure 300 bar, nominal measurement range 0.6 Kg/min.)

- Mixer (M-240)

In order to improve the efficiency of the process, the CO_2 and cosolvent streams should be mixed efficiently in order to improve the mass transfer between the CO_2 and organic phase, and so, facilitate the solubility of the cosolvent (ethanol/ acetate) in the gas dense, which is the key aspect for the concentration of the antioxidant compound in the heavy liquid phase.

– Pre-Heater (E-230)

The heat exchanger E-230 is employed to preheat the CO_2 feed to the required extraction temperature. The equipment has been developed based on a novel design, developed and assembled in the CARTIF labs. The prototype consists in an aluminium block composed by two frames provided with electric band heaters. The tubing is assembled in between the aluminium frames and the whole assembly system is thermally sealed to improve the heating efficiency. The pre-heater is located before mixing the CO_2 and the co-solvent streams instead of placing two separate heaters for each feed; thus the costs are reduced and potential over-heating of the co-solvent feed (and so degradation of antioxidants) are more easily avoided (the cosolvent+antioxidant stream is not in direct contact with the hot surface of the electrical heater, but it is heated by mixing with CO_2 line). Further details of design, materials and construction can be found in the technical drawings.

– Extractor/concentration unit (D-300)

The plant is equipped with a high pressure vessel, (SS 316, 1000 mL internal volume). The unit has been designed to work at the following maximum operating conditions:

- CO_2 mass flow: 20 kg/h.
- Pressure: 200 bar.
- Temperature: 60º C.

In this way, the cosolvent is removed with the sc CO_2 assistance and the compounds of interest are separated and recovered in concentrated solution that can be flow out the process in continuous mode, or retained in the vessel for a batch operation time. The outlet stream, is released and expanded in a multi-stage process in order to lead the CO_2-co-solvent system to the required pressure-temperature conditions for suitable separation of CO_2 and the organic.

The vessel is commercially available, model: 1 Litter EZE-Seal Pressure Vessel, (AUTOCLAVE ENGINEERS). The vessel is designed for a maximum allowable working pressure of 227 barg. It is also provided with 3 inlet/outlet connections (1/4 inch Swagelock fittings), an atmospheric thermowell for the assembly of a 1/16 inch thermocouple (type K), and a 1/4 inch rupture disc (SS 316) as safety element. The Autoclave Engineers' EZE-Seal design consists of a two piece, loose flange designed for ease of assembly and flexibility. The design features a reusable double metal-to-metal seal, designed to permit easy closing of the vessel assembly.

– Separator unit (D-400)

The function of this unit is to separate the CO_2 from the cosolvent (ethanol, acetate) prior controlled expansion of the D-300 outlet stream. The expansion is performed previously by controlled depressurisation in two steps, so two separated streams, one gas (CO_2) and another liquid (solvent) are obtained. Due to the difference in densities a good separation can be achieved. Thus, the gas is ready for the process feedback. The operating parameters of the vessel are initially set at 50 bar and 30 º C, although they could be regulated according to requirements of the process. The temperature of the vessel is regulated by means of a jacket oven coupled to the vessel. The technical features of this unit are similar to the extraction unit D-300, including an internal refrigerating coil for a better control operation.

– Expansion system: V-320, E-330, V-340, E-350

The depressurization of the CO_2-cosolvent system is performed in two steps by means of two electro-pneumatic valves in series provided with intermediate heating. Therefore, the compressed fluid can expanded from a maximum operating pressure of 20 MPa to 5 MPa in two steps. The direct depressurization from 20 MPa to 5 MPa cannot be accomplished in one step since the fluid would reach the sound velocity, (choked flow), and so, the maximum flow rate is limited by this condition.

The first valve is also employed as element of control to regulate the pressure in the extractor vessel, downstream, as it is usual in the pressure-regulation systems.

The control valves were selected according to the operating mass flow rate and the pressure drop required for a suitable operation.

- V-320: Control valve for micro flow rates:
- Model: Badger Meter Research 807, RC200
- Body: AISI316: fittings 1/4 NPTH, teflon packings, size "N"
- Max. pressure: 340 bar, Cv: 0,00024-0,006
- Pneumatic actuator, type diaphragm, 3-15psi
- Eletroneumatic positioner TZID
- Function: pressure regulation in the first extractor unit (D-300) and first expansion stage.
- E-330: Electric heaters composed by an aluminium block provided with two cartridge heaters (235 W each). A bi-metal thermostat prevents the temperature to surpass a maximum set value of 50-60 ºC.
- V-340: similar characteristics to V-320.
- Max. pressure: 340 bar, Cv: 0,0004-0,01, size "M"
- E-350. - Identical to E-330
- Refrigerating/cooler system E-500

A cooler system composed by a compressor, a refrigerant circuit and a water/glycol tank was designed. The main idea of the system function was to obtain an amount of cold mass to be used to liquefy the CO_2stream. So the water/glycol is pumped from this tank to supply coolant to the E-210 unit works. Then the coolant is reintroduced in the water/glycol tank where is diluted in the bulk fluid of the tank, and the heat is absorbed. The thermal inertia of the total mass of fluid allows perform the operation for a long period without using the compressor. At the same time, the process can be supported by the function of the compressor. The lowest temperature of the tank unit is – 30º C.

– CO_2cooler (E-210)

Prior the compression step, the CO_2stream is sub-cooled below 0-4 º C to ensure that it is totally liquefied at 50 bar in the inlet of the pump and there are no cavitation problems. A simply double-tube heat exchanger was employed as cooler unit to liquefy the CO_2 stream. The equipment was designed to provide a duty of 1.6 KW, employing ethylene glycol (30 wt. %) as refrigerant. Further details, (operating conditions, fluid properties and details of construction) are listed in the technical data sheet.

Instrumentation: thermocouples (type K) 1/4 inch and 1/8 inch. Pressure transducers 1/4 inch. Pressure gages (1/4,1/8 inch fitting)

More information about the parameters of design and construction details can be found in the technical data sheets.

General specifications of the pilot plant

A general description of the working parameters and main items of the plant is given:

– Inlet streams:

Feed:

Case a: Solution of Ethanol 96% (for cosmetic uses)
Case b: Solution of Ethyl Acetate (technical grade)

Solvent:

Liquid CO_2 (technical grade)

– Expected capacity:

Case a: 600 g/h of Ethanol feed (assuming a miscibility with CO_2 of 3% w/w)
Case b: 1800 g/h of Ethyl Acetate (assuming a miscibility with CO_2 of 8% w/w)

– Auxiliary requirements:

Air for instrumentation 6 bar.
380, 3 phases AC current.

– General estimations:

CO_2 Cycle:
CO_2 mass flow required: 20 kg/h.
Maximum extraction conditions:
Pressure: 200 bar.
Temperature: 60º C.
Separation conditions:
Pressure: 50 bar.
Temperature: 30º C.
Feed conditions for CO_2 pump:
Pressure: 45 bar.
Temperature: 5º C.
Pumping energy requirements: 6 kcal/kg
Heating energy requirements: 25 kcal/kg
Gas Expansion up to separation conditions: 27 kcal/kg
Condensation of CO_2: 50 kcal/kg

– Vessels definitions:

Extractor:
Extractor unit capacity: 1 litre.
Closure system: Bolted flanged.
Minimum working temperature: - 80º C.
Maximum working temperature: 120º C.
Maximum working pressure: 350 bar
Separator:
Separator unit capacity: 1 litre.
Closure system: Bolted flanged.
Minimum working temperature: - 80º C.
Maximum working temperature: 120º C.
Maximum working pressure: 350 bar

Control loops and process monitorization

Both the start-up and the continuous stationary operation are automated controlled in order to ensure the operating conditions in process: mass flow rate, pressure and temperature parameters.

The wall temperature of the extractor unit, D-300, is controlled too in order to avoid potential heat points (wall-effect) that could degrade the antioxidant capacity of the compounds of interest.

Design of main plant elements

The main items to be considered in the design of the plat are:

Extraction and separation vessels

Heater unit

Expansion Valves

Condensation unit

Extraction and separation vessels

As far is applied the supercritical fluid in this process, the vessels are acting as separation units since in the extractor the antioxidants are depleted from the main stream and in the separator, the solvent in separated from de CO_2.

So the main design features to consider in the design are the maximum process pressure, maximum process temperature and the inner dimensions to achieve good separation efficiency when the maximum mass flow is reached.

In the first case, the extractor, the considered process conditions were: 20 kg/h of CO_2 at 200 bar and 60º C. Then considering the density for that conditions and adjusting the total length of the inlet tube in relation with the diameter of different models of commercial high pressure vessels, it was estimated the residence time. This value should be higher than 40 s to achieve the equilibrium between phases.

For the selected equipment a residence time of 62 s it is expected. The selected vessel was a model EZE-Seal from Autoclave Engineers In order to get the proper heating of the process.

In the second case, the separator, the considered process conditions were: 20 kg/h of CO2 at 50 bar and 60º C. Then, a decrease on the CO2 density is observed. So the residence time for a vessel similar to the extractor, the residence time will be 11 s. Although this value could force some dragging of solvent, conditions could be adjusted during the process to increase residence time up to 17 s if a lower working temperature is used. This parameter will be adjusted during the tuning stage.

The selected equipment was a EZE-Seal model from Autoclave Engineers. The inclusion of a furnace to supply thermal energy was considered at this stage, so the vessel will include an extra heating unit In order to get the proper heating of the process.

Heating unit

To achieve the supercritical conditions of the CO2 a heating of the steam is required. According to the conditions derived from the T-S diagram of CO2 the heating energy requirements are 25 kcal/kg. The mass flow considered for the design was 20 kg/h at

200 bar. The considered design temperature at the inlet was 10º C and the outlet design temperature was 65º C.

The model to proceed with the heating was constant wall temperature. Considering the flow regime for the selected tube diameter (1/4"), it can be considered laminar. So, the item can be stimulated through the Petukhov relation.

The heating method considered was electric heaters coupled to a metal mass. The tube will in direct contact with the metal. The final result of the item were:

- 70 cm of tube required
- wall temperature: 150º C
- total electric power required: 2800 W
- Materials: ANSI 316-L

Since there was no commercial devices which fulfilled the requirements, a complete heater has been designed and build up by CARTIF.

Expansion valves

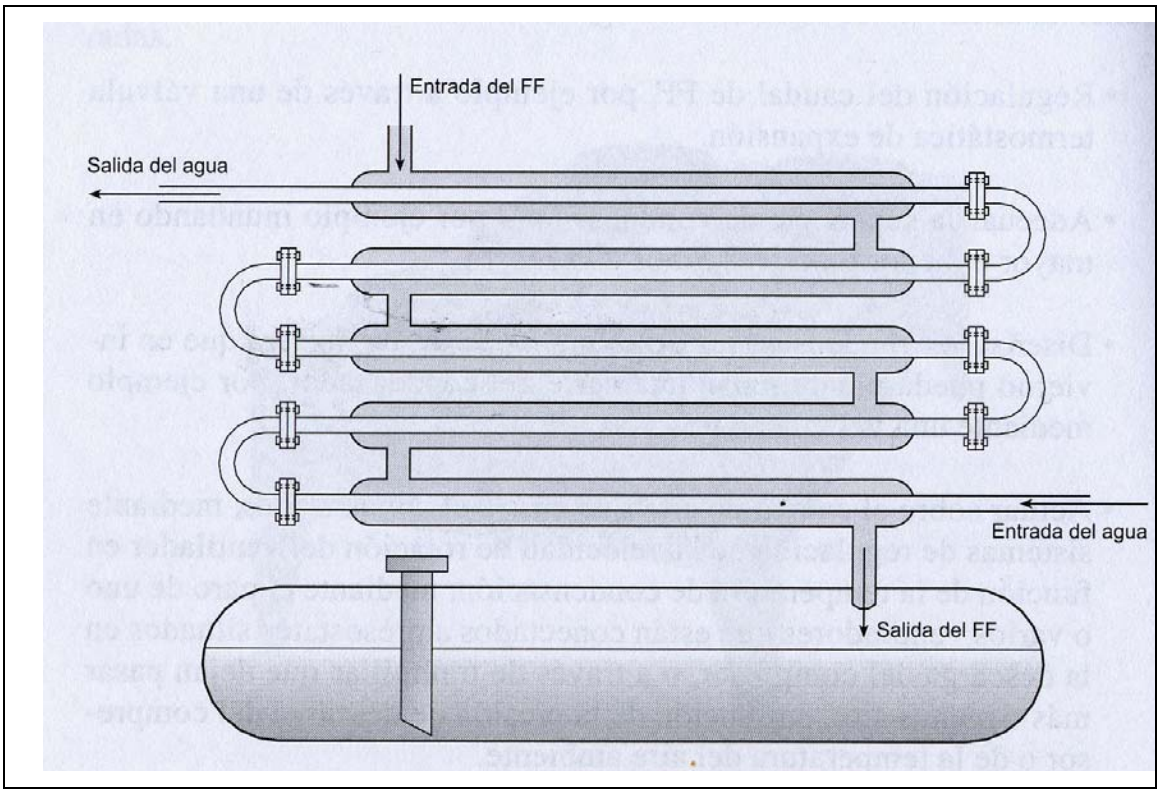
One of the most critical points in the design of the plant is the selection of the control valves, since it will determinate the rangeability of the pilot plant. The selection of the valves was considered based on the following conditions:

- Expansion from extractor to separator: it will be carried out in two stages by operating two in line valves. The first expansion considered conditions were: 200 bar to 100 bar. The thermodynamical trajectory selected for the process was an isoentalpic one. The second expansion was from 100 bar to 55 bar. In both cases it was used the Kv parameter for the selection of the valves. Density, mass flow an pressure drop are required for the estimation of the Kv value.
- Two valves model 807 Rc200 from Badger meter were selected in this case. The body building material was 316-L and they were actuated pneumatically. For the first situation the model was adjusted to a orifice diameter model N. For the first situation the model was adjusted to a orifice diameter model M.
- Control of separator pressure: In order to assist the proper performance of the process, a valve to hold pressure conditions in the separator is required. In this sense valves model 807 Rc200 from Badger meter was selected. The considered design pressure drop was from 55 to 50 bar.

Condensation unit:

The objective of this unit is to liquefy the entire gas stream obtained in the separator vessel. The selected heat exchanger to do this was a system composed by a double tube. In the inner tube is introduced the CO_2 and in through the external tube is introduced the refrigerant o cooler stream.

The next figure shows a diagram of the selected heater:



To estimate the required dimension of the equipment it is assumed that the total heat required by the process of liquefaction is derived from the T-S diagram (from point 3 to point 4).

The total heat will be related with the variation of the temperature through global heat transfer coeficient U as follows:

$$q = \overline{U}\ A_e\ \Delta T_{ml} \qquad \text{eq 1}$$

where temperature is refereed to the outer fluid as B and the inner fluid as A and U is related with the area of the internal tube:

$$U_i = \frac{1}{\frac{1}{h_i} + \frac{Ln\left(r_e / r_i\right)}{2\,\pi\,k\,L} + \frac{A_i}{A_e}\frac{1}{h_e}} \qquad \text{eq 2}$$

h is the heat transfer coefficient

k is the thermal conductivity of stainless steel

r is the radius of the inner tube (internal and external)

A is the area of the tube.

To solve this equation, different methods to estimate h was considered:

Estimation of h_i

During the condensation of a gas in a horizontal tube it can be obtained different types of behaviour of fluid flow. Depending on the behaviour, different correlation equations are required in design.

For all the different correlation equations required it is necesary to estimate the type of flow. This can be done through two parameters: modified mass flow (G^*_V) and the Lockhart-Martinelli parameter (X_{tt}):

$$G_V^* = \frac{x\,G}{\sqrt{D_i\ g\ \rho_V\ (\rho_L - \rho_V)}} \qquad \text{eq 1}$$

$$X_{tt} = \left(\frac{1-x}{x}\right)^{0,9} \left(\frac{\rho_V}{\rho_L}\right)^{0,5} \left(\frac{\mu_L}{\mu_V}\right)^{0,1} \qquad \text{eq 2}$$

Where:

x is the relation between kg of saturated vapour per kg of wet vapour (vapour + liquid).

G Mass flow (kg / m2 s)

Di inner diameter of the tube (m)

μV vapour viscosity (kg/ m s)

To estimate the parameters the conditions of gas it was supposed the that to describe in a better way the overall process x could be considered as 0.5. So the model of flow obtained in the tube is annular.

The models to estimate heat transfer coefficient were:

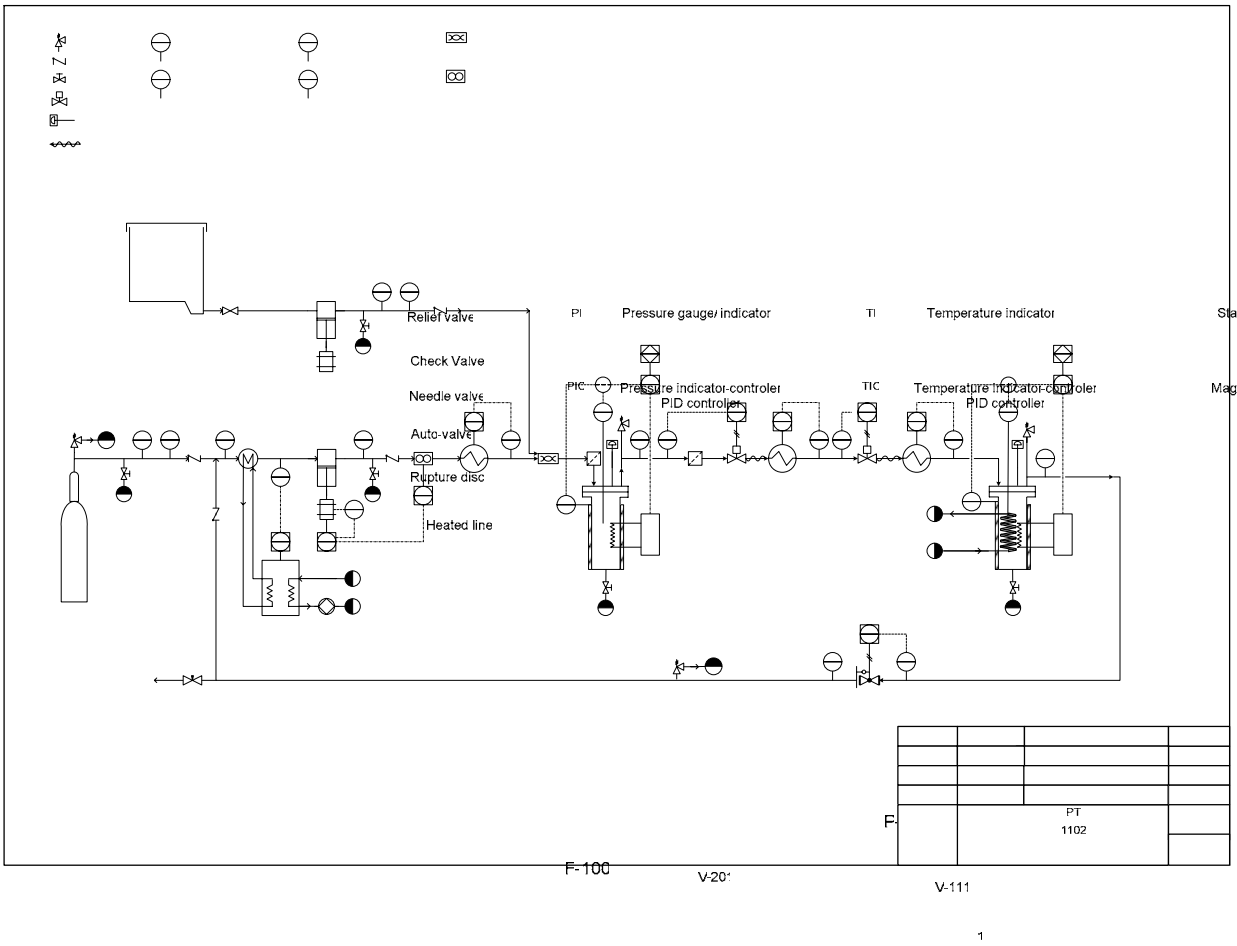
- Boyko y Kruzhilin
- McAbe
- Akers
- Shah
- Travis

In all the situations, different estimations were considered for cooling, condensation and for subcooling.

Finally, the total length of the heat exchanger was selected for the more unfavourable conditions, so 6,5 meters of tube are required for the operation. The expected required heat interchange is about 2086 W for the process.

The selected refrigeration liquid is a solution of ethyleneglycol in water 30%. Total glycol solution needed for the performance is 170 litres/h. The initial temperature of glycol is -10º C and the final expected temperature is 1º C.

Technical data sheets

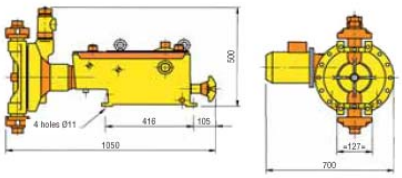


	Technical data sheet ***HIGH PRESSURE PUMP*** Pilot Plant for SCF Extraction of Natural Products
Project: ***EXTRANAT, High Selective and Environmental Friendly Vegetable and Fruit Extraction using Supercritical Fluid Technology***	

FUNCTION			
1	Function: CO2 liquid compression	2	***Identification nº (P&I). P-220***
DESIGNATION			
3	***Supplier: DOSAPRO MILTON ROYAL***	5	***Type/code: Dosing Pump, Milroyal B, Diaphragm-type liquid end/ MB140L12M200/J***
4	***Serial no: 52060150***	6	***Year: 2006***
OPERATION DATA			
7	***Fluid:*** ***CO2***	8	***P max :*** ***20 MPa***
9	***Flow rate:*** ***10 kg/h***	10	***Inlet pressure:*** ***4.5.0 MPa***
11	***Inlet Temperature:*** ***-4 to -5 ºC***	12	***Standard Tests:***

13	***Averaged density:*** ***900-950 (kg/m3), inlet***	14	***Flow rate at max. pressure:*** ***29 l/h***
15	***Averaged viscosity:*** ***0.013665 - 0.128680 (cP)***	16	
17	***Solid/slurry concentration:*** ***no***	18	

DESIGN DATA

1	*Propelling device:*	2	
3	***Max. thrust:*** ***4600 (N)***	4	
5	***Capacity control:*** ***manual***	6	
7	***Min./Max speed :*** ***36-173 (strokes/min)***	8	
9	*Head*	10	
11	***Plunger diameter:*** ***12 mm***	12	
13	***Plunger Seal :***	14	
15	***Inlet/ outlet fitting Ø:*** ***1/4 / 1/4 inch***	16	
17	***Inlet/ outlet valve:*** ***Check ball valve***	18	
19	***NPSH***	20	
21	*Engine*	22	
41	***Motor Power:*** ***1. 5 kW***	42	*Materials*
43	***Speed:*** ***1400 rpm***	44	***Head/body/valve:***
45	***Weigh of motor*** ***15 kg***	46	***Membrane:***
47	***Weigh of mechanical assy (approx.)*** ***76 kg***	48	***O-ring shutter:***
49	***Elec. tension:*** ***380, 3 phases AC current***	50	***Hydraulic liquid***
51		52	***Total weigh of pump (approx.)*** ***115 kg***
53		54	***Volume of hydraulic fluid:*** ***3 l***
55		56	***Volume of lubricating fluid:*** ***1 l***

SAFETY ELEMENTS

57	***Type***	***Safety valve***	58	***Type***	
59	***Quantity***	***1***	60	***Quantity***	
61	***Calibrated pressure***	***230 bar***	62	***Calibrated pressure***	

OTHER TECHNICAL DATA

63		64	

OBSERVATIONS

	Name	Signature	Date
Created	Raúl Piñero Hernanz		
1st check			
2nd check			

	Technical data sheet ***HIGH PRESSURE PUMP*** Pilot Plant for SCF Extraction of Natural Products

Project: ***EXTRANAT, High Selective and Environmental Friendly Vegetable and Fruit Extraction using Supercritical Fluid Technology***

FUNCTION

1	Function: co-solvent liquid compression	2	***Identification nº (P&I). P-110***

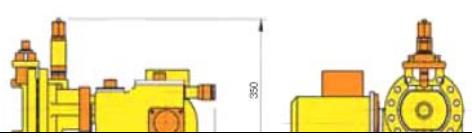
DESIGNATION

3	***Supplier: DOSAPRO MILTON ROYAL***	5	***Type/code: Dosing Pump, Milroyal D, M/X/V Diaphragm-type liquid end/ MD140G4M200***
4	***Serial no: 55060147***	6	***Year: 2006***

OPERATION DATA

7	***Fluid: Ethanol (96 %) ethyl acetate (technical grade)***	8	***P max : 20 MPa***
9	***Flow rate:***	10	***Inlet pressure: 0.1. MPa***
11	***Inlet Temperature: 20-30 ºC***	12	***Standard Tests:***
13	***Averaged density: 789 kg/m3***	14	***Flow rate at max. pressure: 2.2 l/h***
15	***Averaged viscosity: 0.00037 kg/m s***	16	
17	***Solid/slurry concentration: no***	18	

DESIGN DATA

1	*Propelling device:*	2	
3	***Max. thrust: 1100 (N)***	4	
5	***Capacity control: manual***	6	
7	***Min./Max speed : 23-173 (strokes/min)***	8	
9	*Head*	10	

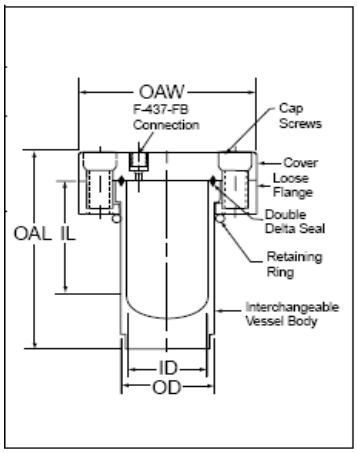
	Plunger diameter: ***4 mm***		
	Plunger seal:		
	Inlet/ outlet fitting Ø: ***1/8 - 1/8 inch***		
	Inlet/ outlet valve: ***check ball valve***		
	NPSH		
	Engine		
41	***Motor Power:*** ***0.37 kW***	42	*Materials*
43	***Speed:*** ***1400 rpm***	44	***Head/body/valve:***
45	***Weigh of motor:*** ***8 kg***	46	***Membrane:***
47	***Weigh of mechanical assy (approx.):*** ***17 kg***	48	***O-ring shutter:***
49	***Electrical tension current:*** ***380, 3 phases AC***	50	***Hydraulic liquid***
51		52	***Total weigh of pump (approx.)*** ***33 kg***
53		54	***Volume of hydraulic fluid:*** ***0.53 l***
55		56	***Volume of lubricating fluid:*** ***0.5 l***
	SAFETY ELEMENTS		
57	***Type*** ***Safety valve***	58	***Type***
59	***Quantity*** ***1***	60	***Quantity***
61	***Calibrated pressure*** ***230 bar***	62	***Calibrated pressure***
	OTHER TECHNICAL DATA		
63		64	
	OBSERVATIONS		

	Name	Signature	Date
Created	Raúl Piñero Hernanz		
1st check			
2nd check			
3rd check			

	Technical data sheet ***HIGH PRESSURE VESSEL*** Pilot Plant for SCF Extraction of Natural Products

Project: ***EXTRANAT, High Selective and Environmental Friendly Vegetable and Fruit Extraction using Supercritical Fluid Technology***

FUNCTION			
1	Function: Extraction vessel	2	***Identification nº (P&I): D-300***
DESIGNATION			
3	***Manufacturer/Supplier: AUTOCLAVE ENGINEERS/ IBERFLUID INSTRUMENTS***	5	***Type/code: 1Liter EZE-Seal Pressure Vessel***
4	***Serial no: 06250223-1***	6	***Date: July, 2006***
OPERATION DATA			
7	Content fluid: CO2, ethanol, ethyl acetate	8	***Operating temperature: 50-60 º C***
9	Salts/ solids concentration: no	10	Max./Min Operating temperature: 120/-80 º C
11	Packing material: no	12	***Operating pressure (Abs): 10 MPa***
13	Position: fixed vertical	14	Max./Min Operating pressure (Abs): 35/0.1 MPa
DESIGN and CONTRUCTION DATA			
VESSEL SPECIFICATIONS			
	Max. Allowable Working Pressure (MAWP): **3300 psig at 850 º C, (227 barg at 454 ºC)**		
	Minimum Design Metal Temperature: **-20 F at 3300 psig (-29 C at 227 barg)**		
	Max. Operating Pressure (MOP): MAWP*0.90		
	Hydrostatic test pressure: 6000 psi at room T (414 barg)		
	Materials: 316 SS		
	Seal/Gasket material: O-ring		
	DIMENSIONS		
	Fluid volume: 1000 ml		
	Inside length: 225.3 mm		
	Inside diameter: 76.3 mm		
	External length: 328.8 mm		
	External diameter: 95,9 mm		
	Wall thickness:		
	COVER TOP/CLOSURE		
	Type: Bolted flanged; ; Cap screws		
	Height:		***ACCESORIES:***
	Thickness:		***Type***



	External diameter:			***Thermowell***
	OTHER ELEMENTS			***Cooling coil***
	Fittings:			***Thermocouple (type K) 316 SS sheath)***
	Supports:			
	Agitation:			
	SAFETY ELEMENTS			
	Type	***Rupture disc (Inconel)***		***Type***
	Quantity	***1***		***Quantity***
	Calibrated pressure	***3158-3300 psi (72 F)***		***Calibrated pressure***
	OTHER TECHNICAL DATA			
	Electrical specifications: general purpose, isothermal extraction conditions			
	OBSERVATIONS			
	• ***Cap screw torque:*** ***- Minimum at MAWP: 35ft-lbs (47 N-m)*** - Max. permitted: 78 ft-lbs (106 N-m)			

	Name	Signature
Created	Raúl Piñero Hernanz	
1st check		
2nd check		
3rd check		

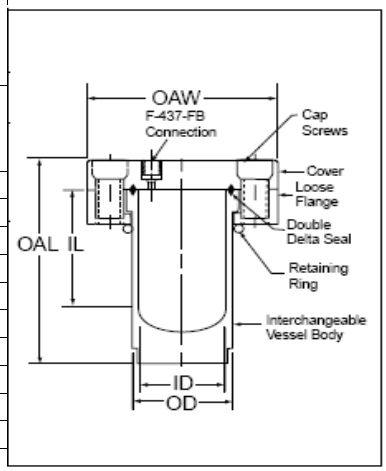
Technical data sheet

HIGH PRESSURE VESSEL

Pilot Plant for SCF Extraction of Natural Products

Project: ***EXTRANAT, High Selective and Environmental Friendly Vegetable and Fruit Extraction using Supercritical Fluid Technology***

	FUNCTION		
1	Function: CO2 expansion and flash vessel	2	***Identification nº (P&I): D-400***
	DESIGNATION		
3	***Manufacturer/Supplier: AUTOCLAVE ENGINEERS/ IBERFLUID INSTRUMENTS***	5	***Type/code: 1Liter EZE-Seal Pressure Vessel***
4	***Serial no: 06250223-2***	6	***Date: July, 2006***
	OPERATION DATA		
7	Content fluid: CO2, ethanol, ethyl acetate	8	***Operating temperature: 30 º C***
9	Salts/ solids concentration: no	10	Max./Min Operating temperature: 120 / -80 º C
11	Packing material: no	12	***Operating pressure (Abs): 5 MPa***
13	Position: fixed vertical	14	Max./Min Operating pressure (Abs): 35 / 0,1MPa
	DESIGN and CONTRUCTION DATA		
	VESSEL SPECIFICATIONS		
	Max. Allowable Working Pressure (MAWP): **3300 psig at 850 º C, (227 barg at 454 ºC)**		
	Minimum Design Metal Temperature: **-20 F at 3300 psig (-29 C at 227 barg)**		
	Max. Operating Pressure (MOP): MAWP*0.90		
	Hydrostatic test pressure: 6000 psi at room T (414 barg)		
	Materials: 316 SS		
	Seal/Gasket material: O-ring		
	DIMENSIONS		
	Fluid volume: 1000 ml		
	Inside length: 225.3 mm		
	Inside diameter: 76.3 mm		
	External length: 328.8 mm		
	External diameter: 95,9 mm		
	Wall thickness:		
	COVER TOP/CLOSURE		
	Type: Bolted flanged; Cap screws: 3/4-10 x 2.0 L		
	Height:		***ACCESORIES:***
	Thickness:		***Type***



	External diameter:			***Thermowell***
	OTHER ELEMENTS			***Cooling coil***
	Fittings:			***Thermocouple (type K) 316 SS sheath)***
	Supports:			
	Agitation:			
	SAFETY ELEMENTS			
	Type	***Rupture disc (Inconel)***		***Type***
	Quantity	***1***		***Quantity***
	Calibrated pressure	***3158-3300 psi (72 F)***		***Calibrated pressure***
	OTHER TECHNICAL DATA			
	Electrical specifications: general purpose, isothermal op.			
	Cooling coil			
	OBSERVATIONS			
	(1) Cap screw torque:			
	- Minimum at MAWP: 35ft-lbs (47 N-m)			
	- Max. permitted: 78 ft-lbs (106 N-m)			

	Name	Signature
Created	Raúl Piñero Hernanz	
1st check		
2nd check		

Technical data sheet

HEAT EXCHANGER- CO2 condenser

PILOT PLANT FOR SCF EXTRACTION OF NATURAL PRODUCTS

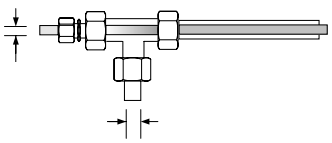
*Project**: EXTRANAT, High Selective and Environmental Friendly Vegetable and Fruit Extraction using Supercritical Fluid Technology***

	FUNCTION					
1	Function: CO2 condenser		2	***Identification nº (P&I): E-210***		
	DESIGNATION					
3	***Manufacturer/Supplier: Cartif***		4	***Type/code: concentric tube, counter-current flow***		
5	***Serial no: -***		6	***Date: July, 2006***		
	OPERATION DATA					
7	***Duty, Heat transfer rate: 1.6 KW***		8	***Type: concentric tubes***		
9	***Total Heat transfer area (A_{TC}): 0.15 m^2***		10	***Size: 3 x 0.5 x 0.1 m***		
11	***Global Heat Transfer coefficient (U_{TC}): 707 (KW/m^2 ºC)***		12	***Nº tubes:***		
13	***ΔT m: 15.13 (ºC)***		14	***Layout: horizontal***		
15			16	***Total Heat transfer area/ tube: 0.02 m^2***		
17			18			
19		units		Outer tube		Inner tube
20	Circulating fluid			Ethylene-glycol 30 wt %		CO2
21				INLET	OUTLET	INLET
22	Vapour flow rate	kg/h		***0***	***0***	20
23	Liquid flow rate	kg/h		***170***	***170***	
24	Operating Temperature	º C		***-10***	***-1***	***30***
25	Operating pressure	bar		***5***	***5***	45
26	Density	kg/m3		***1240***	***1240***	136.56
27	Viscosity	kg/(m s)		***7.67e10-3***	***.67e10-3***	
28	Thermal conductivity	W/ (m ºC)		***0.469***	***0.469***	0.023926

Specific heat	kJ/ (kg ºC)	***3.36***	***3.36***	1.67e10-5
Latent heat	kJ/ kg			2.05
velocity	m/s	0.56	0.56	2.96
Pressure drop	bar	-	-	-
Fouling factor coefficient	***KW/m² ºC***	1000000		1000000

MECHANICAL/ CONSTRUCTION DETAILS

	Outer tube		Inner tube	
	Length	2.5	Length	2.5
	O.D	12.70	O.D	6.35
	I.D	11.27	I.D	4.18
	thickness	1.43	thickness	1.085
	Fittings	model		quantity
	Union T	Hoke Gyrolok 1/2"		6
	Reducer	Hoke Gyrolok 1/2 to 1/4"		6
	Elbow	Hoke Gyrolok 1/4"		4



	Name	Signature	Date
Created	Raúl Piñero Hernanz		
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2nd check			
3rd check			

Cooling fluid inlet

87

T CF 1

T CO

CO2

T CF 2

T CO2 2

Task 3.3: Construction and starting-up of the SFE pilot plant

The technical objective of this task is to perform the construction and set up of the prototype plant according to the design features and operating parameters described in task 3.2 (P&I diagram). The construction of the pilot plant has been accomplished in several stages:

1. Mechanical design and layout of the pilot plant according to the different process units;
2. Equipment sizing and design of prototypes;
3. Purchase of commercial equipment and construction of prototypes;
4. Modifications, final assembly and electric design

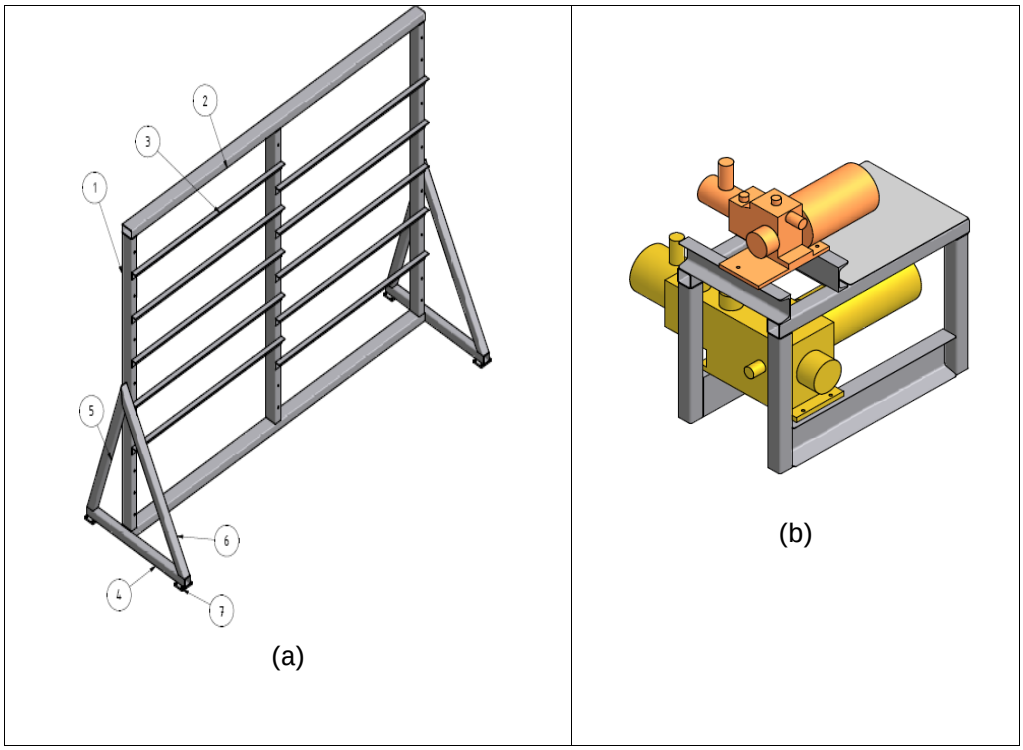
Mechanical design and layout

Mechanical support

The pilot plant has been constructed using an open structure as illustrated in figure 1. The supporting structure was provided with several wings in order to distribute the different units of the process and fix the piping and instrumentation easily. The assembly was accomplished following the indications of the P&I diagram thoroughly in order to achieve both a functional and robust plant with an attractive appearance for demonstrative use too. The assembly was developed in several stages, as shown in figure 2a-b.

The CO2 and cosolvent pumps were fixed in a separate structure in order to protect the magnetic flowmeter from the vibrations caused by pumps heads and motors.

All the elements of the plant are visible and easily accessible. The main structure (next figure) is provided with wheels. The heating and cooling units were thermally isolated for safety reasons and in order to reduce energy costs. The electric and control units were placed separately along with workstation for the process control.



Scheme of the mechanical support employed for the pilot plant, a) main structure;

b) Support and fixing of the pumps

Equipment sizing

The process units were sized and/or designed according to the requirements of the process and the P&I diagram. The pumping units were selected according to the operating range of flowrates and pressures of the process, as well as the power of the refrigerating unit and the heating units of the process. A detailed description of the features for the main process units is listed in the technical data worksheets, included in the previous section (Task 3.2). The piping net comprised high pressure tubing 1/2" (A269-AISI 213 SS 316/316L, max. allowable working pressure 3090 psi), and 1/4" (ASTM 213, max. allowable working pressure, 18700 psi)

Assembly of plant items

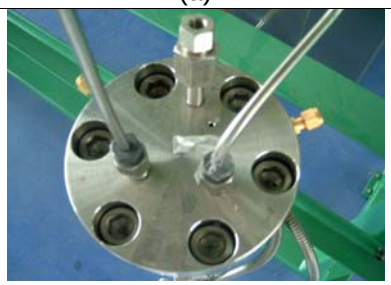
The following images shows some details of the building of the pilot plant as well as some o the main items. Further details of every item can be find in previous WP reports.

Front view of the pilot plant

(a)

(a)

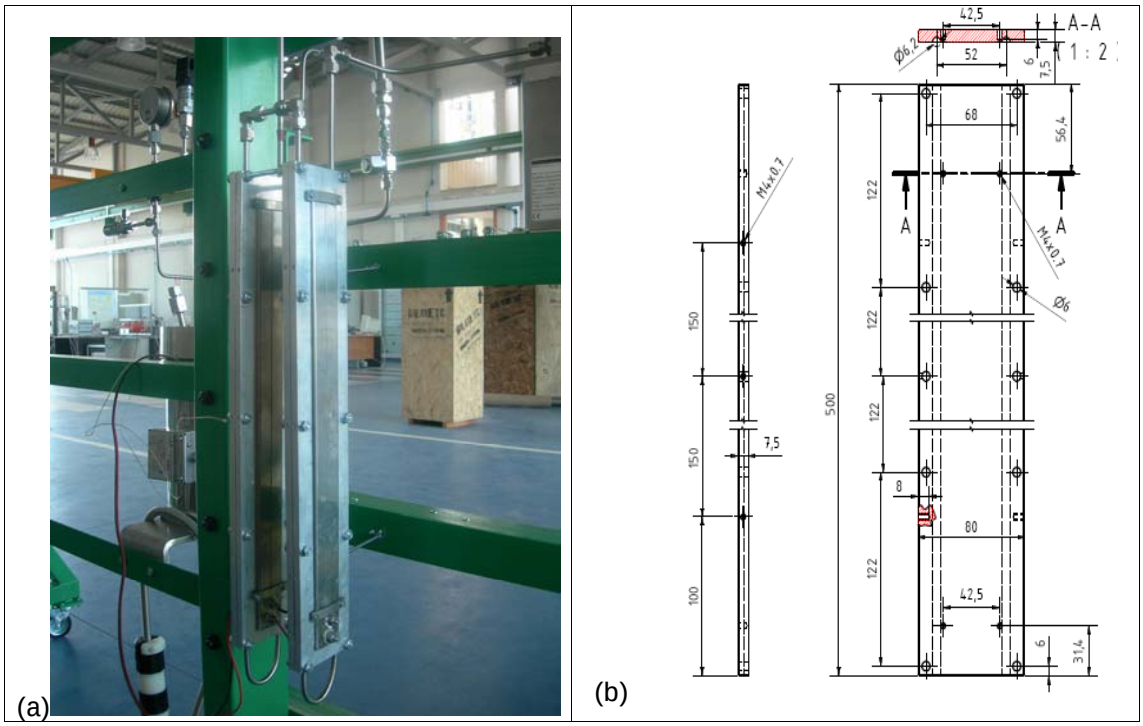
(c)



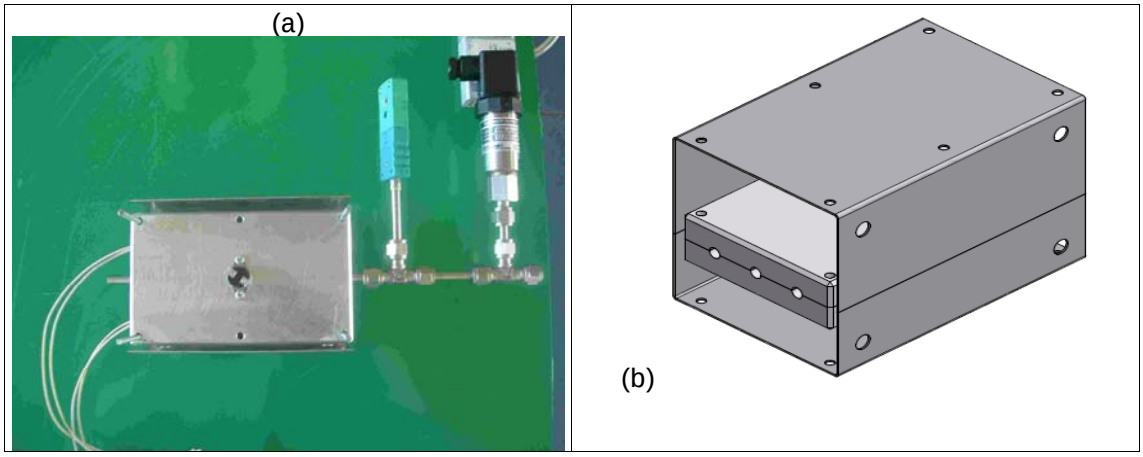
(d)

(e)

Details of construction of the pilot plant. a) High pressure co-solvent pump, b) CO2 and co-solvent pumps and support; c) Extractor vessel D-300 and first expansion stage; d) Vessel closing; e) Front view of the high pressure vessel D-400



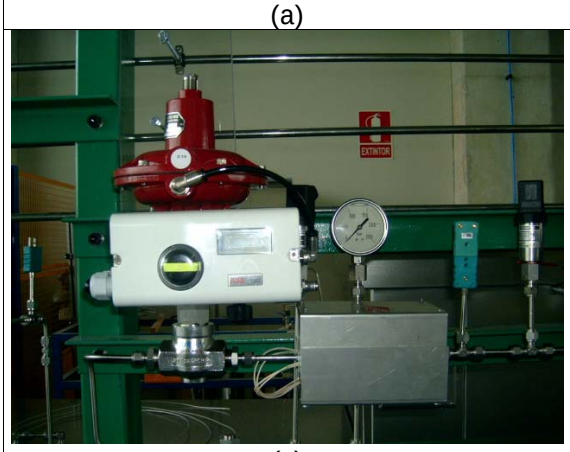
Details of construction; a) electric pre-heater; b) Technical drawing



Details of construction; a) electric heater of multi-step expansion; b) 3D scheme

(a)

(b)

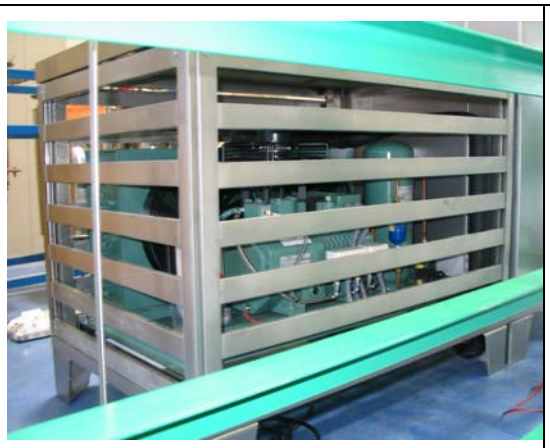


(c)

(d)

Details of construction; a) Extractor vessel D-300; b) Mass flow meter; c) First expansion stage: controlled valve and electric heater, d) View of the two-step expansion system and control pressure of the separator vessel.

(a)



(b)

(c)

Details of construction; a) general view of the refrigerating/cooler system E-500; b) compressor unit of the refrigerating/cooler; c) Tank with water and glycol.

(a)

(b)

(c)

Details of electric power and control switchboard; a) general view of the power switchboard; b) inner view of power switchboard; c) General view of control switchboard.

Once the design of the pilot plant was decided, the different parts to construct this prototype had to be ordered. On the market, several manufacturers are available for this kind of parts and the best offers were selected.

Vessels, Pumps, valves and accessories

Code	Description	Material	Manufacturer	Model	Comments
F-100	Co-solvent feed tank				
F-200	CO2 cylinder				
D-300	Extraction/ concentration vessel	316 SS	Autoclave Engineers	1Liter EZE-Seal Pressure Vessel	
D-400	Separator-expansion vessel	316 SS	Autoclave Engineers	1Liter EZE-Seal Pressure Vessel	
E-210	CO2 cooler-condenser	316 SS		prototype	
E-230	CO2-heater	316 SS		prototype	
E-330	CO2 expansion (Control temperature; 1st stage)	316 SS		prototype	Heating block + band electric resistance
E-350	CO2 expansion (Control temperature; 2 nd stage)	316 SS		prototype	Heating block + band electric resistance
E-500	Cryostat				Oil bath Cooler -Cryostat
P-110	Co-solvent dosing pump	316 SS	***DOSAPRO MILTON ROYAL***	***Dosing Pump, Milroyal D Diaphragm-type liquid end***	co-solvent (ethanol, acetic acid) compression pump
P-220	CO2 dosing pump	316 SS	***DOSAPRO MILTON ROYAL***	***Dosing Pump, Milroyal B, Diaphragm-type liquid end***	CO2 compression pump
VALVES AND ACCESORIES					
M-240	Static mixer for the feed lines before pre-heating	316 SS		prototype	
H-250	Filter after M-240	316 SS	Hoke	6320G4Y	Max. pressure: 5000 psig (345 bar)
H- 310	Filter after D-300	316 SS	Hoke	6320G4Y	Max. pressure: 5000 psig (345 bar)
K-223	Coriolis Mass Flowmeter[3] Nominal mass flow: 0.6 kg/min	316 SS	Rheonik	RHF 07	

Code	Description	Material	Manufacturer	Model	Comments
V-101	Accessory valve for co-solvent line (P-01 inlet)	316 SS	Hoke	3752 G4Y	Needle valve; max .pressure: 5000 psig (345 bar) Temp. range: -54 to 232 ºC CV: 0.07 to 1.1
V-112	Check valve, co-solvent line	316 SS	Hoke	6133G2Y	Check valve; max pressure 6000 psig, 414 (bar) Temp. range: -29 +177 ºC(Viton ring) CV: 0.3 , 2.4
V-111	Purge P-110	316 SS	Hoke	3752 G4Y	Needle valve; max .pressure: 5000 psig (345 bar) Temp. range: -54 to 232 ºC CV: 0.07 to 1.1
V-201	Relief valve, fresh CO2 cylinder	316 SS	Hoke	6548G4Y	relief valve; max pressure 3000 psig, 207 (bar) Temp. range: -29 +177 ºC(Viton ring) CV: 0.1
V-202	Purge of fresh CO2 line	316 SS	Hoke	3752 G4Y	Needle valve; max .pressure: 5000 psig (345 bar) Temp. range: -54 to 232 ºC CV: 0.07 to 1.1
V-203	Check valve, fresh CO2 line	316 SS	Hoke	6133G4Y	Check valve; max pressure 6000 psig, 414 (bar) Temp. range: -29 +177 ºC(Viton ring) CV: 0.3 , 2.4
V-221	Valve to purge outlet of the pump P-220	316 SS	Hoke	3752 G4Y	Needle valve; max .pressure: 5000 psig (345 bar) Temp. range: -54 to 232 ºC CV: 0.07 to 1.1
V-222	Check valve, CO2 line	316 SS	Hoke	6133G4Y	Check valve; max pressure 6000 psig, 414 (bar) Temp. range: -29 +177 ºC(Viton ring) CV: 0.3 , 2.4
V-301	Rupture disc, D-300	316 SS	Autoclave		1/4 '' rupture disc; max. pressure 6000 psi
V-302	Relief valve, fresh CO2 cylinder	316 SS	Hoke	6548G4Y	relief valve; max pressure 3000 psig, 207 (bar) Temp. range: -29 +177 ºC(Viton ring) CV: 0.1
V-303	Valve of discharge of D-300	316 SS	Hoke	7142 G4Y	Ball valve; max .pressure: 1500 psig (103 bar) Temp. range: -40 to +176 ºC CV: 0.40
AV-320	Controlled-valve; (D-300)- Pressure	316 SS	Badger Meter	Research 807,	- Body: AISI316: fittings 1/4 NPTH, teflon

Code	Description	Material	Manufacturer	Model	Comments
	control in D-300 and 1st expansion			RC200	packings, size "N" - Max. pressure: 340 bar, Cv: 0,00024-0,006 - Pneumatic actuator, type diaphragm, 3-15psi - Eletroneumatic positioner TZID
AV-340	Controlled-valve; 2nd expansion	316 SS	Badger Meter	Research 807, RC200	- Body: AISI316: fittings 1/4 NPTH, teflon packings, size "M" - Max. pressure: 340 bar, Cv: 0,0004-0,01 - Pneumatic actuator, type diaphragm, 3-15psi - Eletroneumatic positioner TZID
V-401	Rupture disc, D-300 (relief valve too?)	316 SS	Autoclave		1/4 '' rupture disc; max. pressure 6000 psi
V-402	Relief valve, fresh CO2 cylinder	316 SS	Hoke	6548G4Y	relief valve; max pressure 3000 psig, 207 (bar) Temp. range: -29 +177 ºC(Viton ring) CV: 0.1
V-403	Valve of discharge of D-300	316 SS	Hoke	7142 G4Y	Ball valve; max .pressure: 1500 psig (103 bar) Temp. range: -40 to +176 ºC CV: 0.40
AV-410	Controlled-valve; Back Pressure regulator (D-400)	316 SS	Badger Meter	Research 807, RC200	- Body: AISI316: fittings 1/4 NPTH, teflon packings, size "I" - Max. pressure: 340 bar, Cv: 0,005-0,02 - Pneumatic actuator, type diaphragm, 3-15psi - Eletroneumatic positioner TZID
V-411	Relief valve, CO2 feedback line	316 SS	Hoke	6548G4Y	relief valve; max pressure 3000 psig, 207 (bar) Temp. range: -29 +177 ºC(Viton ring) CV: 0.1
V-412	CO2 purge valve	316 SS	Hoke	3752 G4Y	Needle valve; max .pressure: 5000 psig (345 bar) Temp. range: -54 to 232 ºC CV: 0.07 to 1.1
V-413	Check valve, return line of CO2	316 SS	Hoke	6133G4Y	Check valve; max pressure 6000 psig, 414 (bar) Temp. range: -29 +177 ºC(Viton ring) CV: 0.3 , 2.4

Instrumentation and control

Code	Description	Sensor			Actuator			
		ID.	type	Range	ID.	type	Material	Type of signal (range)
Temperature control systems [1]								
TT-2001	T transducer in CO2 line	TT-2001	thermocouple type K, S310 3.18 x 150 mm	1100 ºC	TT-2001	Electrical heater		4-20 mA
TIC-2101	T control outlet E-210	TT-2101	thermocouple type K; S310 6.35 x 150 mm	1100 ºC				4-20 mA
TIC-2301	T control in E-230	TT-2301	thermocouple type K; S310 6.35 x 150 mm	1100 ºC	TIC-1201	Electrical heater		4-20 mA
TIC-3001	T control in D-300	TT-3001	thermocouple type K; S310 6.35x 300 mm	1100 ºC	TIC-3001	Electrical heater		4-20 mA
TIC-3001	T control in D-300 (wall extractor)	TT-3002	thermocouple type K; S310 6.35 x 300 mm	1100 ºC	TIC-3002	Electrical heater		4-20 mA
TIC-3301	T control in E-330	TT-3301	thermocouple type K; S310 6.35x 150 mm	1100 ºC	TIC-3301	Electrical heater		4-20 mA
TIC-3501	T control in E-350	TT-3501	thermocouple type K; S310 6.35 x 150 mm	1100 ºC	TIC-3501	Electrical heater		4-20 mA
TIC-4001	T control in D-400	TT-4001	thermocouple type K; S310 6.35 x 300 mm	1100 ºC	TIC-4001	Electrical heater		4-20 mA
TIC-4001	T control in D-400 (wall extractor)	TT-4002	thermocouple type K; S310 6.35 x 300 mm	1100 ºC	TIC-4001	Electrical heater		4-20 mA
Pressure control / indicator systems								
PIC-3001	P control in D-300	PT-3001	Ceramic sensor P transducer[2]	400 bar	PIC-3001	auto valve		3-15 psi
PIC-3002	P control in E-330	PT-3002	Ceramic sensor P [2]	400 bar	PIC-3002			
PIC-4001	P control in D-400	PT-4001	Ceramic sensor P transducer [2]	400 bar	PIC-3001	auto valve		3-15 psi
PI-4001	P indicator in CO2 return line	PT-4003	pressure gauge	1-100 bar				
PT-1102	P transducer outlet P-110	PI-1101	Ceramic sensor P	400 bar	PIC-3001	auto valve		3-15 psi

		Sensor			Actuator			
			transducer [2]					
PI-1101	P indicator outlet P-110	PI-1101	pressure gauge	1-250 bar				
PI-2001	P indicator outlet CO2 cylinder	PI-2001	pressure gauge	1-100 bar				
PT-2002	P transducer outlet P-110	PI-1101	Ceramic sensor P transducer [2]	400 bar	PIC-3001			
PI-3001	P indicator outlet of D-300	PI-3003	pressure gauge	1-250bar				
PI-4001	P indicator outlet of D-400	PI-4001	pressure gauge	1-100bar				
PI-4002	P indicator CO2 return line	PI-4002	pressure gauge	1-100 bar				
Flow mass control systems								
FIC-2201	CO2 Mass Flow	FT-2201	Coriolis Mass Flowmeter[3] Nominal mass flow: 0.6 kg/min	0.6 kg/min -20-120 ºC	FIC-2201	**Frequency regulator**		
Other controlled systems								

Note[1]: Supplier: TC direct, Tel 91 840 6695; Fax 91 850 8302 , e-mail info@tcdirect.es
Note[2]: DESIN instruments, model TPR-14/ HP
Note[3]: RHEONIK, model RHF 07

Piping

Designation	***Material***	***Dimensions***	***Max. working pressure [1]***
Tubing 1/2" A269-AISI213 316/316L	*stainless steel 316L*	12.70 x 0.89 mm	*3090 psi*
Pipe 1/4" ASTM213	*stainless steel 316L, seamless*	6.35 x 0,84 mm	18700 psi

[1]Hoke, complete catalogue

Note: All Thermocouple and pressure sensors have NPT fitting

It was found that, for many of these parts, the delivery time was unexpectedly long and that the construction of the pilot plant will suffer a strong delay. In the next charts the order date and the delivery date for some of the parts are shown.

Containers			
Equipment	***Model***	***Ordering date***	***Delivery date***
Extractor	Autoclave Engineers Mod: EZE-SEAL pressure Vessel	01/04/06	15/06/06
Separador	Autoclave Engineers Mod: EZE-SEAL pressure Vessel	01/04/06	15/06/06
Extractor Heating system		01/04/06	15/06/06
Separador Heating system		01/04/06	15/06/06

Pumps			
Equipment	***Model***	***Ordering date***	***Delivery date***
CO2 Pump	Dosapro Milton Roy Mod: MB-140-L-12-M-200/J	20/12/05	15/05/06
Entrainer pump	Dosapro Milton Roy Mod: MD-140-G-4-M-200	20/12/05	15/05/06
Velocity Variator	Variador ACS101-2k7-1	20/12/05	15/05/06
Velocity Variator	Variador ACS101-K75-1	20/12/05	15/05/06

Automatic control valves			
Equipment	***Model***	***Ordering date***	***Delivery date***
Extractor 1 Exit Valve	Iberfluid Mod: Research 807 Rc200	25/04/06	15/06/06
Extractor 2 Exit Valve	Iberfluid Mod: Research 807 Rc200	25/04/06	15/06/06
Separador Exit Valve	Iberfluid Mod: Research 807 Rc200	25/04/06	15/06/06

Manual Process valves			
Equipment	***Model***	***Ordering date***	***Delivery date***
Open/Close valve	Iberfluid-	22/05/06	15/07/06
Needle valve	Iberfluid	22/05/06	15/07/06

Safety valve	Iberfluid	22/05/06	15/07/06
No return valve	Iberfluid	22/05/06	15/07/06

Piping			
Equipment	***Model***	***Ordering date***	***Delivery date***
Filters	Iberfluid	22/05/06	15/07/06
Mixed:	Iberfluid	22/05/06	15/07/06

Instrumentation			
Equipment	***Model***	***Ordering date***	***Delivery date***
Flow-meter	Iberfluid Mod: coriolis Rheonik	10/04/06	15/05/06
Pressure meter	Jesús Suministros	19/05/06	15/06/06
PID	Electrosón Castilla Mod: LS3300DRLC	16/12/05	15/05/06
RS232-RS485 Conversor	Electrosón Castilla Mod: LS3300DRLC	16/12/05	15/05/06

Heater			
Equipment	***Model***	***Ordering date***	***Delivery date***
Electric resistences	WATLOW GmbH Mod: Mica Strip Heater	10/04/06	05/05/06
Metallic bloq	Own development	3/04/06	15/07/06

Task 3.4: Final Tuning

The technical objective of this task was to adjust the working parameters of the control system and to characterise properties of the process. During this task, the prototype has been adjusted to operate in a different range of conditions, which fulfils the required conditions of the "Extranat Process".

It was only possible to develop this task once the plant was totally assembled, and once it was ensured that all was working properly. According to the selected control strategy during design stage, the control of the prototype was decided to be based in a set of a distributed PID controller. Those controllers allow to perform a proportional action on the actuators of the system (valves, pump and heaters) so the operation can be carried out controlling the working variables automatically.

Every PID controller forward different parameters to a CPU to obtain a time register. The main parameters selected to store were: physical value (pressure, flow or temperature), estate of the action (percentage of the action) and state of the alarm (on/of).

Tuning of PID controllers

Although at the beginning it was expected to tune the controllers by focusing on the short-term dynamics of the process time, the results showed that the system turned unstable with the time. This situation could be derived from the non-linear behaviour obtained during the expansion and as a consequence of the use of a compressible fluid such as the supercritical CO2. A so traditional method for PID tuning was applied, and in consequence, it was required more time than the expected to perform this task.

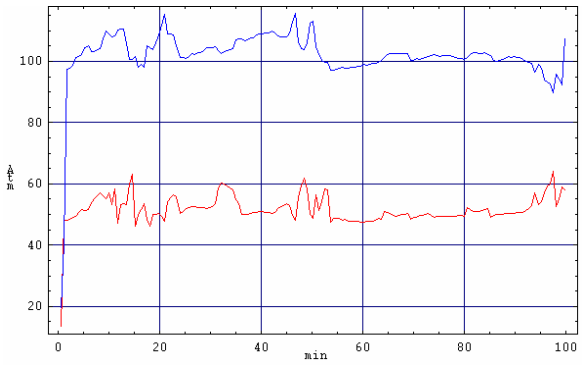
To perform the tuning of the controllers the following method was used:

- Fist step was to ensure that all the equipment was working properly. Safety consideration were specially taken into account since the evolution of the prototype could produce some risky situations (i.e. rupture disk were broken in some of the trials).

- Second step was to stabilize the process according to the set of selected conditions to be used in different extractions. Then the system was disturbed by different methods (mainly "bumping" the controller output), then the system was left evolve to the stabilisation situation. Time functions of the controlled variable with the time were obtained.

- Third step was based on the study of the shape of the functions to determine the more effective parameters to set the configuration of the controller: Proportional band, derivative time and integral time.

Final values were collected in the operations manual as reference but can be modified as a part of the configuration or way of work of the prototype.



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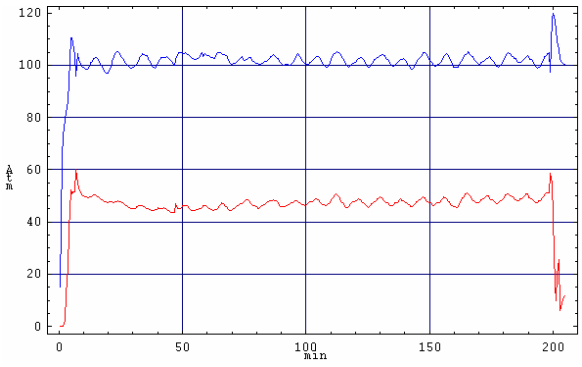

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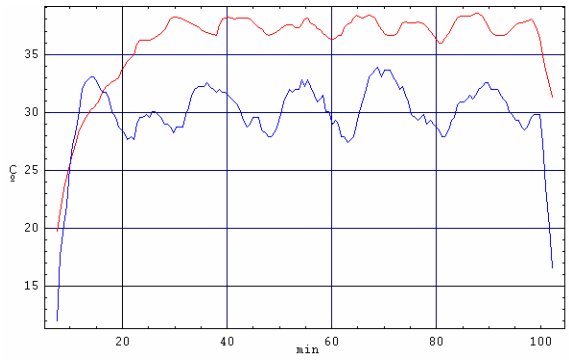


C

Figure:.Examples of some of the improvements in the prototype once the tunning actions were applied. (A) Evolution of the pressure in extractor (blue) and separator (red) before tuning the controllers of expansion valves. (B) Evolution of the pressure in extractor (blue) and separator (red) after tuning the controllers of expansion valves. (C) Evolution of the temperature in extractor (blue) and separator (red) before tuning the heaters controllers. (D) Evolution of the temperature in extractor (blue) and separator (red) after tuning the heaters controllers.

Deviations from the project workprogramme

The development of this workpackage has differed in several ways from what was firstly programmed.

Its starting point was supposed to be month 3, right after the finalisation of Workpackage 1 and in parallel to workpackage 2. When this time came, June 05, the workpackage started but it was early clear that not much advances could be done before some conclusions were reached in WP 2. This caused a delay of several months. The length of the tasks was not compromised and it was intended to be maintained.

Thus task 3.1 started in Month 6 (September'05) and lasted until Month 7 (October'05), while task 3.2 started at the same time and it lasted until Month 9 (December'05).

Task 3.3 was affected in the same way by this delay, it started on month 9 instead of in month 6. But this task also suffered another delay. This delay was caused by circumstances from outside the consortium. Once the parts to build the pilot plant were chosen among those offered in the market, it was found that in some of them, mainly the pumps, the delivery time was much longer than expected. In some of them this period went up to 6 months. Therefore, this deley impeded the starting on time of task 3.4: Final tunning before the end of the first period.

In order not to stop the technical development of the project, it was agreed to continue with the characterisation of the extraction, including both the process and the extracts. The extraction tests were carried out using the Supercritical fluid extraction plant located at Cartif facilities. The results achieved in this plant were found succesfull, and so it was decided to design the Extranat pilot plant taking as a starting point the design of the existing plant. This facilitated the assumption that the extracts obtained from the future plant will be very similar to those yielded by the Cartif's plant.

The analysis made over the samples of extracts were made thinking on those that are programmed to be done on the extracts from the pilot plant in Workpackage 6. This way the delay on the construction of the pilot plant was absorved by working in advance in tasks programmed after this construction.

As it was explainede before, Task 3.4 was only possible to develop once the plant was totally assembled, and once it was ensured that all was working properly, what happened in August 2006.

List of Deliverables and Milestones

Only Milestone 2: *Design Parameters for the SFE Pilot Plant* was assigned to the development of this Workpackage.

Milestone no.	Milestone Name	WP no.	Date due	Actual delivery date	Lead contractor
2	Design Parameters for the SFE Pilot Plant	3	Month 12 (March'06)	Month 12 (March'06)	Envip

No Deliverable was programmed for this Workpackage.

Workpackage 4: Manufacturing process establishment at laboratory scale

The purpose of the workpackage 4 was to reproduce the results of the extractions carried out in the WP2 and to identify possible failures and problems when applying to different substances. The development of the workpackage has been based on the following points:

Supercritical fluid extraction of representative liquid fractions obtained from different raw materials: carrot, tomato and grape pomace.

Verification of the best process conditions for yield improvement for the required product (alternative conditions were also evaluated)

Adaptation of prototype to perform the final process.

Task 4.1. Supercritical Fluid Process Running

The initial extraction process expected at the beginning of the project was varied, so the initial solid-liquid supercritical extraction has been substituted by a double operation composed of two stage: direct solid-liquid extraction with grass solvent followed by the liquid to supercritical fluid extraction. The advantages of this operation mode have been reported in previous workpackages.

In this sense, the number of extractions considered in the technical annex was adapted to the actual workload and new process. The extraction of carrot, grape pomace and tomato was decided in this stage.

Next tables shows some examples of the most relevant conditions used during the development of this task for some of the raw material.

Grape pomace		Preparation of origin extract Solvent: Ethanol Time 20 h Temperature 40º C Nitrogen atmosphere		Antisolvent SF extraction Extractor temperature 40º C Expansion temperature 35º C Separation temperature 35º C	
Sample	Raw material pre-treatment	Mass/solvent ratio	Origin extract feed	Extraction pressure	Separation pressure
Number	Conditions	g/ml	ml/min	Bar	bar
3	Fresh	0.33	3	90	75
4				150	90
5				120	90
6	Freeze dried	0.1	3	90	75
7	Freeze dried +H20	0.1	3	90	75
8				120	75
9				150	90
10	Fresh	0.33	6	90	75
11			9		75

12			12		75
13	Fresh	0.33	15	120	90

Tomato		Preparation of origin extract Solvent: Ethyl acetate Time 20 h Temperature 40º C Nitrogen atmosphere		Antisolvent SF extraction Extractor temperature 40º C Expansion temperture 35º C Separation temperature 35º C	
Sample	Raw material pre-treatment	Mass/solvent ratio	Origin extract feed	Extraction pressure	Separation pressure
Number	Conditions	g/ml	ml/min	Bar	bar
T1	Freeze Dried	0.33	3	90	75
T2					
T3	Freeze Dried	0.04	5	90	70

Task 4.2. Result analysis and readjustments

The technical objective of the task was to analyse the result obtained in previous task. In order to assist a better comprehension of report, the results are commented for every raw material.

CARROT

Although different extractions were performed using carrot ethyl acetate extract, it couldn't be determined the antioxidant capacity (AO) of final extracts. Several trials were done using TEAC method but at the end it failed, since the effect of the oil faded carotenoids antioxidant effect.

It is needed to add sunflower oil to the initial ethyl acetate solvent for SFE process, in order to trap the compounds of interest. However, AO of sunflower oil was really much higher than the one due to carotenoids, so their AO was faded by the own AO of oil. DPPH showed to be equally failed.

Therefore, we try to set up ORAC method protocols, since Huang D. et al analysed lipophilic antioxidants using randomly methylated beta-Cyclodextrin (RMCD). Finally, it was no possible to solubilise carotenoids with RMCD since it was observed turbidity in all samples for this method. On the other hand, the equipment acquired to perform fluorescence measurement didn't work properly and it was needed to acquire a different model. Currently we are under development of the proper methodology to apply ORAC to non polar method.

However, carotenoids quantity was successfully measured by HPLC DAD to obtain a measurement of the behaviour of the process and prototype. The obtained results showed behaviour similar to the observed with previous work packages.

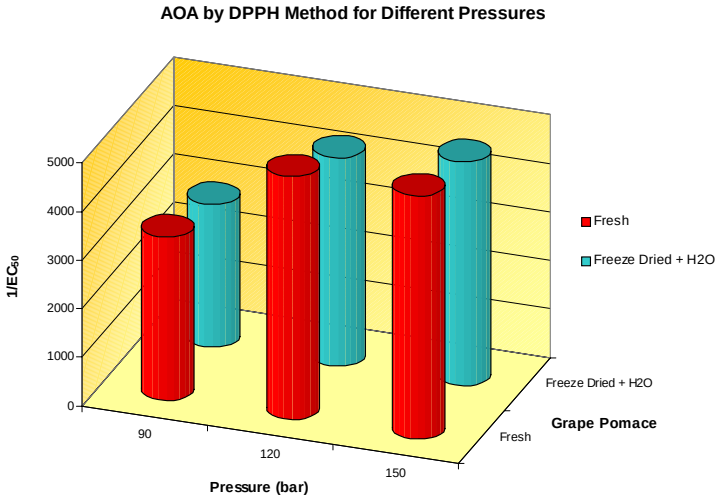
GRAPE POMACE

Several activities were done with the grape pomace: Antioxidant Activity, Water Content, Ethanol Content and Colour for conditions of extraction with the supercritical pilot plant.

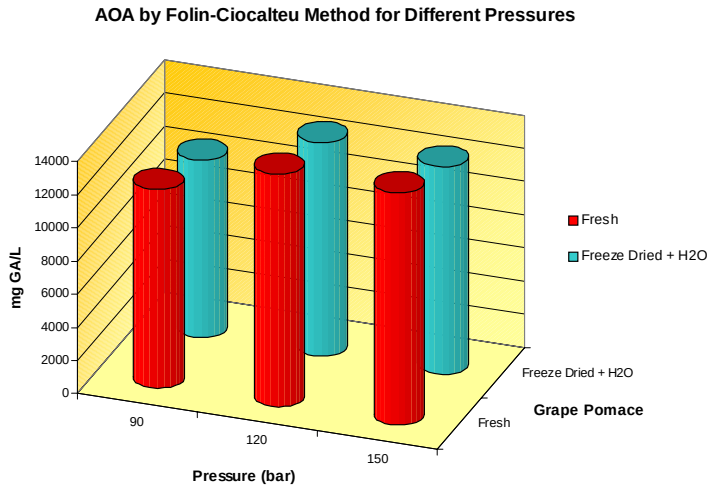
Antioxidant Activity (AOA)

Measures were done by DPPH method (Brand-Williams W. et al.,1995, protocols) and Folin-Ciocalteu method (Waterhouse A. et al., protocol).

Antioxidant activity was measured by two methods in order to validate and compare results. The methods used were Folin-Ciocalteu and DPPH, following the protocols described in previous reports. No modifications were made.

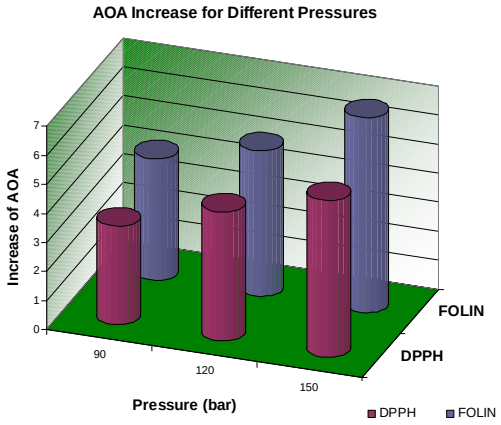


AOA by DPPH method for different pressures and for fresh and freeze-dried + H2O grape pomace



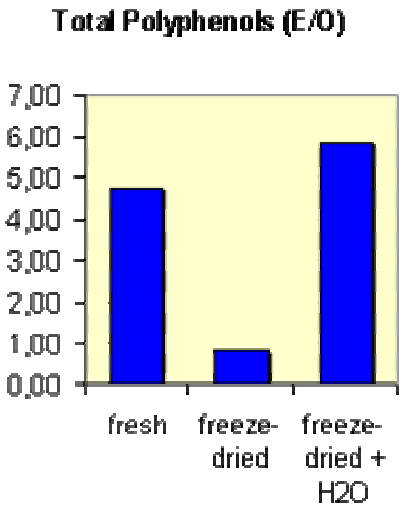
AOA by Folin-Ciocalteu method for different pressures and for fresh and freeze-dried + H2O grape pomace

AOA in the extract recovered from the Extractor Unit increases with pressure for both Folin and DPPH. (See following fig.)



AOA Increase for different pressures for Fresh Grape Pomace.

When fresh grape pomace (GP) was used applying different pressures with the SFE plant (90, 120 and 150 bar) an increase in the AOA was produced. These results are show in the fig.2, whose data have been obtained dividing the Extractor AOA by the Origin AOA in both methods. It can be appreciated that there is an increase of the AOA in the extract for fresh GP between 4-7 times the one of the origin for Folin and of 3-5 times for the DPPH method. When the GP is freeze dried the variation of the increase with pressure is less noticeable than with fresh GP (between 5,9-6,5 for Folin and 3,5-4,1 for DPPH).

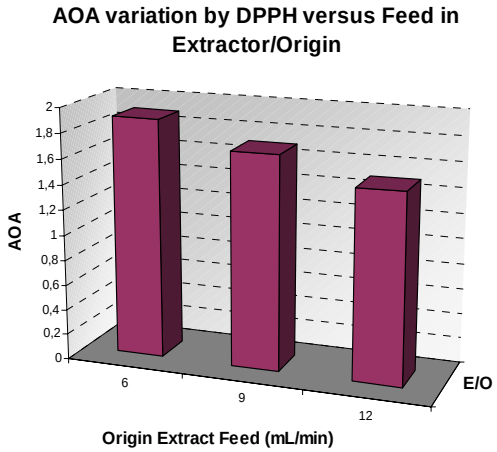


Variation of polyphenols content using Folin method vs. raw material pre-treatment

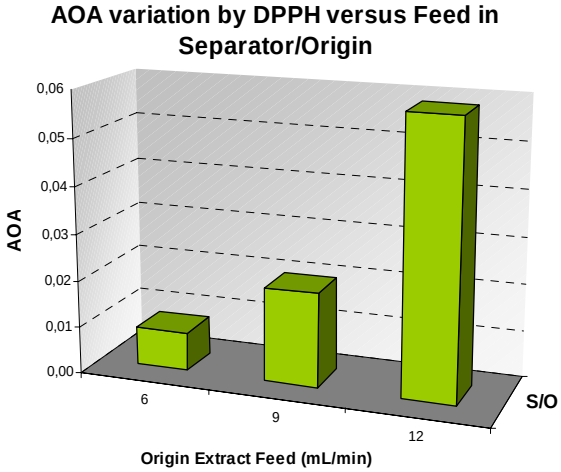
Water is required to retain the polyphenols compounds in the extractor unit: if the Origin Extract is obtained from freeze-dried grape pomace and no water is added, the increase of

concentration of the antioxidant compounds in the Extractor Unit is not achieved (TPExtractor/TPOrigin ~ 0,8).

Then, the variations of co-solvent-CO2 flow rate ratio were studied for fresh GP at 90 bar of pressure and compared with their AOA variations. We found that when feed of co-solvent-CO2 increases AOA decreases for extractor/origin and the opposite behaviour was found for the separator/origin.



AOA variation by DPPH method vs.Co-solvent feed in Extractor/Origin

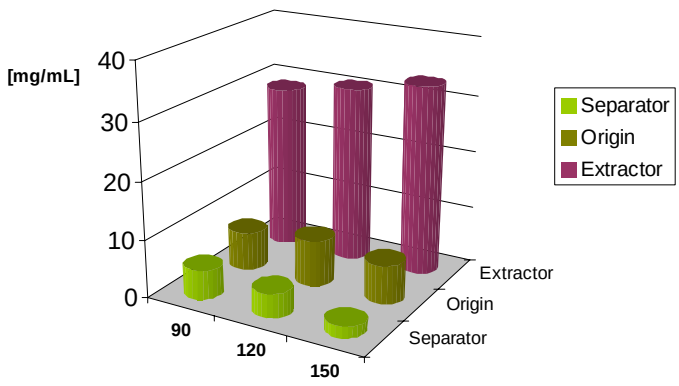


AOA variation by DPPH method vs.Co-solvent feed in Extractor/Origin

Dry extract

To estimate dry content, measures were taken weighting 2 mL of sample in a vial and keeping it into the oven at 105ºC for until all the water was removed (once the weight keeps constant). Difference between initial and final weight of sample is the total content of water.
The weight of the dry sample was divided by the used volume in order to calculated the g of dry extract/ mL solution.

Dry Extract [mg/mL] from Fresh GP



Final A

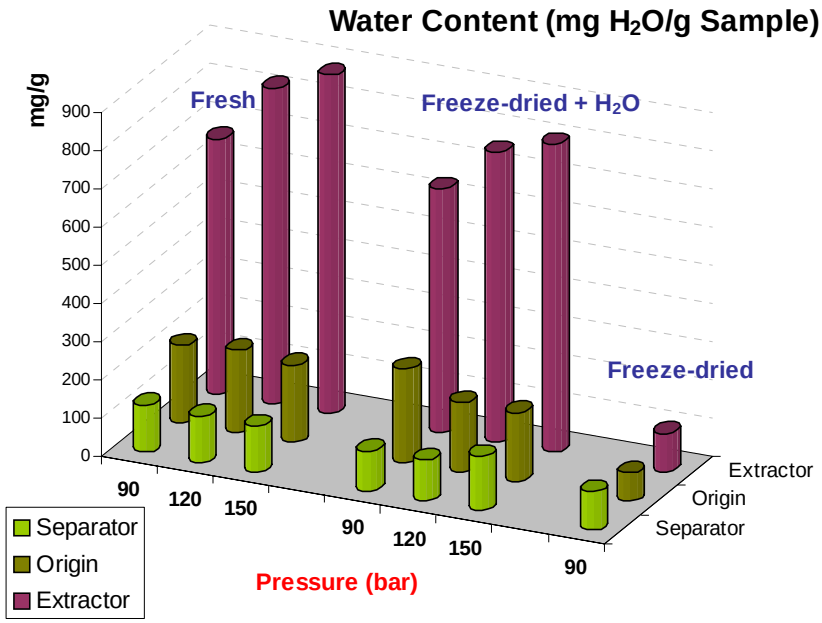
Dry extract from fresh GP

When pressure increases, the dry extract is higher in the Extractor extracts and decreases in the separator extracts.
The Total Dry Extract increases about 4 times in the extract recovered from the Extractor Unit

Water Content
It was required to determine the water content in extractor. It was achieved using Karl-Fischer method with a Tim 880 Titration manager from TitraLab, Radiometer Analytical apparatus. Reagents: Aquagent R Titran 2, titrant component for volumetric K-F titration; Aquagent R Solvent CM, solvent component for volumetric K-F titration both from Scharlau. Hidranal R Water standard 10,0, from Riedel-deHaën was used as standard.

Procedure:

Sample was diluted 100 times in solvent (methanol).
The quantity of sample introduced (around 1 ml) in the equipment was measured.
Then, this data was introduced in the apparatus and it calculates automatically the mg of H2O /g of sample introduced.
A blank of MeOH was done to consider possible presence of water in the solvent.
A correction factor made with the standard of Hidranal was carried out dividing theoretical value of the standard by real value found for the standard.
Quantity of water given by the apparatus was multiplied by the correction factor, in order to have the real value for the H2O content in the sample.



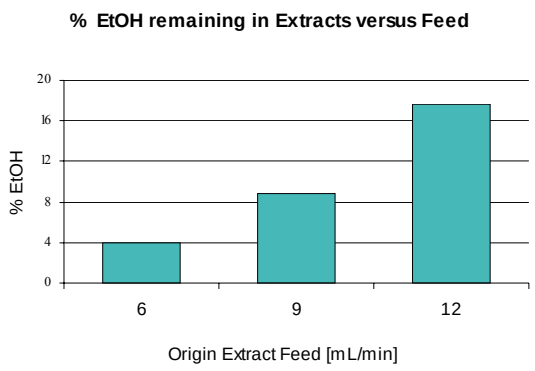
Water content for Fresh, Freeze-dried + H2O and Freeze dried GP extracts.
Both in the case of fresh GP and freeze-dried GP when water is added to the Origin Extract, between 55 and 65% of the total water is recovered in the Extractor Unit.
For origin, extractor and separator extracts coming from freeze dried grape pomace, no differences were found and separation was not really produced.

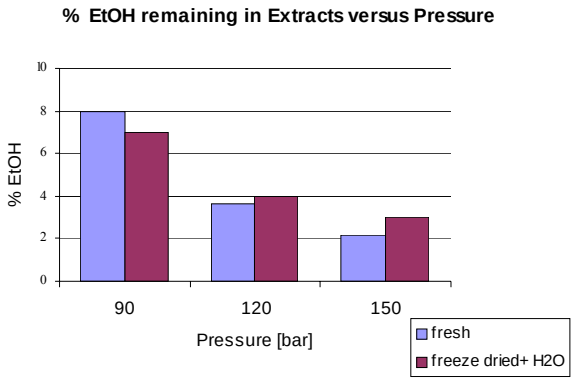
Ethanol Content

Ethanol content was obtained discounting the water content value from the whole extract.

For the prototype and during Extranat Project it was found that EtOH is removed from the extract more effectively with lower feed and higher pressure.

This effect can be observed in figures 9 and 10.





%EtOH remaining vs. Feed.

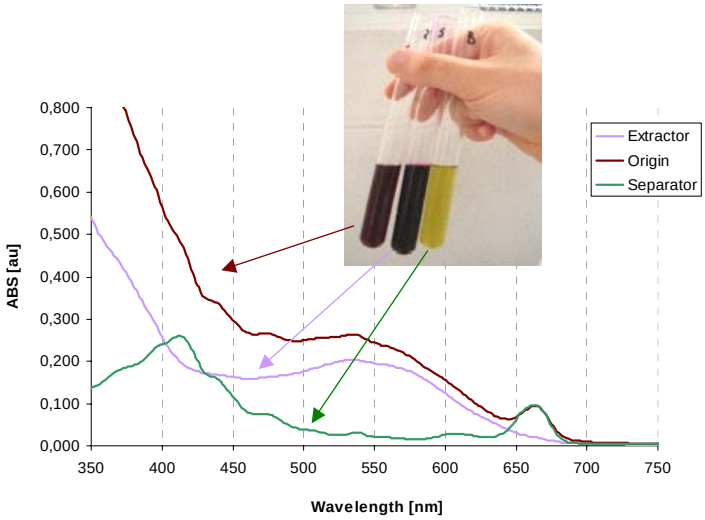
%EtOH remaining in extracts vs. Pressure.

Colour

Optical differences in colour in extractor, separator and origin extracts were found, due to the separation process for the different vessels (see photograph and figure below)

Different colours for the extractor, separator and origin extracts.

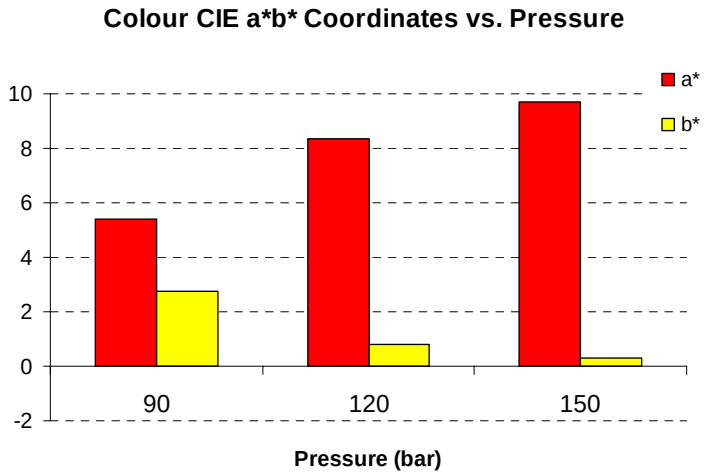
Spectra: origin, separator and extractor samples.



Spectra for Origin, extractor and separator extracts

A quantitative separation between antioxidant compounds (polyphenols) and pro-oxidant compounds (chlorophylls) was achieved in the extractor and separator units respectively. In this picture, chlorophyll absorbance peak can be seen around 660 nm for separator and origin extracts while this peak is not present in the spectra of the extractor extract.

Colour was measure with CIE L*a*b* system, which is a mathematical derivative of CIE XYZ (1931) that describes colours using three synthetic primaries. The standard illuminant was D65 and 10 degrees for the observer were used. % of transmittance was measured with the spectrophometer (UV-Pharma Spec 1700 Shimadzu) and then L* (lightness or brightness), a* (red-greenness) and b* (yellow-blueness) parameters values were calculated.



Colour CIE a*b* coordinates vs. Pressure.
When applying increasing pressures (90, 120 and 150 bar), an increase in the red colour and a diminution in the yellow component is appreciated (see previous fig).

TOMATO

Before introducing the ethyl acetate solution obtained from tomato in the SFE plant, sunflower oil was added to favour the retaining of carotenoids in the extractor. The idea was to introduce a substance with a higher tendency to retain non-polar compounds than CO2 while the ethyl acetate was removed completely. By this method a liquid food grade product can be obtained ready to use.

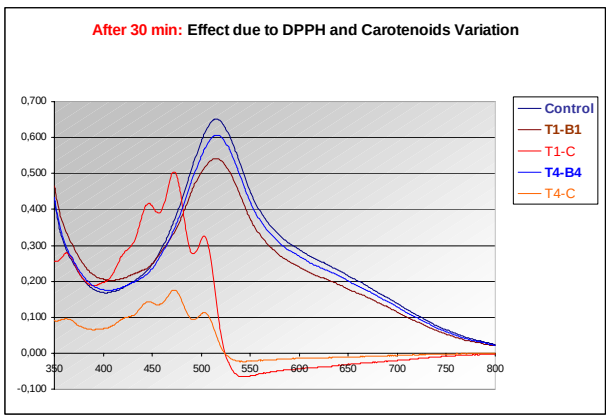
Antioxidant Activity (AOA)

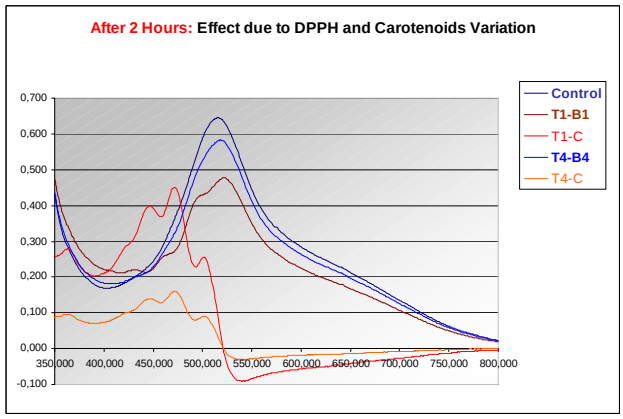
AOA for freeze-dried tomato extracts was 0,09 (1/EC50, mg dry Tomato/ mL solution) in the origin extract in ethyl acetate (EtAc) and before adding sunflower oil.
DPPH method was used with a blank, since carotene and DPPH absorbance overlaps and some fading can be observed.

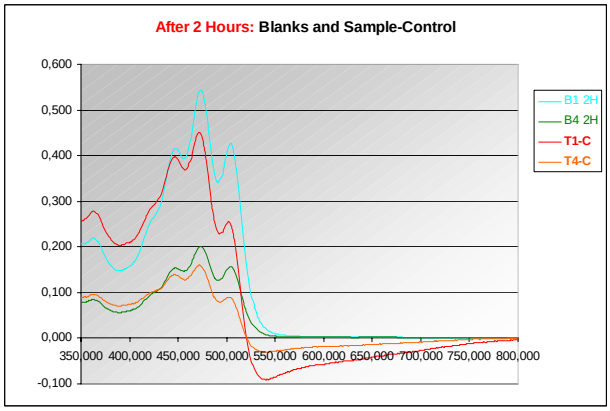
Although blank samples didn't change in 2h, samples containing lycopene varied and this was related with certain lycopene degradation. AOC was a bit overestimated. See fig 14.
However, AOC was not very different when other wavelengths were used, in which both absorbance did not overlap (ie. 560 and 580 nm). Although the selected wavelengths were not corresponding to the maximum absorbance peak of DPPH, they could be fairly used for this purpose.

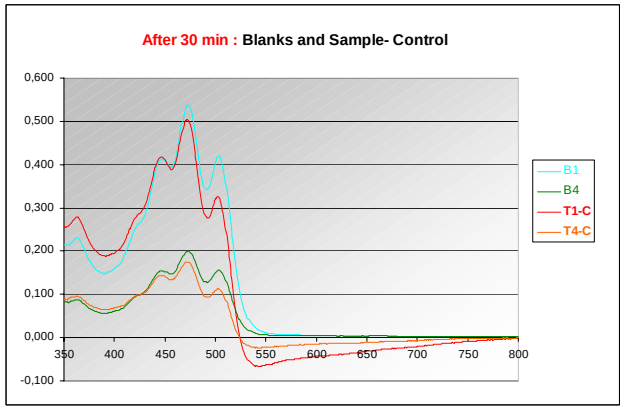
Table : AOA of Freeze-dried Tomato for different wavelengths at 30 min.

T 3-O	AOA (mg dry tomate/mL solution)		
t min	1/EC50516	1/EC50560	1/EC50580
30	0,09	0,08	0,08





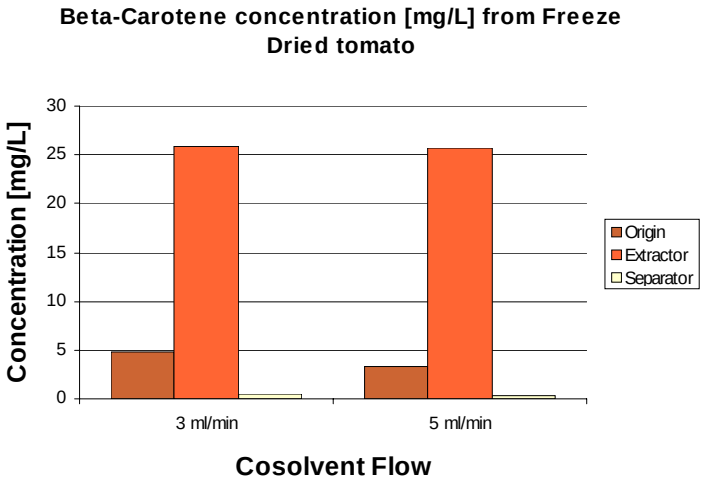




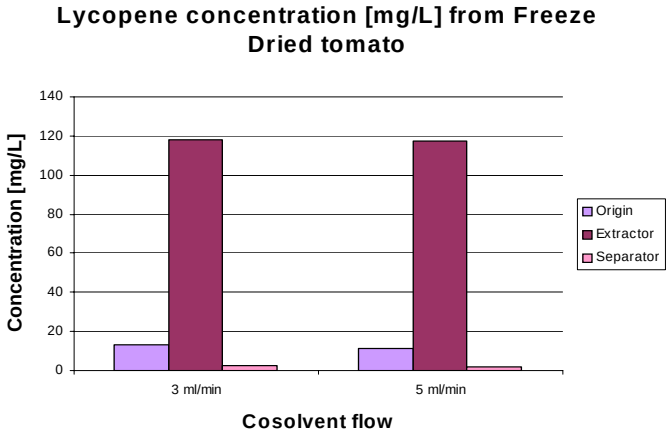
Spectra of DPPH and carotenoids variation with time.

It was not possible to measure AOC in oily samples with DPPH method, since the effect of the oil fades lycopene and carotenoids effect, (at least for the oil-carotene concentrations used), which were the same obtained with SFE under working conditions.

Quantification of Compounds of Interest



Beta-carotene concentration vs. Co-solvent flow

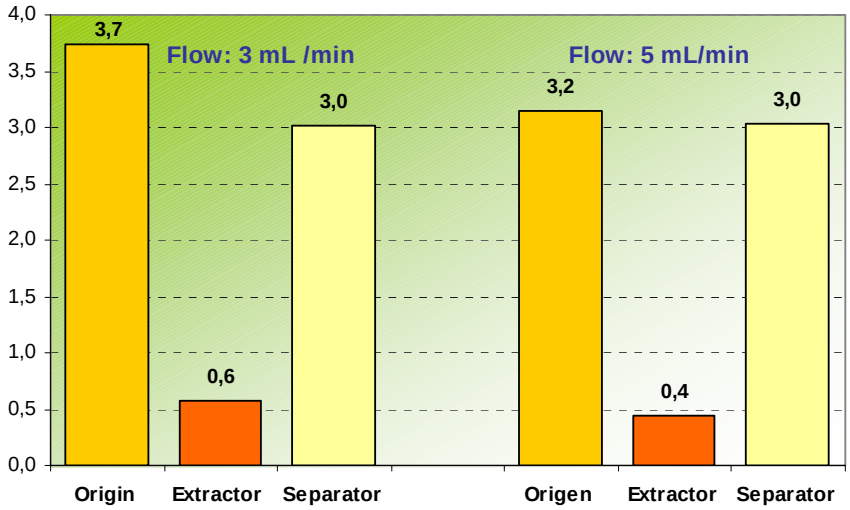


Lycopene concentration vs. Co-solvent flow

Beta-carotene concentration in Extractor is around six times the one of the Origin.
The optimum Co-solvent Flow was 5 ml/min for these trials

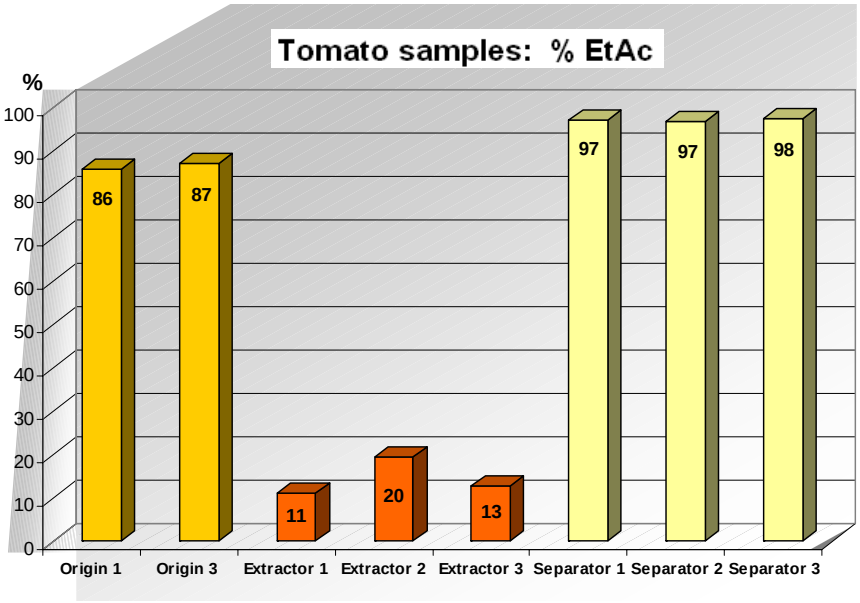
Water Content
There are not significant differences in water content concerning these CO2 flow variations.

In the extractor (Flow 5), there is less water, however its initial content in water was lower in the origin too.



Water Content (mg H2O/ g Sample) for freeze-dried tomato samples

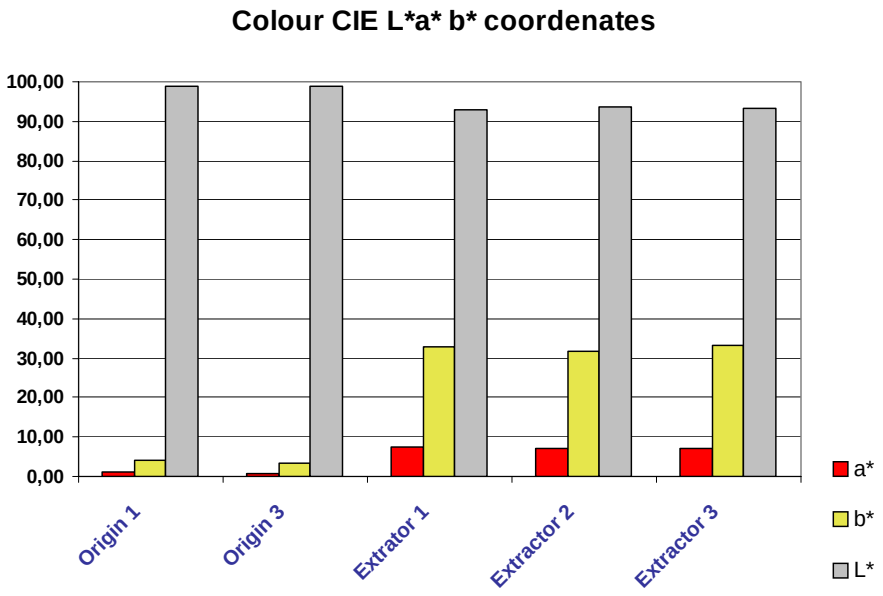
Ethyl Acetate Content



Ethyl acetate content in tomato extracts.

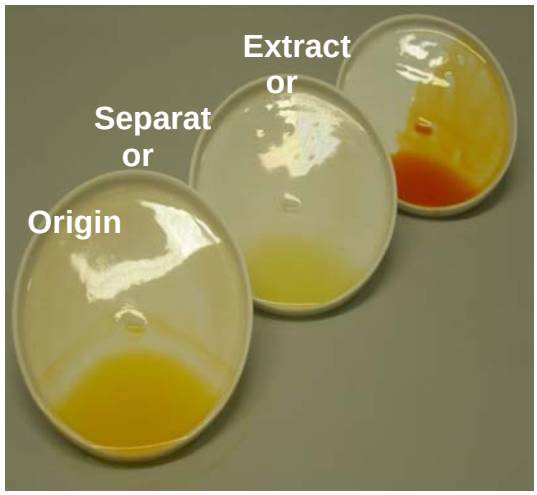
There are not significant differences in the % Ethyl acetate for the samples taken. Although, huge differences were found concerning the extractor, separator and origin extracts.
The percentage of Ethyl acetate of the extractor has diminished a 80% when compared with the origin.

Colour

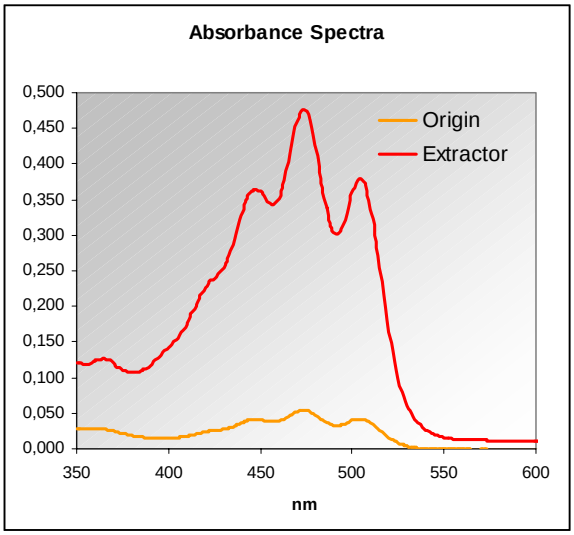


Colour analysis for tomato samples

Extractor extracts were really stronger orange coloured, while origins were more pale yellowish coloured. This is due to the higher carotenoids concentration in the extractor extracts. See following figures:



Colours of origin and extractor and separator vessels extracts.



Spectra for tomato before and after SFE.

Task 4.3. Set up of the optimised system

During this task different correction were carried out to optimise the final selected process. As it has been shown, the process consists on concentrating ethanolic solution for the grape pomace and a solution of ethyl acetate for the tomato and the carrot using supercritical fluids. The objective is to remove the maximum quantity of solvent, remaining a watery solution for the grape pomace and an oleaginous solution for the carrot and the tomato.

Several trials to study the CO2 solubility with the two types of solvents used were carried out. The solvents used in the process were ethanol and ethyl acetate. The aim of these experiences was to determine for each operation pressure the best co-solvent flow.

The optimum co-solvent flow for the specific operation conditions is defined as the necessary flow for the CO2 to extract the whole solvent used in the process. This is a basic point, since we are able to determine the operation time of the extraction plant minimizing the operation costs.

The goal of the experiences was to determine the solubility in CO2 of the co-solvents used in our process. The experience was carried out by replicating, varying the co-solvent percentage, the extractor pressure and its application or not inside the extractor with a spray nozzle.

Co-solvent	Flow (g/min)	Pressure (bar)	Co-solvent Flow (mL/min)	Spray Nozzle
Ethanol 96%	100	120	3	Yes/No
Ethyl Acetate		90	5	Yes/No
			10	No

Table : Experimental parameters and their values

At the end, an optimum rate of co-solvent flow per 100g CO2/min was obtained. Characterization was carried out for the ethanol and the ethyl acetate and for different pressures (between 90 and 150 bar), different percentages of solvent per CO2 flow (3%, 5% and 10%), as well as a modification in the extractor applying or not a spray nozzle.

Determining the effect of ethanol in the process.
The operation process consisted on introducing the co-solvent in the CO2 line just after impulsion pump of the liquid CO2, the process was developed in open loop. The fixed operation conditions were: the flow of 100 g/min, pressure in the separator of 30 bar and temperature of 50ºC, except in the separator, which is of 40ºC.

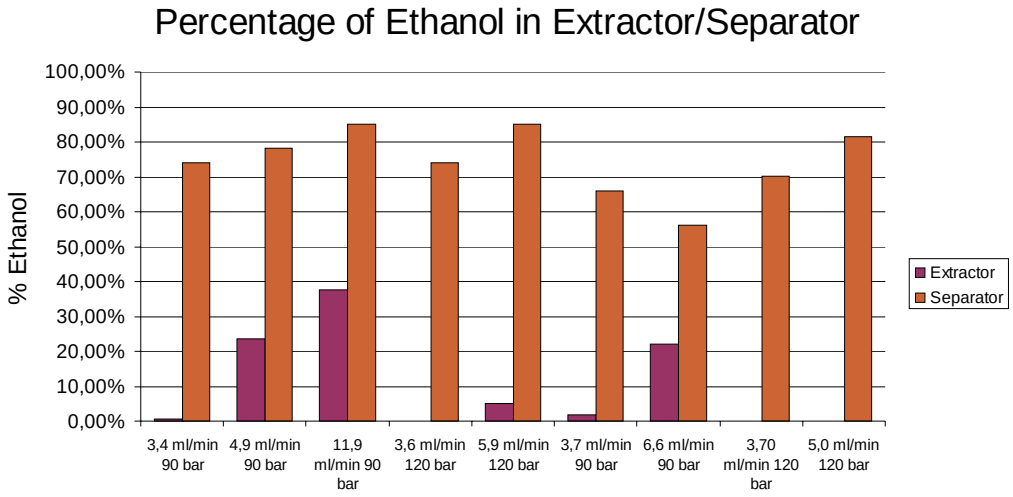
Measures of the solvent (ethanol) retained in the extractor and in the separator for several conditions were performed, with and without spray nozzle in the entrance of fluid to the extractor will be carried out. The operation variables was obtained by setting the CO2 flow at a fixed value at 100 g/min and varying the co-solvent flow for introducing a percentage of 3, 5 and 10 % in main CO2 stream, and for pressures ranging between 90 and 150 bar.

In order to estimate properly the effect of different working conditions, the plant was initially charged of clean CO2 and later, the free co-solvent (or co-solvent containing vegetal extract) was feeded during constant time periods. Total CO2 costs was quantified and as well as the solvent volume obtained in the separator and in the extractor. Next table shows some of the obtained results.

Pressure (bar)	Cosolvent Flow (ml/min)	Spray Nozzle	Extractor Solubility (ml/min)	Separator Solubility (ml/min)	Total Solubility
90	3,4	No	3,4	0,9	Yes
90	5,0	No	3,8	1,1	NO
90	11,9	No	7,4	1,8	NO
120	3,6	No	3,6	0,9	Yes
120	6,0	No	5,7	0,9	Yes
90	3,7	Yes	3,7	1,3	Yes
90	6,6	Yes	5,2	2,9	NO
120	3,7	Yes	3,7	1,1	Yes
120	5,1	Yes	5,1	0,9	Yes

Table : Solubility of Ethanol for different parameters.

Values, which gave a total solubility in the extractor are in blue colour. This means that the whole solvent has been extracted. As it can be observed, it can be obtained the complete solubility at 90 bar when co-solvent is feed at 3,5 mL/min. If working pressure is increased (i.e. 120 bar) the total feed of co-solvent can be risen up to 5,7 mL/min.



Percentage of Ethanol in the Extractor and the Separator

In conclusion, when working with ethanol or ethanol solutions, the increase of the co-solvent flow causes an increase of solubility in the extractor. The increase of the operation pressure causes an increase of the solubility in the extractor.

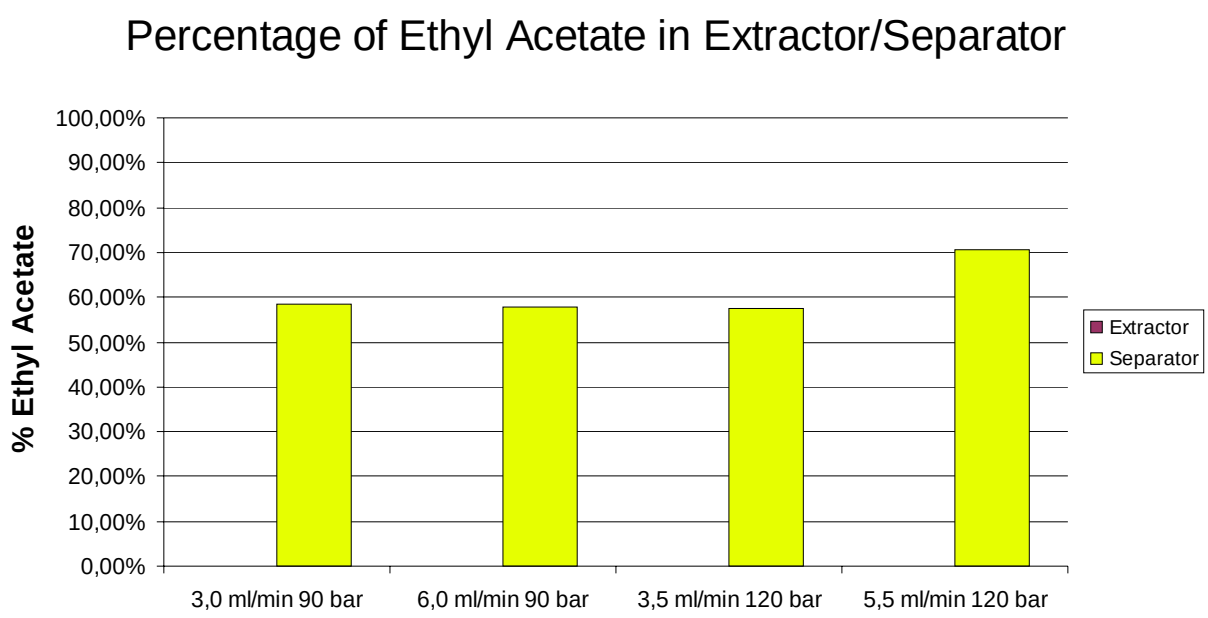
The co-solvent percentage modifies the capacity of solubility of the CO2. An increase in pressure gives a greater solubility. The used mouthpiece system is either ineffective or the drive process in the container is not effective due to the little surface in the containers to take place an effective atomisation.
As we can observe the solubility increases in the extractor with the pressure and with the quantity of co-solvent to introduce. The CO2 has a bigger solvatation capacity for the ethanol at a bigger pressure. The increase with the quantity of introduced co-solvent can be due to the biggest solvent readiness to extract and to drag. The application of a system with mouthpiece in the extractor at 90 bar improves the solubility, probably because the mixture between the CO2 and the solvent improves, while at 120 bar this effect is not noticed.

Determining the effect of ethyl acetate in the process.
As it was proceeded with ethanol, different conditions were used to determine optimal conditions

Pressure (bar)	Cosolvent Flow (ml/min)	Spray Nozzle	Extractor Solubility (ml/min)	Separator Solubility (ml/min)	Total Solubility
90	2,9	Yes	2,9	1,2	Yes
90	6,2	Yes	6,2	2,6	Yes
120	3,5	Yes	3,5	1,5	Yes
120	5,4	Yes	5,4	1,6	Yes

Table : Solubility of Ethyl Acetate for different parameters.

As we can see with the ethyl acetate the limits of solubility are bigger than those obtained for the ethanol, not being detected remains of solvent in the extractor for the studied relationships of co-solvent.



Percentage of Ethyl Acetate in the Extractor and the Separator

In conclusion, when working with ethyl acetate, the increase of the operation pressure causes an increase of the solubility in the extractor, although it was not possible to reach the upper limit. The mouthpiece use apparently doesn't produce changes.

The increase of the co-solvent flow causes an increase of solubility in the extractor.

This conclusions are similar to those obtained with ethanol but the acetate possesses a bigger solubility.

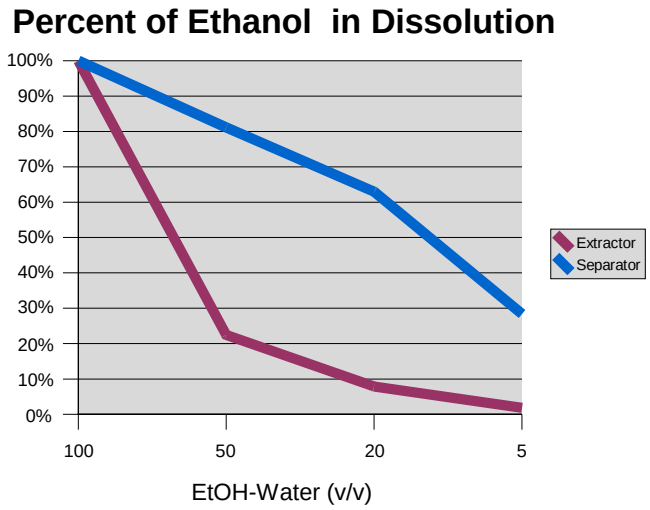
Cosolvent	Pressure (bar)	Solubility limits (ml/min)
Ethanol	90 120	3,5 6,0
Ethyl Acetate	90 120	6,0 6,0

Table . Solubility limits for Ethanol and Ethyl Acetate

These experiences were developed in order to obtain the best relationship co-solvent-CO2, with the objective of optimising the process. While for the ethanol we can apply a flow of co-solvent of 3 ml/min for the acetate this flow is above 5 ml/min. An increase of pressure has the inconvenience of increasing the extraction of compounds of interest in the separator being less selective the extraction process.
Therefore, it must be a commitment between the pressure and the flow to obtain the best conditions for the extraction.

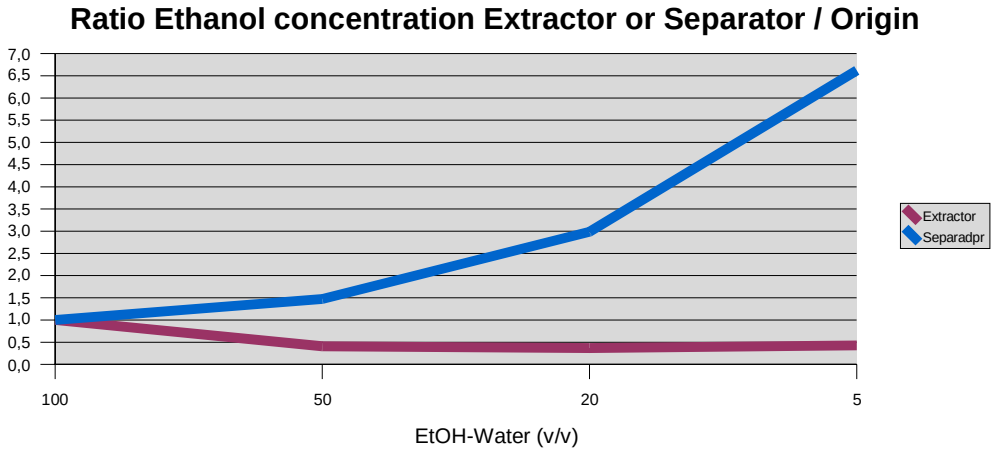
Finally some comments will be done on the use of hydroalcoholic solution in the process

Once the best operation conditions were determined, the next step was to characterise a hydroalcoholic solution with different percentages of Ethanol-water in order to know the behaviour in the pilot plant.
The process consists on introducing hydroalcoholic solutions varying the ethanol percentage in them for operation conditions of 90 bar and 50 ºC in the separator with a co-solvent flow of 3 mL/min.

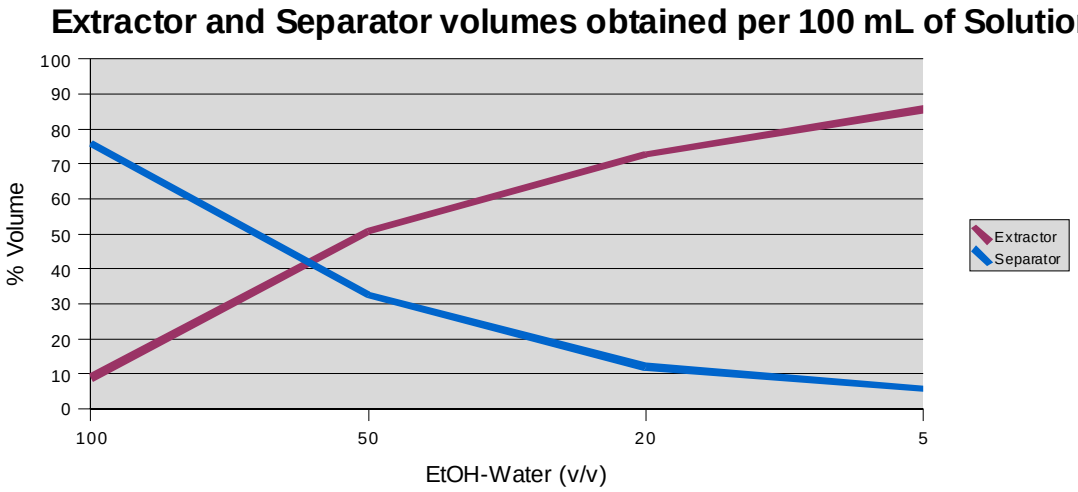


Percentage of ethanol in dissolution.

The percentage of ethanol from the solution in the separator is bigger than in the extractor and in the original dilution. This shows an extraction of the solvent. On top of the ethanol being extracted some water is retained too, so there is not a 100% of ethanol in the solution extract.
For a fixed pressure, the efficiency of ethanol elimination in the extractor is similar for the different concentrations used of ethanol-water in the original solution. It can be presumed that for a specific concentration, the concentration process is of 40%. In the separator, the concentration varies depending on the ethanol percentage of the solution.



Ratio Ethanol concentration Extractor or Separator divided by Origin.



Extractor and Separator extracts volumes obtained per 100mL of Solution.

All this results were considered in the elaboration o the operations manual used during the training tasks

List of Deliverables and Milestones

Deliverable no.	Milestone Name	WP no.	Date due	Actual delivery date	Lead contractor
4	Samples of extracted compounds to initiate optimisation of the system	4	Month 22 (January 07)	Month 22 (January 07)	CARTIF
6	Definition of Procedures and Working Methods	4	Month 28 (July 07)	Month 28 (July 07)	CARTIF

Milestone no.	Milestone Name	WP no.	Date due	Actual delivery date	Lead contractor
4	SFE Pilot plant optimised at experimental scale	4	Month 21 (December 06)	Month 21 (December 06)	CARTIF

Workpackage 5: SME Implementation. In Situ Measurements and Final Validation

The purpose of the workpackage 5 was to implement the prototype in one of the SE's facilities and to reproduce the results obtained along the project. Due to the modification happened during the projects as well as the delays in the execution of previous workpackage it was decided to **modify the order of the tasks** to be more efficient in the development of the tasks. So the it was decided to perform the training in CARTIF facilities and then to proceed with the rest of the tasks. This allowed to be more effective in the definition of requirements of the facilities in Matarromera.

Task 5.4 Training on the operational performance of the pilot plant

Technicians from Matarromera which would be in charge of the operation of prototype were instructed in different themes such as: basic principles of supercritical fluids and relevant aspects on the extraction with GRAS solvents. Especial considerations were taken into account considering safety condition when working with high pressure process.

The working methodology developed in previous workpackages contained in the operations manual of the pilot plant was fully explained in this task. Practical examples on the starting up and function of plant were provided.

Finally, the proper description on the methodology for assembly and disassembly of the plant was discussed. Maintenance was considered as a part of this topic.The technicians from Matarromera achieved the required skill to work with the prototype. This experience was very useful for the development of the following task.

Task 5.1 Transport and installation of the system at the SME facilities.

The objective of this task was to dispose of the proper space suitable for the installation of the prototype. In this sense it was necessary to provide the required auxiliary services such as socket and air connections.

At the same time, considering the information gained in previous tasks, different prototype lay out were considered to ensure a good integration of the supercritical extraction plant with rest of the facilities in Matarromera. Safety considerations as well as restriction in the access to the prototype was considered.

Finally, required information for the fulfilment of PED directive was collected.

Next figure shows some images of the final disposition of the prototype:

Final lay out of the prototype at Matarromera's facilities

Task 5.2 - Performance of measurements during processes and Task 5.3 Analysis / Validation of the measurements and final adjustments

The objective of these tasks was to start up the prototype and to proceed with the extraction of different solutions so relevant information was collected at industrial level. From the obtained data some small adjustments were carried out. Finally, the prototype was ready to be used for the extraction conditions selected in the extranat process.

The results were obtained according to the expected plan and incidents (or especial comments) were not worthy enough to be commented.

It can be considered that as a consequence of this task, the deliver of the prototype to the consortium was completed.

List of Deliverables and Milestones

Only Deliverable 8: Supercritical *Fluid Extraction Prototype,* was assigned to the development of this Workpackage.

Deliverable no.	Milestone Name	WP no.	Date due	Actual delivery date	Lead contractor
8	Supercritical Fluid Extraction Prototype.	5	Month 27 (June 07)	Month 27 (June 07)	MATARROMERA

No Milestones were assigned to this workpackage.

Workpackage 6: Production Test

The purpose of the present workpackage 6 was to make analysis on the obtained extracts to determine the possibility of using in food industry or in cosmetic and/or pharmaceutical sectors. Results and conclusions of the tasks are summarised now.

Task 6.1. Final product conservation and treatment: Definition of optimum concentrations

It should be commented that, at the beginning of the project the obtaining of extracts from direct solid to liquid extraction with supercritical CO2 was expected, finally, and as a consequence of the important variation on whole process conditions, the final product obtained in Extranat project is mainly a liquid product.

Depending on the type of extract obtained, the liquid could be an aqueous solution or an oil based solution. The conservation strategy should be considered from this point of view. Anyhow, as far it was observed, the obtained solutions showed a good conservation behaviour, once they are obtained by the extranat process.

According to the Extranat process, those solutions obtained for polar compounds are based in aqueous solution. In this sense the total removal of water by spray drying can be achieved in order to get a stabilisation in the long term. Some trials were carried out in this sense to obtain a solid powder. In this sense, different extracted samples obtained from grape pomance were dried by means of spray drying in laboratory. The main result that can be observed is that the use of some new additives to avoid the generation of a paste is required, which in turn is very difficult to handle. This situation, on the other hand is very usual when it is working with plant extracts and spray drying.

Figure: On the left the spray drying device used in stabilisation of grape extracts can be observed. The image of the right shows the powder obtained. The colour distribution is

homogeneous and remains clearly the colour of the starting material.

Previous figure shows the aspect of the solid extract obtained when spray drying is used for the stabilisation of the product. The advantage of this formulation type is that long term conservation can be obtained.

Although a small reduction in the antioxidant power is observed, the majority of the activity is maintained by the dry extract. The main drawback it can be remarked is that, when the product is intended to be applied in liquid formulations new problems arose due to the dispersion capacity. This point should require to be solved. It can be commented two important aspects. First a clear and fast solution of the product is required but this is not always achieved in an easy form. Second, the final taste of this product could be "sand-like" when using the product in some formulation.

Concerning to the formulation of non-polar extracts, it should be considered that the extract is obtained finally mixed with oil due to the necessity of the use of a fixing agent. This situation suggests that the final form of the product should be in liquid state. This will allow making easier the addition of this kind of product when perfect mixing is required (for instance in cosmetics)

On the other hand, the total quantity of oil to be added could affect to the properties of the final product, since it will be required to achieve a determinate quantity of active principle to obtain the antioxidant effect. Since this will affect to the total quantity of oil used, some techniques for emulsifying formulations should be required. Anyhow, it is difficult to say the quantity to use since it will depend on the application and the final product to use.

In this sense, and according to the initial proposed task objectives, it can be observed that the optimum concentration should be selected depending on the process. It is very important to mention that, when Extranat process is applied to vegetal wastes with a high content in licopene (for example tomato or watermelon) there is a limitation in use.

European Directives 64/36/CE and 95/45/CE will be mentioned briefly. They are related with the use of colorants in food products and with the purity of the colorants used in food products. According to the normative, the term colorant is used for those substances that give or recover some colour to foods. It can be included inside this concept natural compounds obtained from food or other substances, which are not usually used as food. Normative refers too, those colorants obtained by means of physical or chemical extraction.

According to the normative, carotenoids are included like substances that can be used as colorant for foods. The non-polar compounds extracted in this project can be related with the following categories: E160a for blends of carotenes and E160d for licopene.

The extract obtained in this project from carrots, which could related with colorant type E160, and this could present a limitation due to the use of ethyl acetate in the initial stage. Although ethyl-acetate is considered a GRAS solvent and its use is allowed for food uses, the present normative (95/45/CE) limits the uses of solvents for food uses when working with carotenes to acetone, methyethyl acetone, methanol, ethanol, propan-2-ol, hexane, dichloromethane and carbon dioxide.

It can be commented that, the Extranat process developed in this Project is robust in this sense. The situation can be solved using acetone in the first solid to liquid extraction, and adjusting pressure and temperature to force the removal of acetone by the CO2. Finally the final concentration of organic solvent should be checked to be under 50mg/kg of sample. Anyhow, the toxicity of extracts has been checked out in task 6.3. and the extracts can be used with no limitation in cosmetics products in the way they were obtained.

The extracts obtained from tomato could be related under the denomination E160d. In this case, there is no limitation to the use of ethyl acetate for food uses during the extraction. There is a limitation in the maximum final content of ethyl acetate allowed in the extract which, should be under 50 mg/kg of extract. At the same time, to be considered as colorant it should be obtained a total concentration in colorant (lycopene or other) above 96 %. So, our final product could be used for foods but the term colorant should not be used. For example, it could be considered as oil highly enriched with licopene. The effect should be quite similar and normative is accomplished. Finally, the use of the extranat product could be easier for correct dosage since at the end a homogeneous solution is provided.

Any way, as far normative limits the maximum quantities of licopene in foods, these values could be used to check the total dosage of oil to be used. The next table shows the values according to the Annex V contained in directive 94/36/CE.

Task 6.2. Characterisation of the final product composition

According with the proposal, the main objective of this task was to determine quality and degradation profile of final products. The reports of results are described withing task 6.5 due to the strong relation with execution of both tasks. It can be appreciated too, that the analysis of properties of final products according to task 6.4 are required to achieve results on task 6.2.

Task 6.3. Analysis of antioxidant activity and toxicity

Sets of representative samples have been assayed for different chemical and biological activities in order to obtain relevant information on obtained extracts. A brief description on properties of extracts is given bellow:

Grape waste extract (GE= Grape Extract) obtained in the extractor of the SFE pilot plant, pressure 90 bar.

Grape skin extract before undergoing SFE, 26/4/06

Grape skin extract after SFE at 90 bars, 27/4/06

Grape skin extract after SFE at 120 bar, 27/7/06

Grape pomace before undergoing Supercritical Fluid Extraction, sample 5-Or (25/5/07)

Grape pomace obtained in the extractor of the SFE pilot plant, pressure 120 bar, sample 5-Er (25/5/07).

Grape pomace before undergoing Supercritical Fluid Extraction, sample 11-O (8/6/07)

Grape pomace obtained in the extractor of the SFE pilot plant, pressure 90 bar, sample 11-E (11/6/07)

Ext Co2 SFE obtained from Carrots soaked with 600 ml of ethyl acetate for 2 hours, filtered, additioned with 75ml of sunflower oil and finally extracted with the supercritical fluid plant (CAR).

Carrot extract before SFE

Carrot SFE at 90 bar extracted in ethyl acetate and paraffin 20/10/06.

The following actions were carried out

Cell cultures

Two cell lines were used. First, the rat hepatoma cell line MH1C1 was used. It was obtained by the Istituto Zooprofilattico (Brescia, Italy) and grown in nutrient mixture Ham's F-10 medium supplemented with 20% foetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin.

Secondly, the human colon adenocarcinoma. Caco-2 cell line was selected because also of the possibility to be used for bioavailability studies. Cells were obtained from the European Tissue Culture Collection and cultured in D-MEM with GlutaMAX™–I supplemented with 10 % fetal bovine serum and 100 IU/ml penicillin and 100 □g/ml streptomycin (all from Gibco-Invitrogen). Cells were grown at 37°C in a humidified atmosphere containing 5 % CO2.

Cytotoxicity assay

In order to find the concentration of grape and carrots extracts not able to induce cytotoxicity, cell viability was determined by the MTT colorimetric assay [1], measuring the mitochondrial function using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT). Briefly, cells were incubated in 24-well tissue culture plates for 24 h with grape extracts at a concentration of 100, 50, 10, 5, 1, 0,5 ml/l and then 0.4 mg/ml (0.9 mM) of MTT dissolved in PBS was added to the medium in each well, and incubated for 2 h at 37°C. The medium was then removed, the formed blue formazan dissolved in 1 ml of DMSO, and quantified by absorption measurements at 570 nm using a microtiter plate reader (Biorad-Mod. 550). Cell viability was calculated measuring the difference in optical density of treated samples with respect to control cells.

Antioxidant effect

The antioxidant activity of extracts was evaluated in vitro by the DPPH method [2] and the citronellal thermo-oxidation inhibition test [3]. In addition, antioxidant activity was assessed in cells by measuring lipid peroxidation inhibition, and cellular ROS level.

The cells were incubated for 2 h with the extracts at the concentration of 10 ml/l or 1 ml/l and then treated for 1 h with 200 µM/1 mM Fe2+/ascorbate (FeAsc) or tert-butyl-hydroperoxide (TBHP)to induce oxidative damage.

DPPH reduction method

Extracts (50 µl) were added to 2950 µl of a 0.1 mM DPPH solution in methanol [2]. The exact initial DPPH concentration in the reaction medium was calculated from a calibration curve. The decrease in absorbance was determined at 515 nm at 0 min, at 30 sec, every 1 min for 15 min, and every 2 min until the reaction reached a plateau (about 30 min). Antiradical activity was expressed as the EC50, i.e. the antioxidant concentration necessary to decrease the initial amount of DPPH by 50 %.

Citronellal thermo-oxidation

In this test, the aldehyde citronellal is used as the oxidation substrate: it is subjected to heating and intensive oxygenation in chlorobenzene, and its disappearance with the consequent formation of its degradation products are monitored by gas chromatography. Fifteen ml of a chlorobenzene solution,containing 150 µl of dodecane (Aldrich) as internal standards, were poured into a two necked flask equipped with a condenser to prevent evaporation. Extracts, dissolved in chlorobenzene, were added to the solution to reach final concentrations. The mixture was then heated at 80°C and intensively oxygenated by bubbling in O2 at a flow rate of 10 ml/min. At time zero, 300 µl of citronellal (Fluka) were added to the reaction medium. Immediately and at periodic intervals, 0.1 µl samples were withdrawn and analyzed by gas chromatography. The antioxidant power of samples was measured by determining the efficient quantity (EQ), i.e. the concentration required for each compound to double the halflife with respect to control reaction (citronellal without antioxidant.

Lipid peroxidation assay

After treatment of cells with FeAsc or TBHP ± extracts, lipid peroxidation was measured by a fluorometric method for the determination of hexanal using 1,3-cyclohexanedione reagent and HPLC (HP1090 Hewelett-Packard Walbronn, Germany) according to Yoshino et al. [4] and Cabré et al. [5].

Cellular redox status 2’7’-dichlorofluoroscein (DCF) assay

Cellular ROS level was quantified by the 2,7-dichlorofluorescein (DCF) assay. After the treatment, cells were incubated for 10 min at 37°C with 50 µM dichlorofluorescein-diacetate, then washed, harvested with a policeman and resuspended in cold physiological solution. Cells were analysed with a Coulter Epics XL (Coulter) flow cytometer.

Antiproliferative effect

Clonogenic assay

Cells were treated for 24 h with the extracts at the concentration of 10 ml/l or 1 ml/l, then washed twice with PBS and fresh medium was added. After 10-15 days, the colonies were stained with crystal violet and counted. The clonogenic efficiency was calculated as the mean percentage with respect to control cells.

Cell cycle analysis

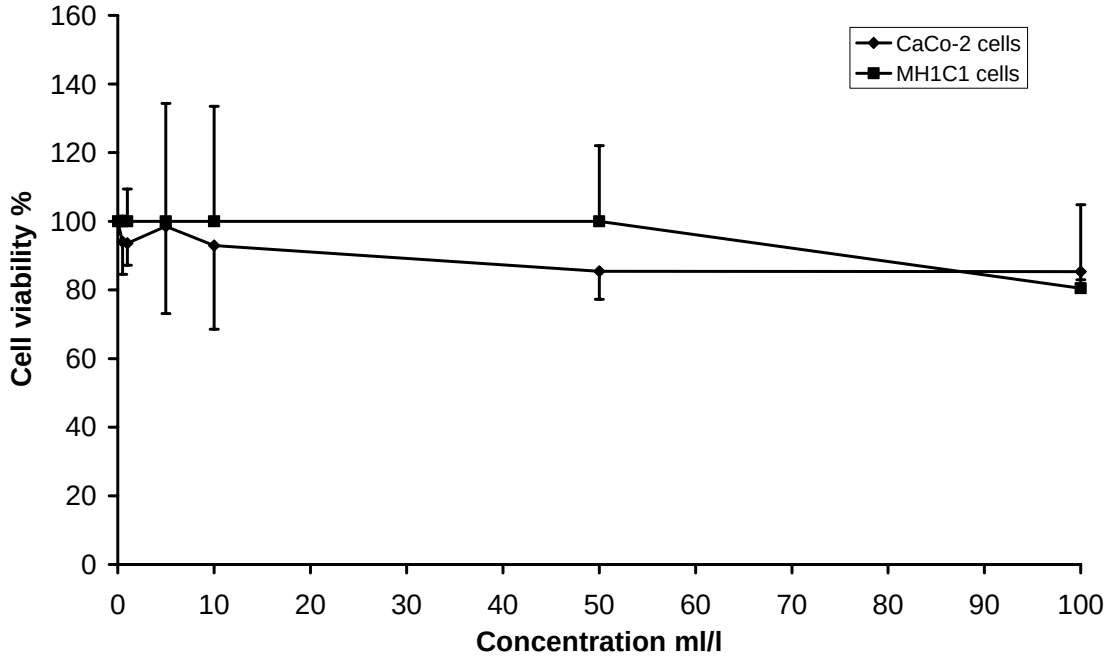
Cell cycle distribution was assessed by determining BrdU incorporation versus DNA content. Caco-2 cells were incubated with 30 □M BrdU during the last hour of 24 h treatment with 10 ml/l or 1 ml/l grape extract, harvested and fixed in cold 70% ethanol. Fixed cells were washed in PBS, resuspended in 2N HCl for 30 min at room temperature, pelletted, and then resuspended in 0,1 N sodium tetraborate for 15 min. The samples were then washed in PBS, incubated for 15 min in PBS containing 1% bovine serum albumin and 0,2 % Tween-20 (PBT), and then for 60 min in 100 □l of anti-BrdU monoclonal antibody (Becton Dickinson) diluted 1:20 in PBT. After two washes with PBT, cells were incubated for 30 min with 100 □l of FITC-conjugated anti-mouse antibody (Amersham) dilute 1:100 in PBT, then washed twice and resuspended in PBS containing 5 □g/ml propidium iodide (PI) and 1mg/ml of RNase A. Cells were analysed with a Coulter Epics XL (Coulter Corp.) flow cytometer. Ten thousand cells were measured for each sample. Computer statistical analysis of mean fluorescence intensity (MFI) and graphic representation were performed with the XL2 software (Coulter).

RESULTS

Citotoxicity

Effect of grape extract on cell viability

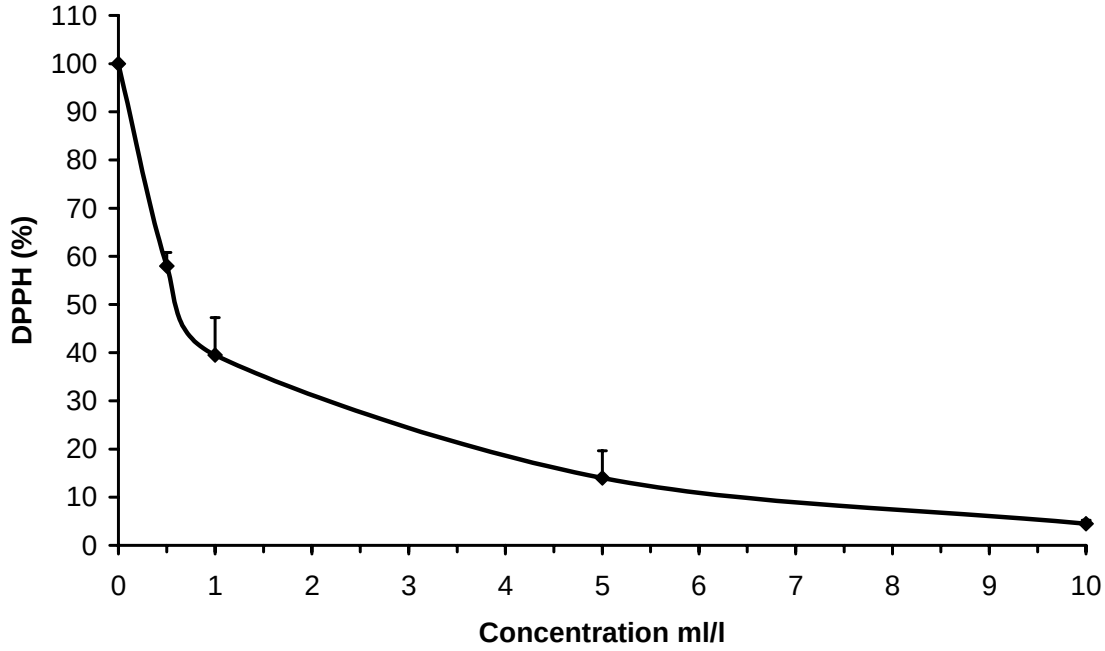
Preliminary experiments were carried out to determine the cytotoxic effect of the grape extract on MH1C1 and CaCo-2 cells by the MTT test. The grape extract induced a weak cytotoxicity only at the highest concentration (100 ml/l) in MH1C1 cells with a cellular survival of 80 % . It was devoid of any toxicity in CaCo-2 cells up to 100 ml/l. (Fig. 1).



Effect of GE on MH1C1 and CaCo-2 cell viability as measured by the MTT assay.

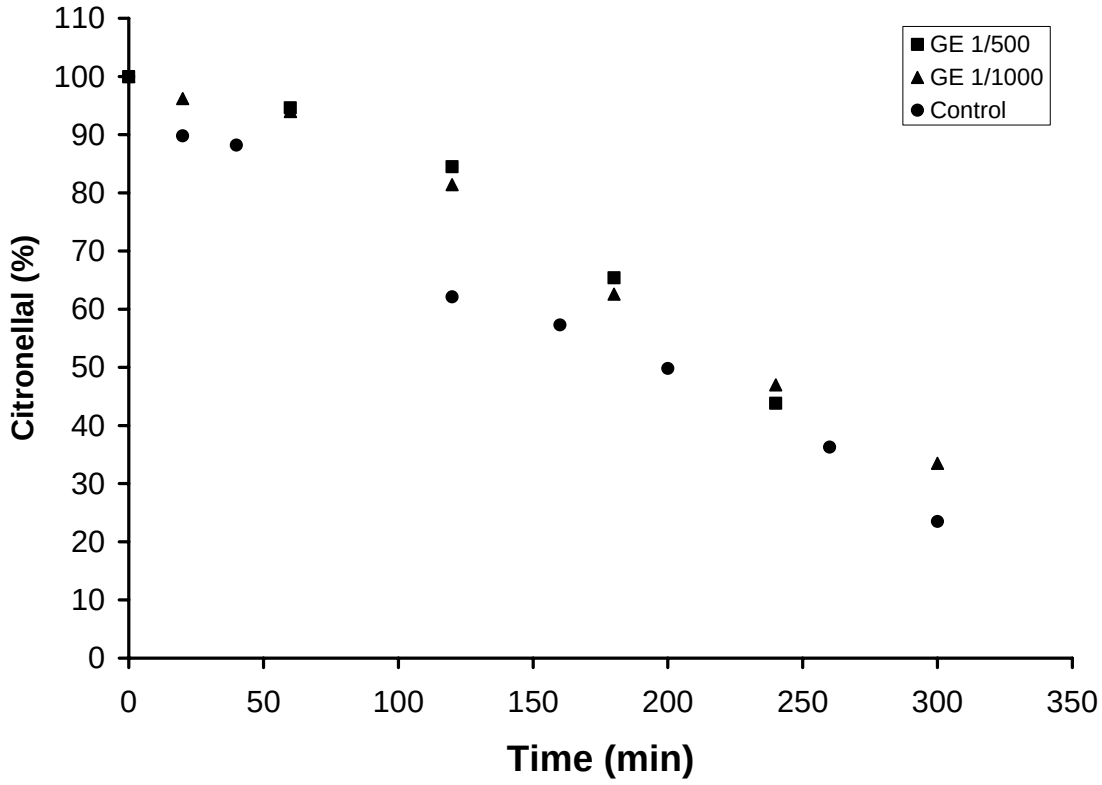
Antioxidant/anti-radical activity of grape waste extract.

Fig. 2 reports the dose-dependent anti-radical activity of GE. Antiradical activity, expressed as the antioxidant concentration necessary to decrease the initial amount of DPPH by 50% (EC50.) is 0.65 ml/l.



Antioxidant activity of GE as measured by the DPPH assay

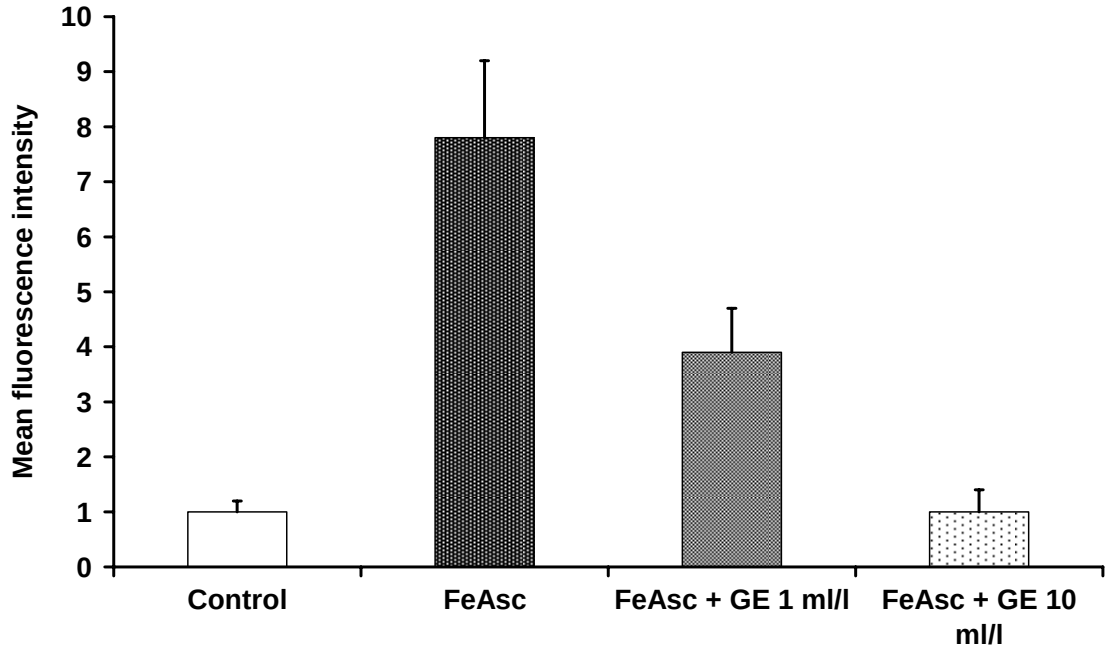
In contrast, by the citronellal thermo-oxidation inhibition test the extract did not present present a significant antioxidant effect.



Oxidation kinetics of citronellal alone or with GE.

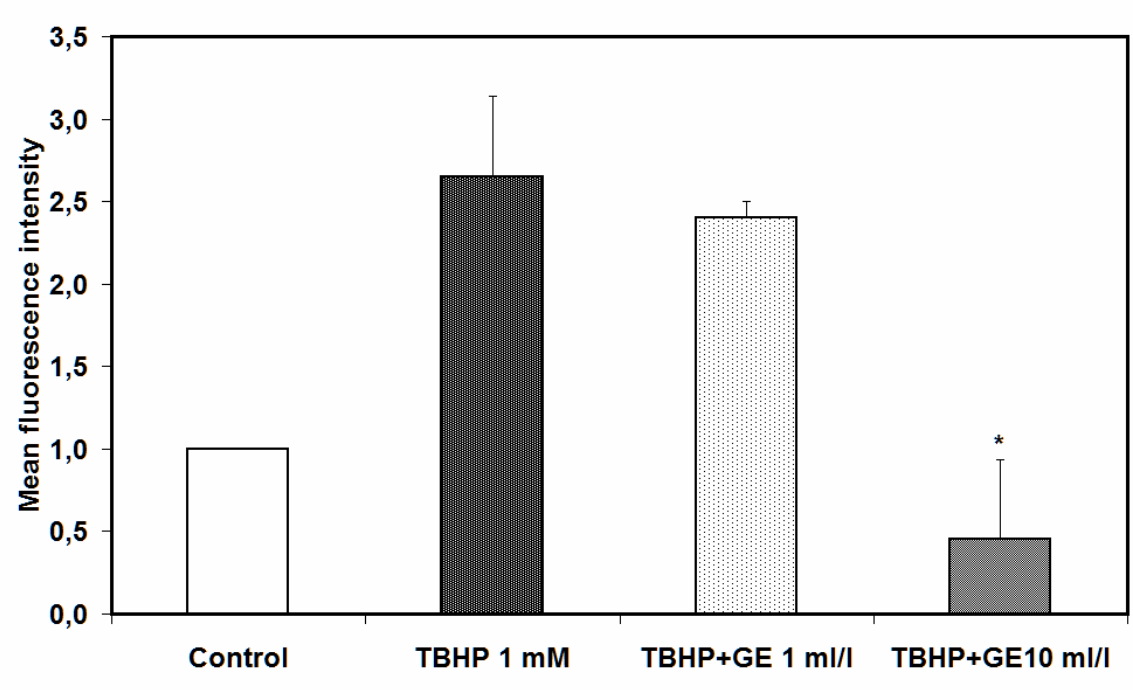
Reactive Oxigen Species (ROS) quenching activity of grape waste extract

The grape waste extract reduced Fe2+/ascorbate-induced ROS production in MH1C1 cells in a dose-dependent manner (Fig. 4). In particular the highest concentration (10 ml/l) reported the ROS to the level of control cells.



GE activity in quenching ROS induced by Fe2+/ascorbate.

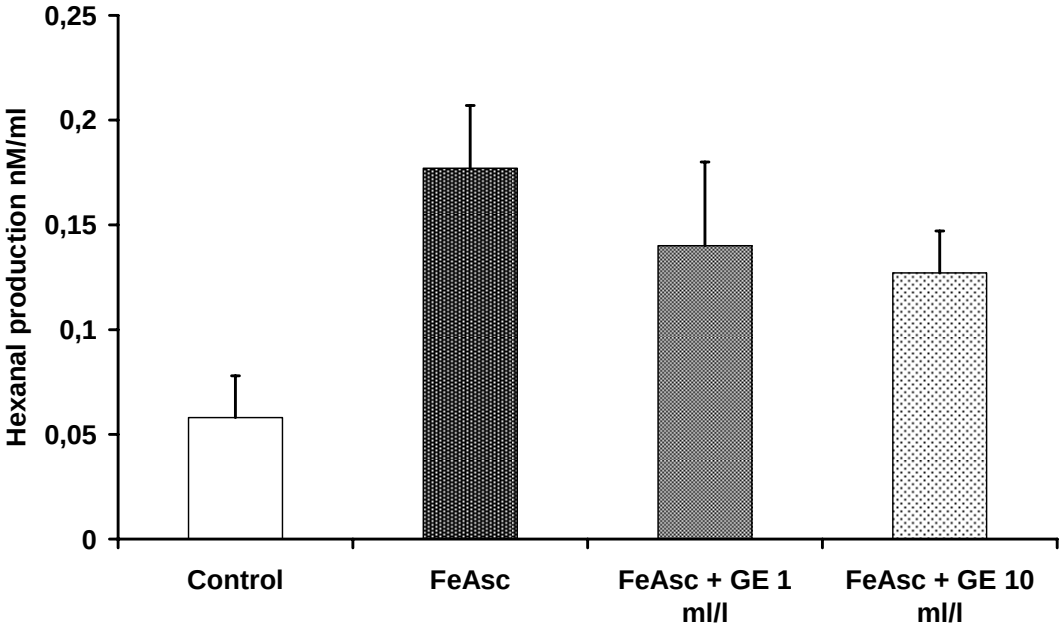
The same behavior was found in TBHP-challenged CaC0-2 cells.



Inhibition of TBHP-induced ROS production by GE in CaCo-2 cells.

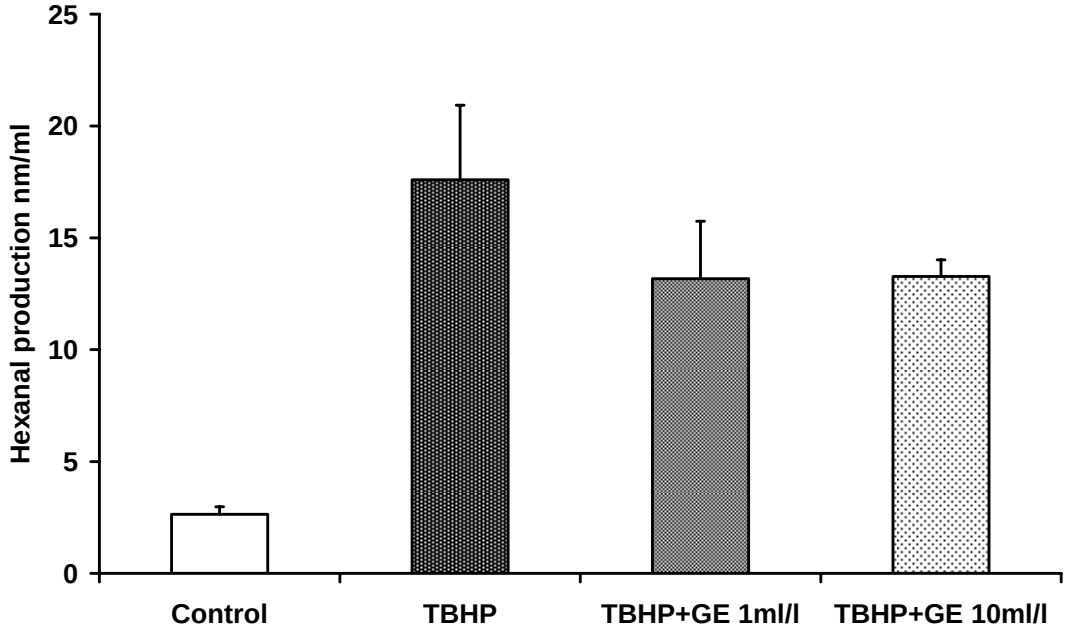
Anti lipid peroxidation effect by grape waste extract

Figure 6 shows the effects of grape extract on lipid peroxidation induced by Fe2+/ascorbate in MH1C1 cells. Incubation of the cells for 1 h in the presence of 200 μM/1mM Fe2+/ascorbate increased membrane lipid peroxidation, raising the hexanal production to 0.177 nM/ml from the level of 0.058 nM/ml measured in untreated control samples. Grape extract inhibited the hexanal production in a dose-dependent manner by about 20% and 30% at 1 and 10 ml/l concentration respectively.



Anti-lipid peroxidation activity of GE in FeAsc challenged MH1C1 cells.

Next figure shows the effects of the GE on hexanal production induced by TBHP in Caco-2 cells. Incubation of the cells for 2 h in the presence of 1mM more efficiently increased membrane lipid peroxidation, raising the hexanal production to 17,6 nmol/ml, from the level of 2,6 nmol/ml measured in untreated control samples. The grape extract, at both concentrations used, showed a slight and not statistically significant protective effect.

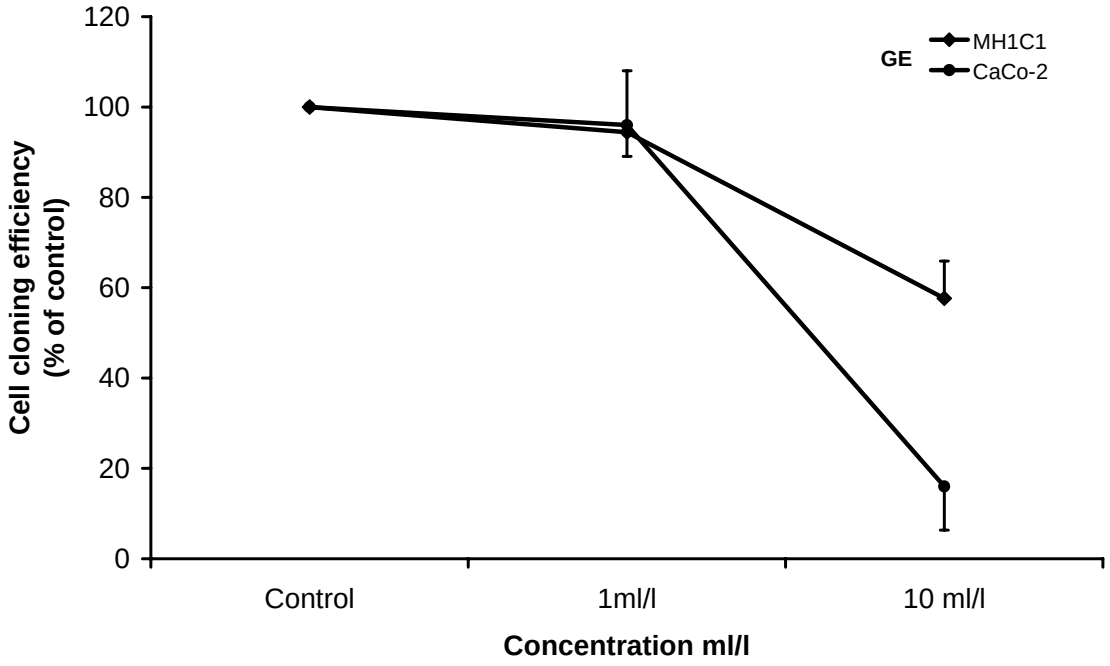


Anti-lipid peroxidation activity of GE in TBHP challenged CaCo-2 cells.

Antiproliferative effects

Effect of grape waste extract on clonogenic efficiency in MH1C1 cells

To investigate the effect of the GE on cell proliferation, we analysed the clonogenic efficiency in MH1C1 and CaCo-2 cells and the distribution of CaCo-2 cells in each phase of the cell cycle.

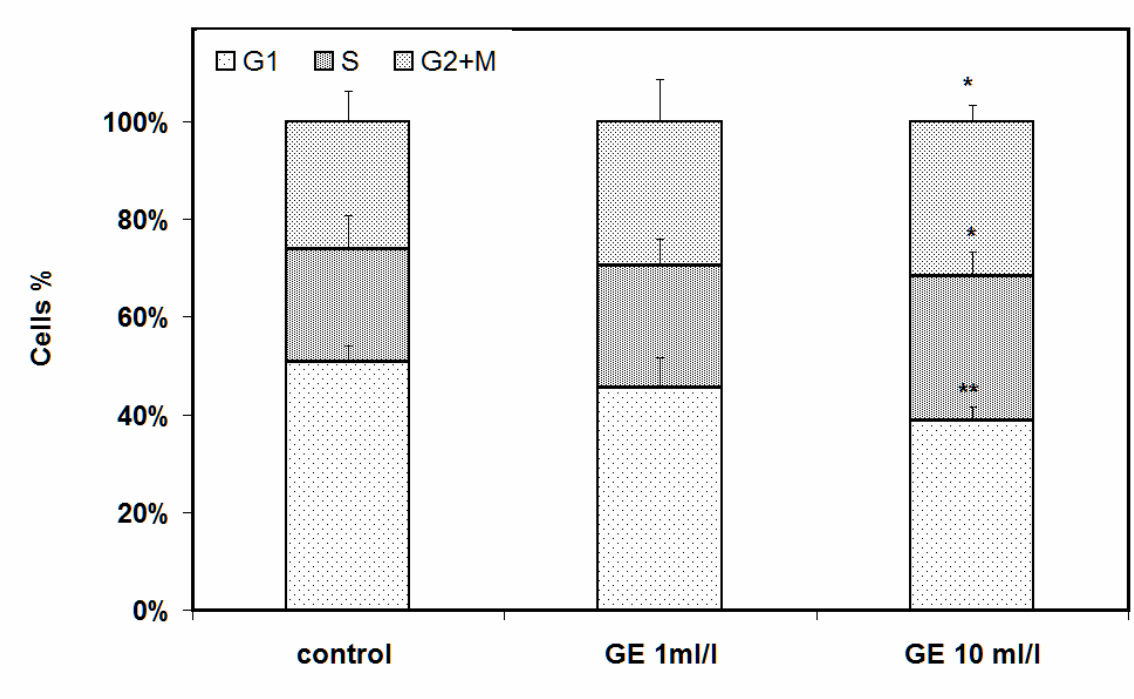


Reduction in clonogenic efficiency GE in MH1C1 and Caco-2 cells.

Results show that GE is highly effective as an antiproliferative substance in neoplastic cells, reducing clonogenic efficiency up to 80% in CaCo-2 cells.

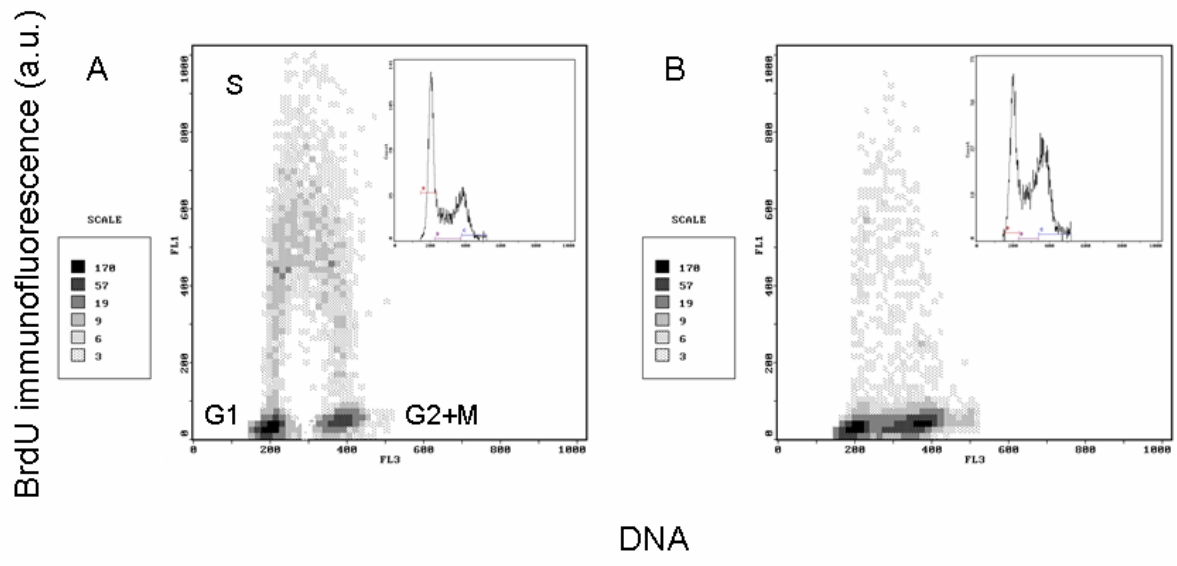
Cell cycle analysis

The distribution of cells in each phase was analysed by determining the DNA content with flow cytometry. As reported in Figure 9, a significant reduction ($p < 0,01$) of cells in G1 phase as a consequence of an accumulation in the S and G2+ M phases was observed in Caco-2 cells treated with 10 ml/l GE. In contrast, no significant effect was detected in cells treated with 1 ml/l GE.



Effect of GE extract on cell cycle distribution.

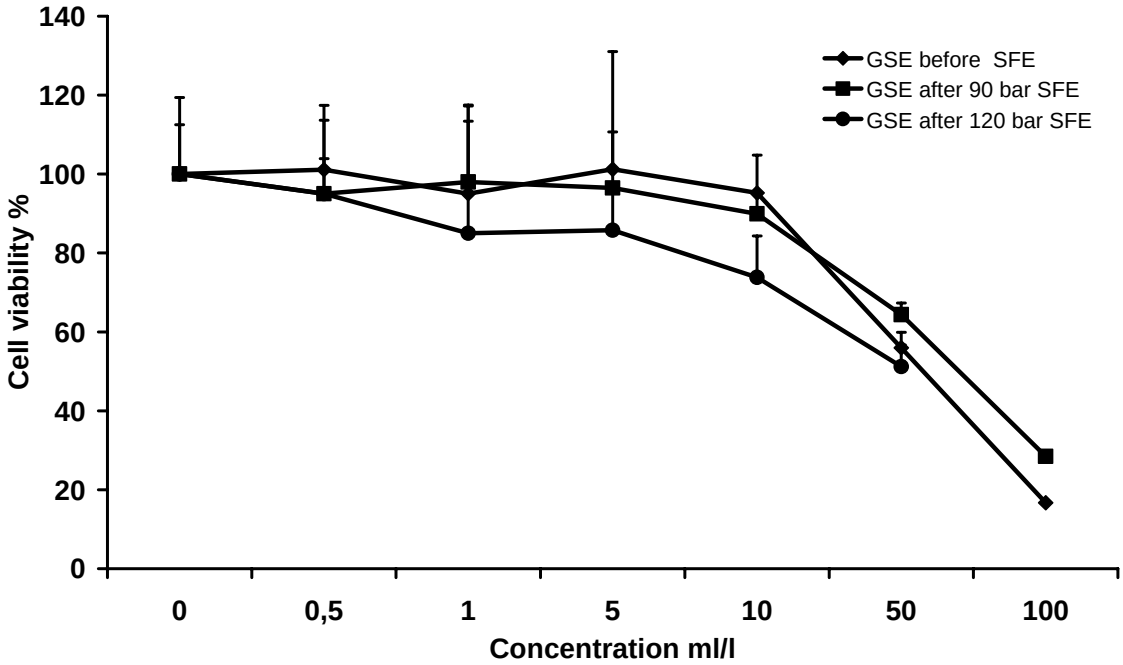
Next figure shows the dot blots of BrdU immunofluorescence vs DNA content in control Caco-2 cells and in cells treated with 10 ml/l GE. The results show that cells treated with GE incorporate a significantly lower amount of BrdU than control cells. In fact a quantitative analysis of BrdU immunofluorescence, in the region corresponding to cells in S phase, indicated an inhibition in BrdU incorporation.



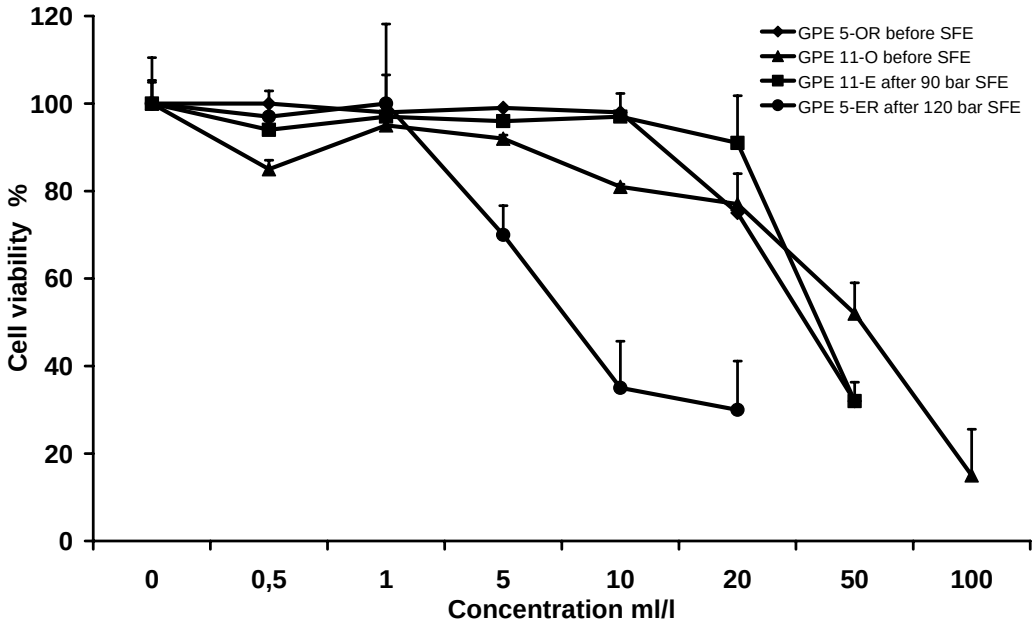
Effect of GE on BrdU incorporation in CaCo-2 cells.

Cytotoxicity / safety of grape skin (GSE) and pomace (GPE) extracted with SFE at different CO2 pressures.

Grape skin and pomace SFE extracts were assayed for cytotoxicity in MH1C1- cells.



Citotoxicity of grape skin before and after SFE at different pressures.

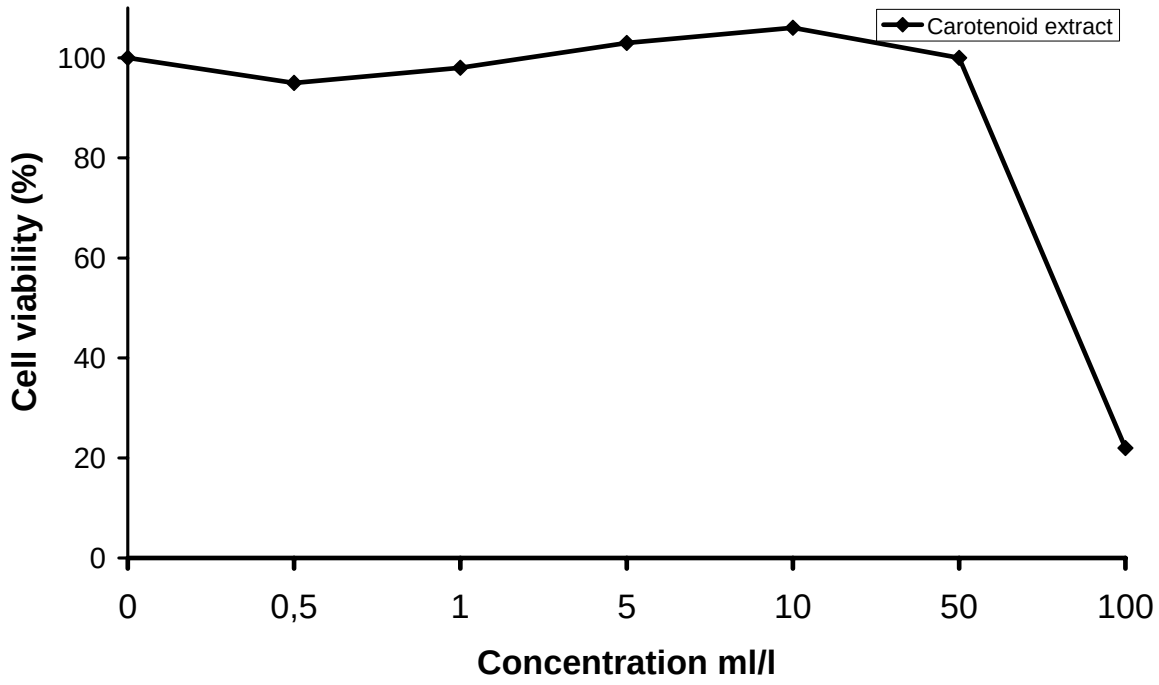


Citotoxicity of grape pomace before and after SFE at different pressures.

Citotoxicity of grape skins and pomace extracts seems to be dependent to SFE conditions. In particular SFE at 120 bar seem to be the most effective in induce citotoxicity in CaCo-2 cells, most probably due to the most effective extraction procedure of, among others, potentially cytotoxic constituents.

CARROT SUPERCRITICAL FLUID EXTRACTS (CAR)

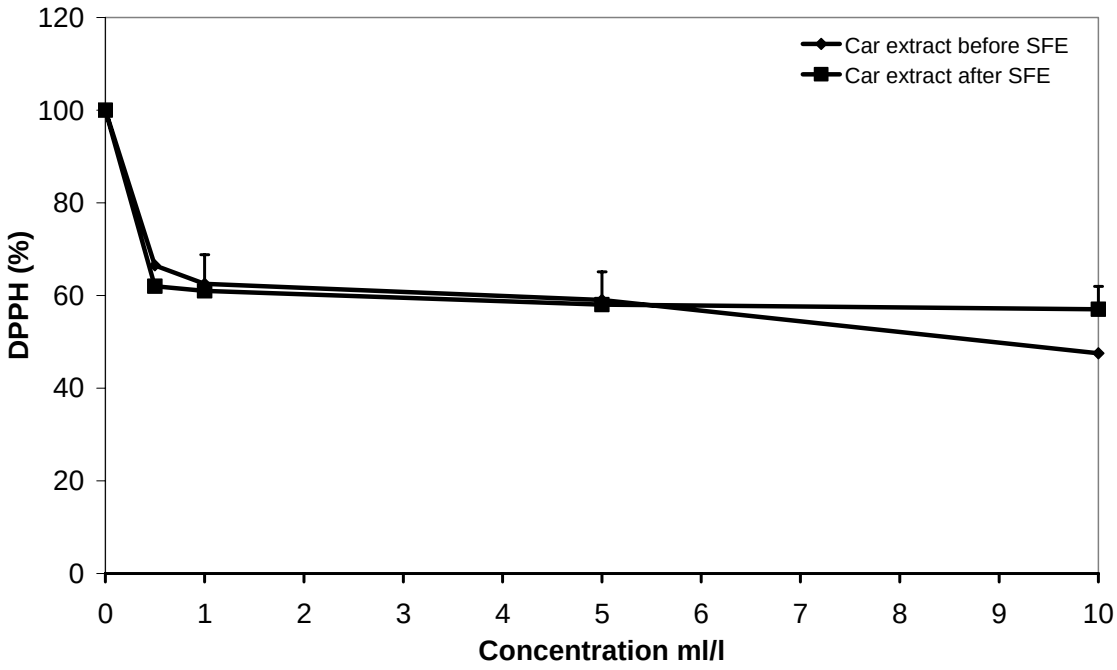
As far as carrot extract is concerned, it is to point out that the first sample received for Partner 9 was dissolved in sunflower oil after SFE, due to technical reasons. This was not an issue for toxicity assessment (Fig. 13) but precluded further investigations since sunflower oil is rich in vitamin E, one of the most effective chain breaking antioxidant/free radical scavenger by itself.



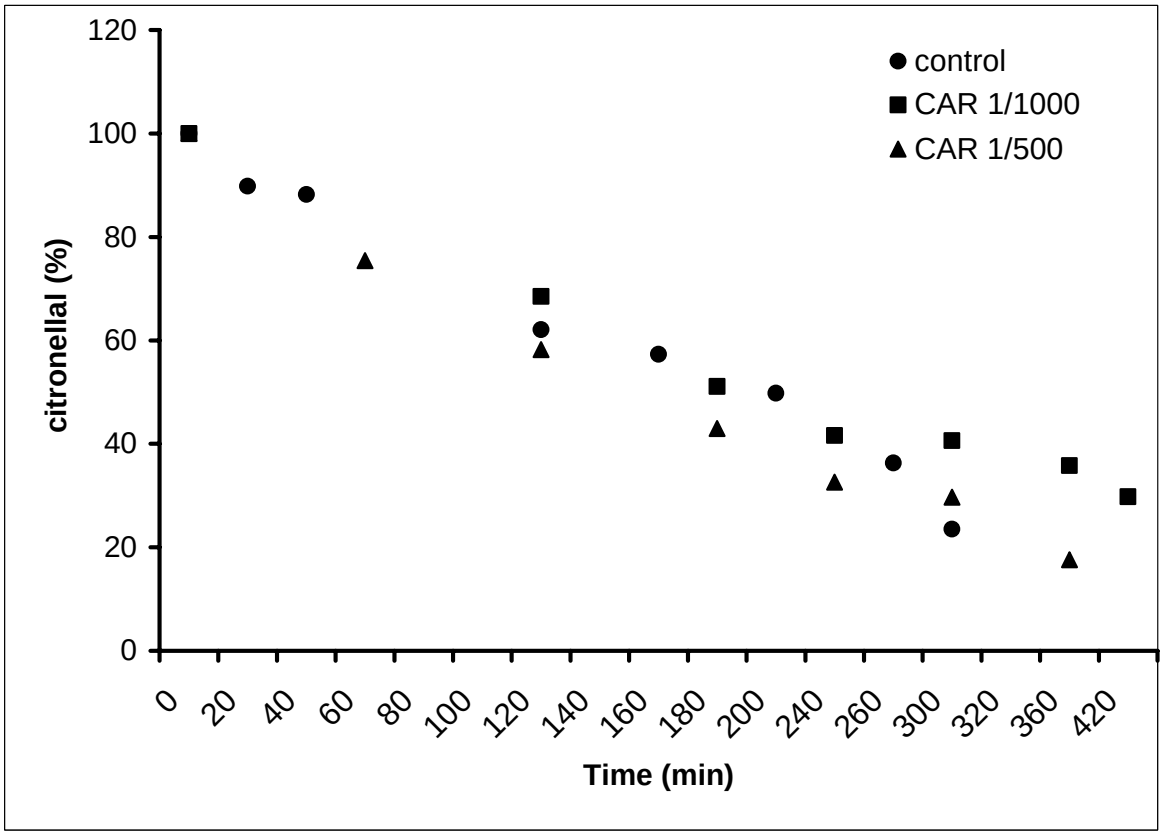
Effect of ethyl acetate /SFE/ sunflower oil carotenoid extract on MH1C1 cell viability as measured by the MTT assay

CAR extract seems to be non-toxic at concentrations below 50 ml/l. Nevertheless, sunflower oil presence impaired measurement of antioxidant, anti lipid peroxidation and anti free radical activities since it is by itself a powerful antioxidant containing relevant amounts of vitamin E.

A further sample, extracted by SFE at 90 bar and resuspended in ethyl acetate and paraffin was assayed for its antioxidant/anti free radical activities. Unfortunately, it was impossible to test the sample in biological experimental models (cell cultures) because it resulted unmiscible in an aqueous medium since organic/aqueous phase separation occurred.



Antioxidant activity of carrot extract before and after SFE at 90 bar and resuspended in ethyl acetate and paraffin. No significant difference can be observed after SFE.



Citronellal thermo-oxidation kinetic in the presence of different concentrations of carotenoid extract.

In this experimental system, it is to point out that CAR shows an antioxidant effect only at the lowest concentration, whereas such an antioxidant effect lacks at the highest concentration used. This should not surprise taking into account the unusual antioxidant properties of beta-carotene (6,7) and that these experiments are carried out at an high O2 relative pressure.

On the whole, it is to stress out that grape waste extract obtained by Supercritical Fluid Extraction (GE) seems to be a very promising compound in terms of antioxidant and antiproliferative effects. Carrot extracts suffer from the usual technical problems inherent with their lipophilicity and peculiar characteristics of carotenoids as antioxidants.

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Task 6.4. Analysis and effects of the organoleptic qualities

The main objective of the task 6.4 was to evaluate procedures for the evaluation of the organoleptic properties of final products. As it was defined in task 6.1 most of the uses of the obtained extract should be as colorant. However, during the elaboration of the proposal, it was

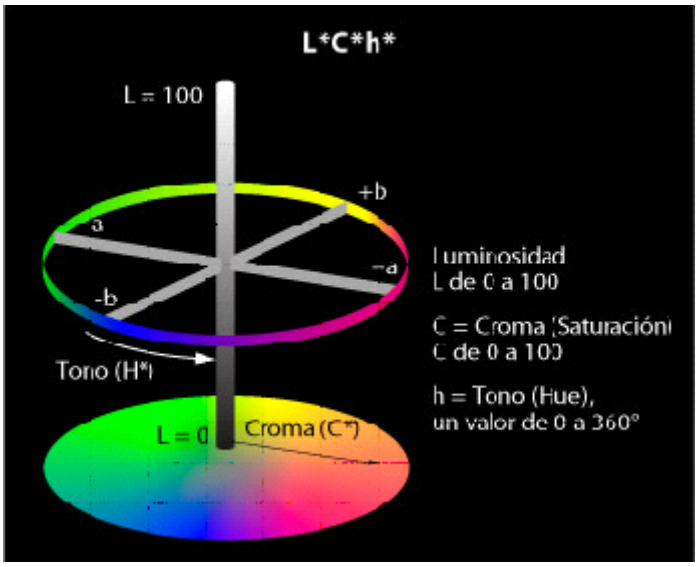
proposed some standard methodologies based on the ISO 6564 and ISO 4120 norms for the evaluation of the use on foods. This was because it was considered like viable the production of 'aromas' from vegetables.

Finally, the evolution of the Project has derived to the production of extracts with antioxidant properties (on polar and non polar base). Although, they could be used fairly in foods as additives, the dosage limitations imposed by normative points that the more important aspect to be considered is the colour when studying organoleptic properties. This is due to the small quantities of extract that would be required. At the same time, other uses beyond foods should be considered.

Then, the initial proposed ISO methodologies would be poor to evaluate the properties of interest of the product. It will be explained that the ISO 6564 is a set of methods for describing and assessing the flavour of food products. Finally an overall impression from the food (in terms of intensity) is obtained to describe the product. On the other hand ISO 4120 describes a procedure for determining whether there is a perceptible sensory difference or similarity between samples of two products.

At the end, it was decided to evaluate the colour by means of instrumental analysis using CIELab system. A quantitative value of the colour can be obtained by this method, and this could be getting a good agreement with the general requirements of industry colorant sector.

The method defines the three essential components for perceiving a colour: light source, object and observer. The spectral power distributions is considered in the estimation of colour. Any colour thus can be measured and described by a set of tristimulus values (XYZ) which indicate amounts of reference red, green and blue lights, respectively, required to match a colour. The method allows exchange of colour information by numbers.



The figure shows a three dimensional CIELab space. The neutral scale is located in the centre of the colour space. The L values of 0 and 100 represent a black and a reference white, respectively. The a and b values represent the redness–greenness and yellowness– blueness attributes, respectively. The C* (or Croma saturation) scale is an open scale with a zero origin (including all colours in the neutral scale that do not exhibit hue). The hue angle, h, lies between 01 and 3601. The colours are arranged following the sequence of the rainbow colours. The four psychological hues, which are pure red, yellow, green and blue colours, do not lie exactly at the hue angles of 01, 901, 1801 and 2701, respectively.

Once the methodology was identified, the identification of properties on final products was carried out. Those products included the extracts as a part of additives in the formulation. Next task describes the results.

Task 6.5. Finally quality parameters

As it was commented previously, those results obtained from execution of task 6.2 are related in this task report. The aim of the task 6.5 was to determine possible uses of the extract in cosmetics products. The degradation profile on active principles was followed through the evolution of colour and through the estimation of antioxidant activity.

The first action was the selection of the cosmetic formulation to be used. It was decided to use preparations intended for using after showering or bathing. The reason for the selection was that the main purpose of these products is the delivery of active principles. In this way, It was expected to study the colouring effect along with functional properties (like antioxidant activity) when using the obtained extracts. So the formulation of a afterbath gel base was selected

The composition of the formulation contained the following components: Polisorbate 80 (Tween 80), triethanolamine, carbomer 940, ethanol (alcohol denat), dimethicone copolyol, castor oil and water. This formulation produces a clear, thick gel. According to the industrial formulation, the addition of preservatives or other ingredients (like fragances and colour) depends on the use (“quantum satis”). In this sense, the quantity was adjusted considering:

results of task 6.4. and total solvent remaining (especially ethyl acetate)

colour obtained

maximum quantities of extract allowed to produce a stable formulation

Some comments will be made on the elaboration of the gel. Since the mixing of the components required working temperatures above 80º C, it was possible to promote degradation somehow in the extracts. Then, the addition of extracts was made below 40º C. At the same time, viscosity reduction was rerquired to produce a homogeneous mixing in the gel when working at low temperatures. Viscosity was reduced by using lower levels of triethanolamine and carbomer 940 than the recommended in the industrial formulation.

The addition of the extracts was made considering the type solvent used in the extranat process. So when working with polar compounds (like grape extracts) the addition was made, making a correction in the total water content. In the same way, when working with non-polar compounds (like tomato extracts) a correction in the total content of castor oil was done. In the last case, castor oil was fully substituted by sunflower oil in order to get the maximum quantity of antioxidant.

Finally it was considered not necessary the addition of other preservatives like paraben derivates, since the former ones are used to avoid long term microbiological degradation.

Next figure shows the aspect of the obtained gels:

(A) (B)

Figure: Aspect of the obtained gels. (A) gel obtained by addition of grape extracts (B) gel obtained by addition of tomato extracts

It can be appreciated that it was necessary to modify the viscosity in sample B to obtain a good mixing. A difference in thickness is obtained as a result of that.

The colour measurement was made using a double beam standard spectrophotometer (UV-1700 Shimadzu) and from standard solutions of gels. The determination of antioxidant activity was made using DPPH or Foling tests.

Results

First gel produced was obtained with the extract of tomato. Once the gel was obtained, colour was defined the according to CieLab coordinates: a=1, b=4, L=93 and the Hue value was considered of 10º.

Samples of gel were introduced in plastic cuvettes (1x1 cm) and then samples were stored under standard conditions. Measurements of the colour and antioxidant activity were made every week.

It was observed that

the antioxidant activity remains constant or no variation is observed

the absorption at 444, 470 and 502 nm shows strong decrease

Lost of colour is obtained

Next figure shows the degradation profile of lycopene with the time. Values of degradation were based in the relative decrease of absorption of samples at the three maximum wavelength for lycopene.

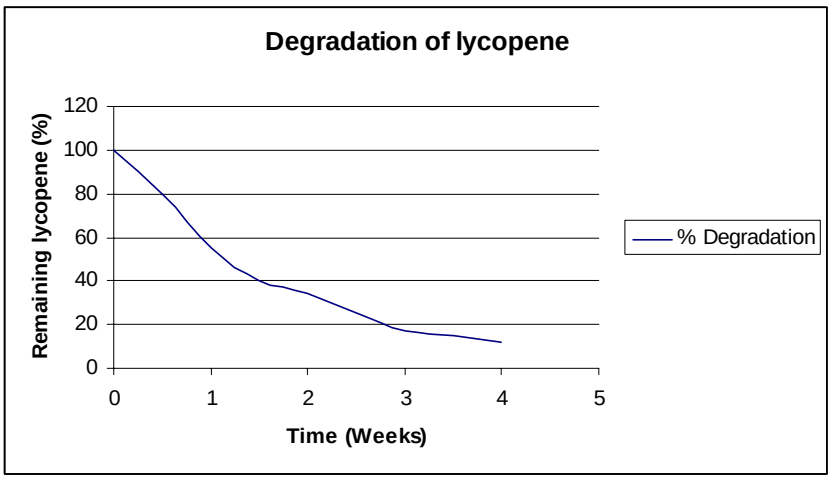


Figure: Stability of lycopene with time.

The antioxidant activity observed can be associated to the sunflower oil mainly (where it was contained the lycopene). So, these results points to a degradation due to other factors than oxidation since tocoferol (contained in sunflower oil) would be acting as a preservative for lycopene. The lost of lycopene can be related with the exposition to the light, since no thermal variation were promoted.

In this sense, if gel is stored protected from the light, degradation occurs but is in a range very different. Next figure shows the evolution of the samples.

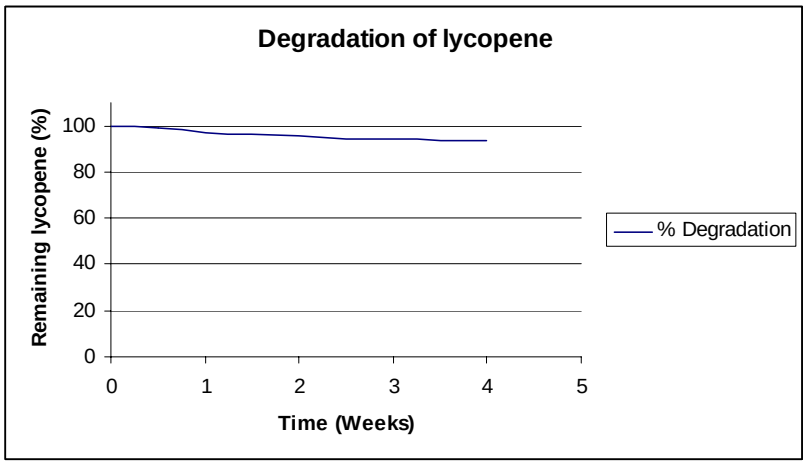


Figure: Stability of lycopene with time without light exposition.

After that, a new gel was produced using the extract of grape pomance. Once, CieLab coordinates observed were: a=3, b=-6, L=93 and the Hue value was considered of 10º.

In the same way that previous gels with lycopene, samples of gel were introduced in plastic cuvettes (1x1 cm) and then they were stored under standard conditions. Measurements of the colour and antioxidant activity was made every week.

Measurement at 530 for the evolution of the degradation profile was carried out. The next mathematical expression was used according to the method described by Francis, F. (1989). Food colourants: Anthocyanins. Critical Reviews in Food Science and Nutrition, 28, 273–314.

CPolyphenols = (Absortion530 * Dilution factor) / 98.2

A good stability of the product was observed in this case. No important variations in colour and antioxidant capacity were observed. In order to quantify properly the degradation profile of the samples the relation of samples with the initial concentration (expressed as ln(CPolyphenols / C0Polyphenols)) was represented in the following graphic.

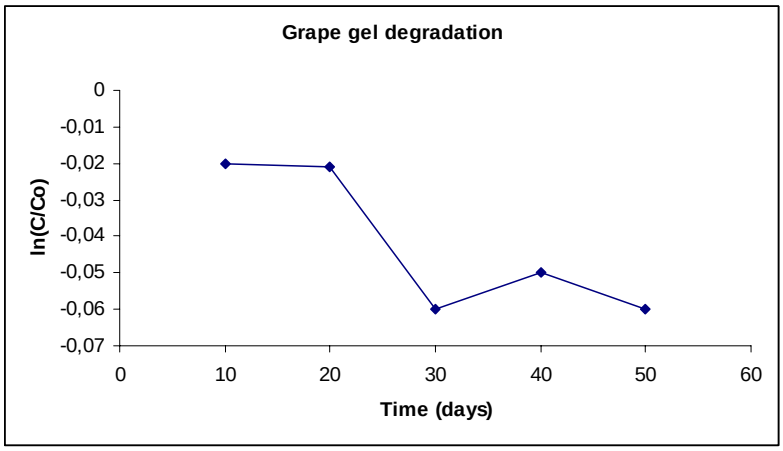


Figure: Evolution of the concentration of polyphenols with the time in the gel formulation.

Although some degradation occurs, the total value of variation it is not so important to be considered.

Conclusion

Some characterisation and uses of the obtained extracts have been developed during this task. The results show that there are different exploitation ways for the extracts obtained in the process. Although the products could be applied in food, some considerations of adaptation in the production of extracts according to the present normative would be required.

The use of extract in cosmetic formulations has been applied and, in the case of grapes, it is possible to produce a stable after-bath gel.

List of Deliverables and Milestones

Only Deliverable 10 was assigned to this Workpackage. No milestones were assigned.

Deliverable no.	Milestone Name	WP no.	Date due	Actual delivery date	Lead contractor
10	Final Product Quality Parameters and Composition	6	Month 29 (August 07)	Month 29 (August 07)	ALDIVIA

7.- Workpackage 7: Exploitation and Dissemination.

This workpackage has a two-fold objective. On one hand it aims to achieve agreements for the further exploitation of the results of the project. On the other hand it comprises the tasks devoted to make the project information available to the general public as well as to interested role players from the sector. Both of these objectives are focused on reaching a good level of impact.

These objectives can be explained as follows:

Exploitation Plan: to design a marketing strategy for the potentially marketable results, accounting for each element of the marketing-mix scheme:

- Production definition, clear definition of the marketable results;
- Price, determining the adequate price strategy depending on cost/benefit analysis;
- Distribution, main distribution routes and agents will be identified and contacted;
- Sales strategy, an analysis of the diversity of commercial formulae will be carried out;
- Merchandising, identification of the best ways for advertising: brochure, commercial fairs;
- Promotion policy, use of financial schemes at national and/or European level for first users.

Dissemination Plan: The objectives of the dissemination plan are two fold:

- Design and start up a dissemination strategy to make the results available to the related sectors in Europe, demonstrating the competitiveness advantages that the implementation of this technology would imply in the industries that finally incorporate it.

Task 7.1: Study of the user needs and socio-economic aspects of the potential market and marketing analysis.

The main objective of this task was to collect and extract information from target sectors. Since the technology should be used by the SME's involved in the Consortium, main research was devoted on to the perception of public acceptance of extracts enriched on antioxidants refereed as functional products. The main targets of the research were focused to gain knowledge on perception of the population on these products, the reasons to acquire these products or to refuse them, the knowledge about the uses.

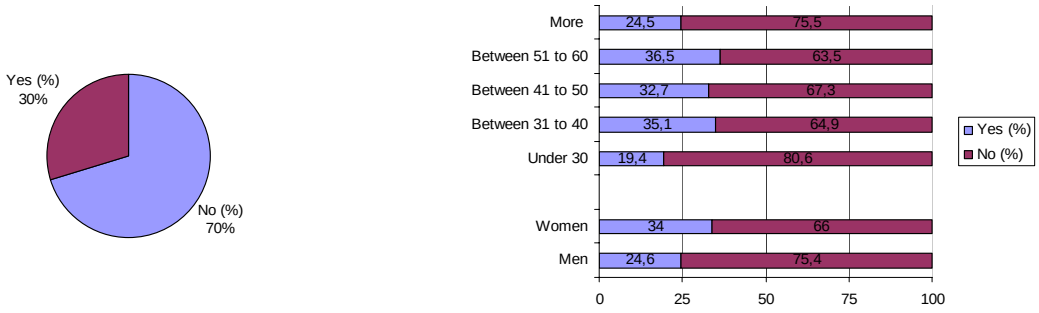
Questionnaires were based on the following points:

- Recognition on functional foods
- Shopping rate and habit (where, frequency and for whom)
- Perceived reasons for the acquisition (advantages or objections)
- Influence on buying decision: brand, product or component
- Use of the product
- Valorisation on properties for similar products (price, distribution, packaging, physical properties like colour and flavour)

Results

Recognition on functional foods

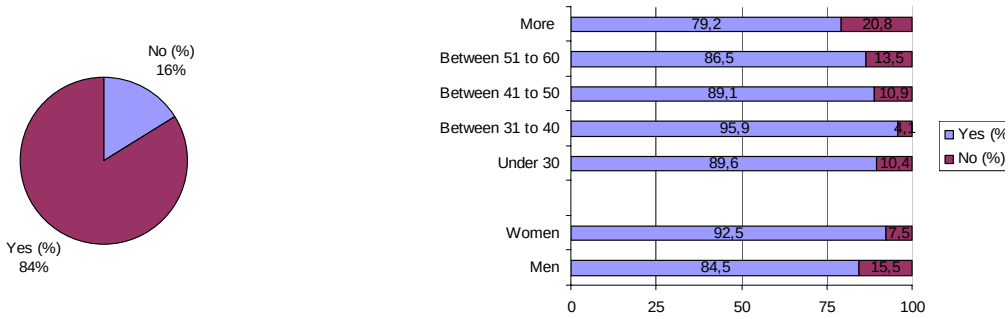
It can be concluded that there is a remarkable lack of information on the functional foods in an initial stage when the population is consulted. Perception can be tagged as low. Next figure shows the results:



Results on recognition of functional foods

It can be observed a bigger interest on the functional products of the population between 31 and 60. At the same time, as a general remark it can be observed that women are better informed than men on this matter.

The situation change, once the functional foods and products are described or presented 'in brief'. Next figure shows these results:



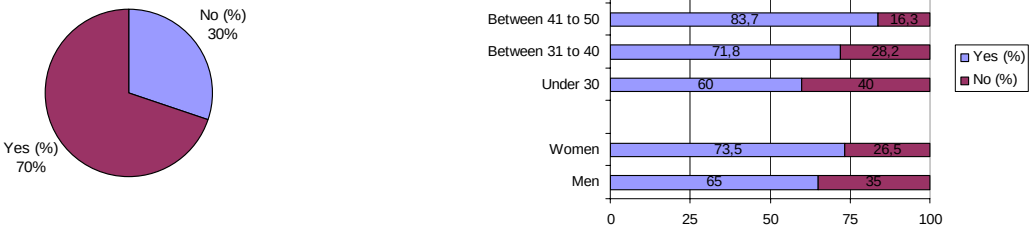
Results on recognition of functional foods once their are described

It is clear that once a brief introduction or explanation about the properties of these products is offered the most of the population is able to recognise them. This shows that a hypothetical product should consider the way it offer the information on the functionality gained due to the use of the extracts.

Shopping rate and habit (where, frequency and for whom)

Those who were able to recognise the functional products were asked for the way they consumed and the way they acquired the product:

The results are showed in next figures:

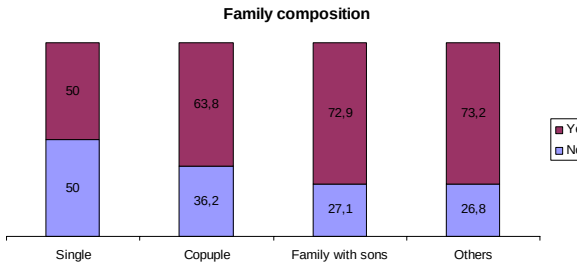


Population and the consume of products (considering at least one member of the family)

The differentiation of the consumer is centred in the people between 41 and 50. This sector with the people between 51 and 60 are related with this niche of the market which is more concerned about the importance of the diet and the relation healthy

According to the results, it can be observed that when the responsible of the family for shopping is the man (or somehow it is involved in the decision) the consume is increased, although the previous figure shows that use of the product is higher among women. Considering the composition of the family unit (from a single person to several people) it can be remarked that when total number of fellows is increased consume is increased too.

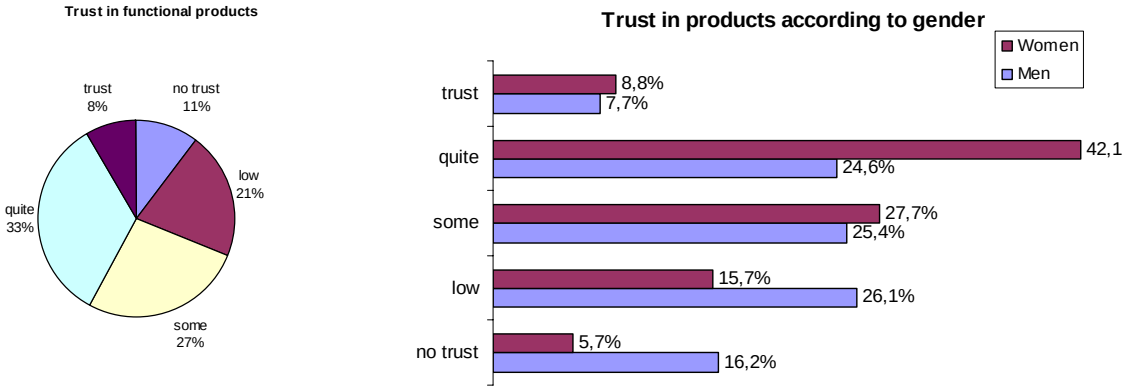
These results can be observed in the next figures:



Description of consume of products according to family composition.

Perceived reasons for the acquisition (advantages or objections)

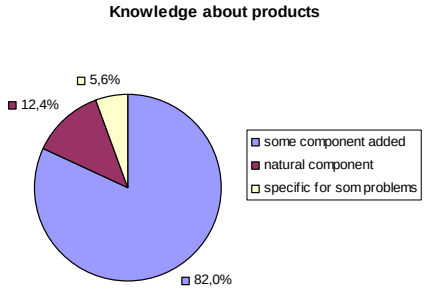
As first approximation people was consulted for their trust in functional products. The scale was ranged according five values: no trust, low, some, quite and trust. The next figure shows the obtained results:



Trust in the functional products

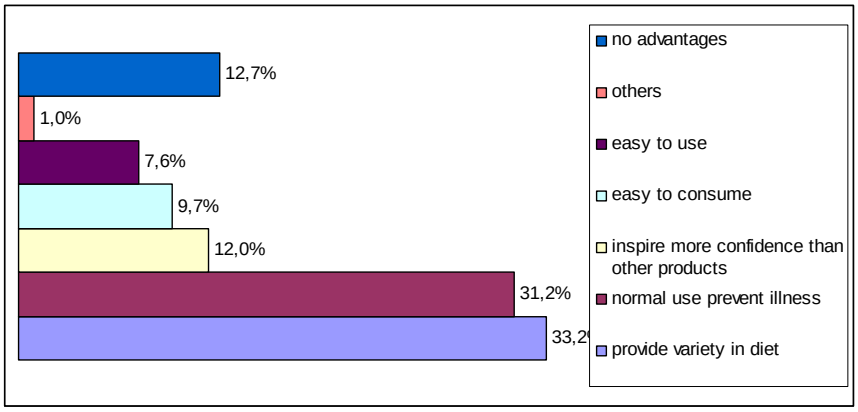
It can be observed that there is a clear differentiation between the trust in the products of women and the tendency in the shopping which is clear higher in men. At the same time, the consume of functional products is higher in women.

For those who identified the functional products without any further explanation, a new consult was made about the description of the product. The results were related mainly with inclusion of determinate compound in the product or with natural products. Surprisingly some person related these products with the use for specific medical problems.



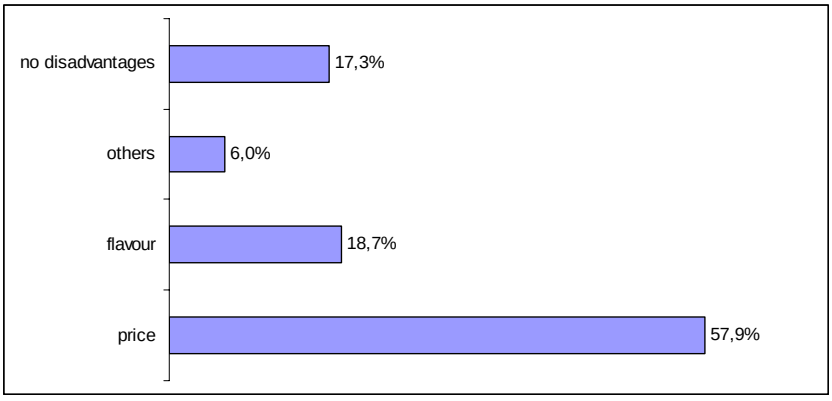
Knowledge about the products

The observed advantages were focused in the intake of these products rather than other uses. At the same time a clear identification with the wellness of the product is observed. Next figure shows the results:



Observed advantages

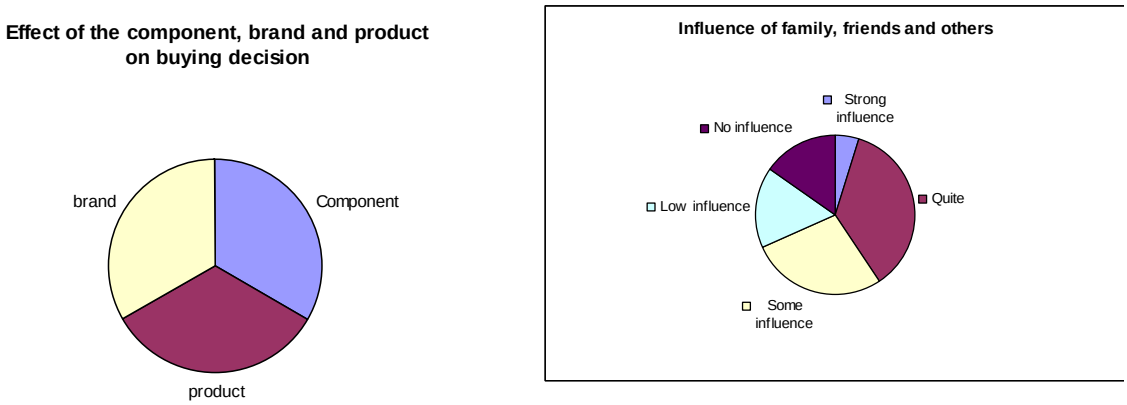
Concerning to the objections (or disadvantages) of the products it was identified the following precisions:



Observed disadvantages

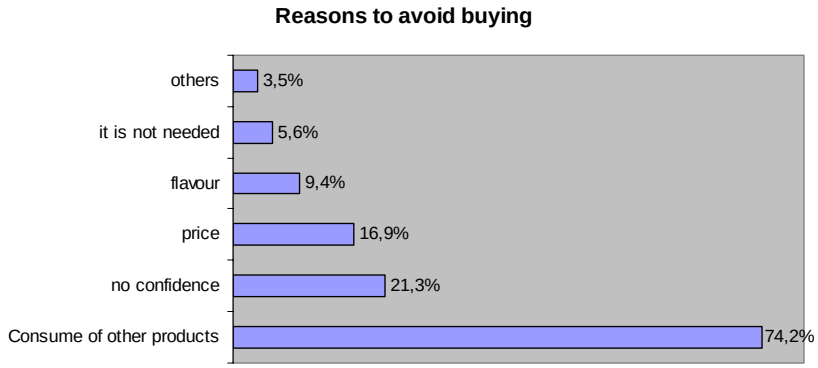
Influence on buying decision (brand, product or component)

The effect of the component, brand or product on the decision of the buying of functional products is balanced. It seem that there is a major influence in the decision of buying from others (family, friends, etc)



influence on buying decision

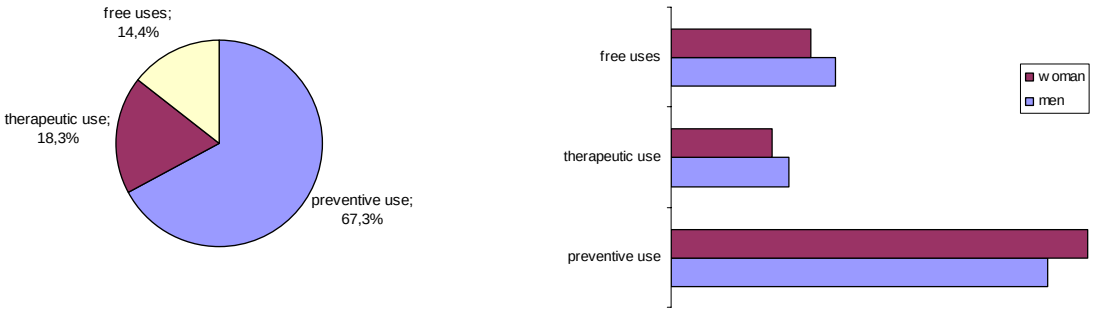
When people were consulted about the reasons for not to buy functional products, the reasons can be divided in five categories related with confidence, price and flavour. The main reason for not buying was that the consumers rather preferred use other products.



Other influences on buying decision

Use of the product

The people were consulted about the use of these functional products according three possibilities related with their properties: preventive use, therapeutic use, free use (not related with wellness). Most of the results was focused in the preventive use of these products which is a good agree with the correct use of their properties.

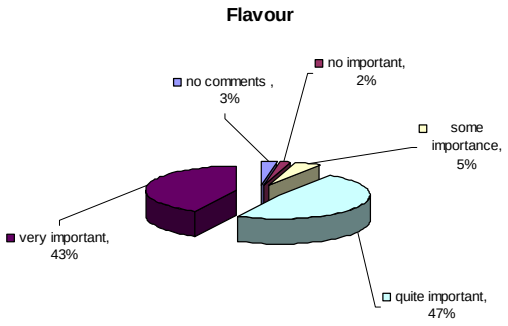


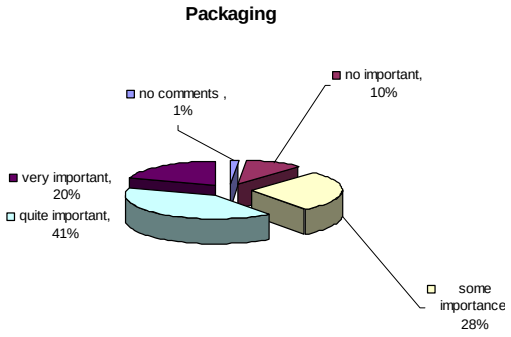
Intended uses of the products

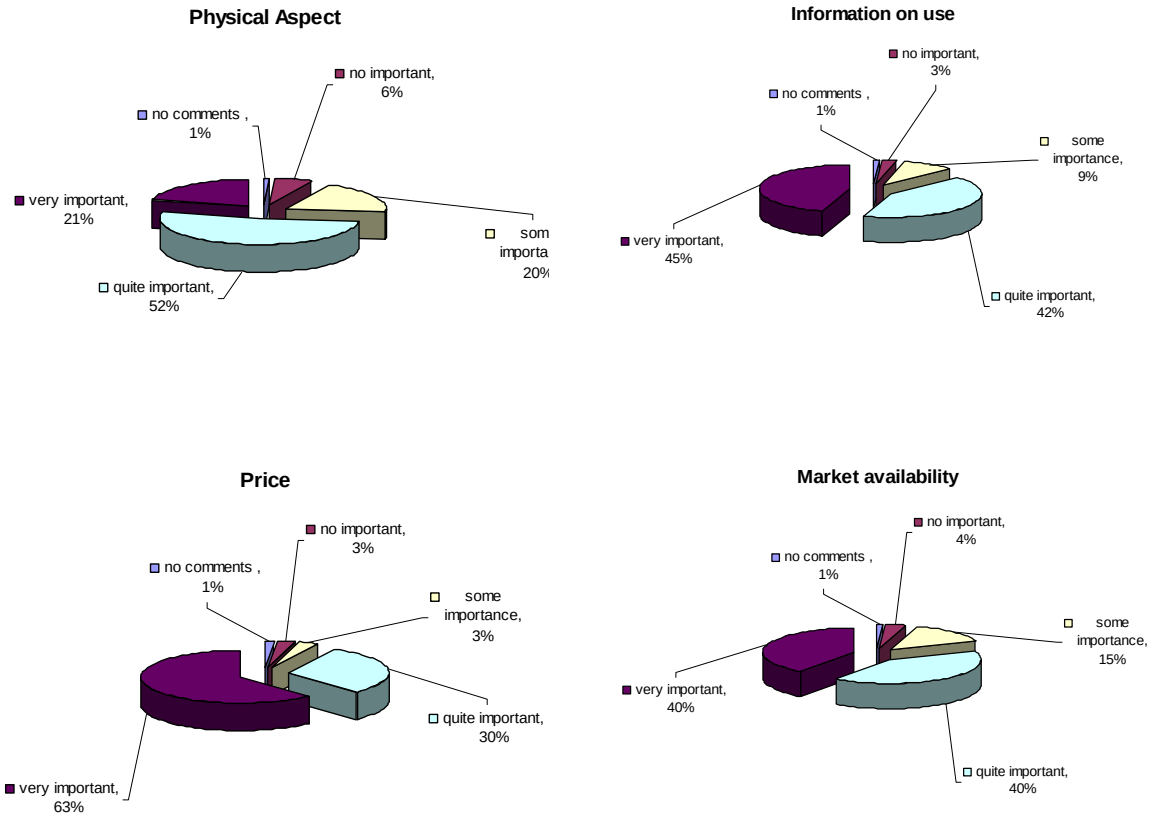
Valorisation on properties for similar products

It was handled the following concepts on properties of the functional products: flavour, packaging, physical aspect, information on the use, price and availability of those products in the market. For every concept was it was assumed a scale ranged in five relative values: No comments, No important, some importance, quite important and very important.

In the next figures it can be observed the obtained results:





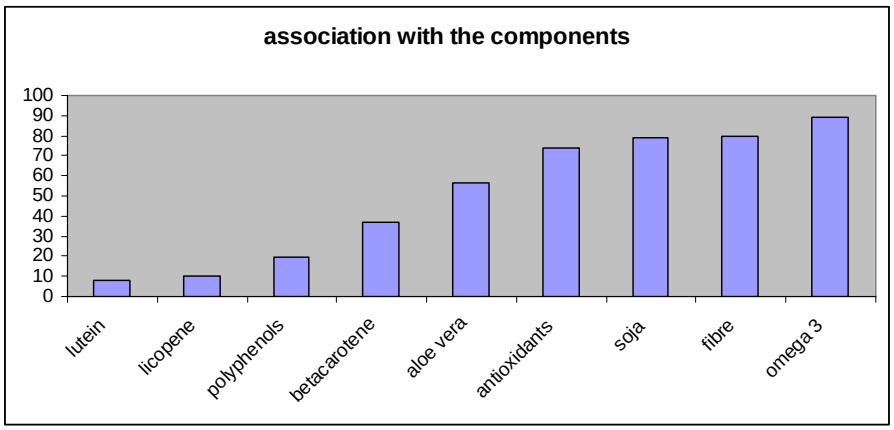


Valorisation on properties for similar products

Finally people were questioned about the knowledge and the association with the components. The following set of components was used: omega 3, fibre, soja, antioxidants (generic), aloe vera, betacarotene, licopene, polyphenols, lutein

It can be observed that, although the term antioxidant is clearly recognised as functional compound, the precision on the antioxidant properties of the polyphenols, licopene and betacarotene is not so evident for the general public. Last compound (betacarotene) was identified mainly due to their uses in the sunscreens topic creams. Those compounds more clearly identified by the people were those related with foods, where marketing campaigns have been more active.

Next figure shows these results:



Association of compounds with functional products

Conclusion

The conclusion of this task that relevant information has been gathering from market. Main aspects could be summarised in the following points

- The spontaneous recognition of functional products is quite low, however, if some comments are suggested on properties, the situation changes and recognition capacity is increased.
- This situation points that although the recognition of the product is high the denomination of the category "functional product" is low.
- The influence of the factors in the decision of the acquisition of the product is influenced in equal terms by brand, product and component. At the same time, the influence of the family and friends is high. It can be said that the consumer knows the products by the mass media (mainly commercials) and the decision for buying is highly determined by people from the near environment.
- The price and flavour are the most relevant aspects that consumers valorise. Availability is another important factor.
- Although the possible effect of the product is identified (antioxidant), there is a poor relation with the compound which helps to achieve this property (licopene and polyphenols)

Possible actions to consider in the use of the extracts obtained by means of the Extranat process should consider that end consumer should be informed better on the property rather than the compound.

Finally it will be commented that although the main consumers are women, the acquisition of the product is higher in the case of men. This could be considered in the definition of some strategies for marketing.

The main remarkable result is that people relates the use of functional products with the prevention of illness, so they are used to hold a wellness state.

Task 7.2: Exploitation plan.

This task had as objectives to design a Technology Exploitation Plan making use of the collected information and taking into account all elements of the marketing-mix scheme. Methodology to be used should include:

- Target market analysis
- Pre-assessment of market value and selection of the best technology transfer practices.

During the first period of the project, the main efforts within this task were used to reach a first draft of the plan for the use and dissemination of the knowledge, attached to this report as an Annex. This first draft is also considered as Deliverable number 5.

This draft plan was firstly designed in the first months of 2006 and then it was amended and improved by all members. The consensus was finally reached during the mid-term meeting.

ECONOMIC ASSESSMENT

1. Introduction and scope.

The purpose of this study is to make an economic balance of process developed in the EXTRANAT project for the extraction and concentration of antioxidant compounds from grape pomace residues.

Both equipment and operating costs were considered in order to asses the feasibility of the process and determine the potential economic benefits of the pilot plant. The market price of the final product, (polyphenol-enriched dry extract) was estimated and included in the economic balance of in order to estimate the net benefit of the process. A process scale-up was performed in order to estimate the total costs and benefit of running the process when increasing the plant capacity and, therefore, check the potential feasibility of the process at industrial scale. Finally, an estimated market price of the final extract product was calculated in order to make the process economically viable within a scale-up factor ranged from 50 to 100.

Main regards

Costs have been estimated from the results obtained in the pilot plant developed in the EXTRANAT project.

The advantages of the *economy of scale* were considered to evaluate the feasibility of the process. A continuous flow process was considered to optimise the capacity of the plant.

The main contributions considered in the overall cost of the process are the following:

1) Operating costs

1a) Energy cost: pumping, heating, refrigerating and drying costs

1b) Raw material cost: grape pomace residue and reagents.

2) Equipment costs: process units, (pipes, valves, pumps, tanks,..); instrumentation and control

Economic viability of the process depends strongly on the quality of the final product (total polyphenol content and antioxidant activity) to estimate its market price. Accuracy in the market price of the final product is essential for come up to sound conclusions about the feasibility of the process.

- Market price of raw material and final products.

The incomes corresponding to the sales of the final product is the key point to determine the economical viability of the process. A previous market study was performed in order to estimate the market price of the final product (spray-dried extract) and so calculate the net benefit of the process. Market prices of the final product were estimated according to the quality of the extract especially referred to the overall polyphenol content present in the samples, (Next Table).

Table. Market prices of polyphenols spray-dried extracts, (courtesy of Exxentia S.A)

Polyphenols content [wt. %]	Market price (euros / kg)
< 20	20-30
20 -50	50
50-80	70
> 80	100

In order to make a preliminary assessment of the economic feasibility of the process, both the incomes of the final product, (spray-dried extract) and the cost of the raw materials/re-agents were addressed. However, the main costs are associated to the equipment and build-up of the plant (capital investment cost) as well as the operating costs, (energy consumption and reagents). In principle, the cost of the raw material, (grape pomace) is assumed to be negligible as long as it is considered as a non- valuable residue ready for disposal in most of the wine-making factories.

Some additional regards have been considered for the cost estimation:

Grape pomace residue is a non-expensive raw material.

A preliminary solid-liquid extraction with ethanol is performed in order to exhaust the solid matrix and obtain a first raw extract. Solid-liquid (S-L) extraction is performed at 40 ºC and ambient pressure prior crushing of the frozen solid.

Costs associated with S-L are, in principle, not considered.

The CO_2 consumption is significantly reduced in continuous operation since the CO_2 stream is separated from the organic solvent and fed back to the process. The recovery of the entrainer solvent, (ethanol in this case) has been considered in the mass balance. A recovery of 60-80 wt. % of ethanol could be assumed in the sc CO_2 process, (losses are produced in the S-L and SC extraction).

The content of dry extract present after the the sc CO_2 extraction-concentration process is *ca* 0,020-0,030 g/mL. Weight % of polyphenols present in the dry extract is ranged from 50 to 80 wt.%. According to the market study, the price of the spray-dried product was fixed at 70 euros/kg, although could reach 100 euros/kg eventually if polyphenol content reach 80

wt. %, (table 1). Anyhow, the extraction with sc CO_2 produces an effective isolation and selective separation of polyphenols of interest that may enhance the quality of the final product and wide its scope in high level applications, so that the market price could rise.

Net benefits of the process were calculated as follows:

Net Benefit = Incomes for product - (Operating costs + Capital investment costs + cost of raw materials and reagents)

2. - Operating conditions

An initial economical study was performed for the pilot plant developed by CARTIF. Operating costs of the **supercritical extraction plant** were estimated from experimental results at the following operating conditions:

CO2 feed. Close loop operation with recovery and CO_2 feed back.

The CO_2 stream is pumped and then mixed along with the co-solvent feed. The plant is assumed to operate under near maximum capacity, (12 kg/h of CO_2 flow rate). CO_2 is separated and then fed back to the process continuously. In practice, a purge of 2-5 wt. % should be performed to ensure the stability of the operation plant. Thus, the CO_2 consumption comprises the amount of CO_2 needed for the start-up period and the purge stream along the continuous operation.

Raw extract feed.- (co-solvent stream)

Co-solvent/ CO_2 ratio was set at **5,6 wt./wt. (%)**, (0,72 dm3/h of feedextract/12 kg/h of CO_2). Usual operating conditions were set up with a co-solvent/ CO_2 flow ratio *ca* 4-5 %.

Process parameters:

Solid-Liquid Extraction:

T = 40-45 ºC

P = 1 bar

Mass grape pomace / co-solvent volume = 0, 33 kg/dm3

Ethanol loss (remained in solid after filtration) = 20 wt. %

SC Fluid extraction:

T = 40-45 ºC

P = 90 bar

CO_2 Flow rate = 12 kg/h

Feed Extract Flow rate = 0, 9 dm3/h (*ca* 0,72 kg/h)

CO_2 separation/ ethanol recovery conditions: 30-35 ºC, 50-60 bar.

Raw material consumption :

CO_2 consumption / day = 0, 6 kg /day (2 % purge stream)

Ethanol consumption / day = 27 dm^3/ day (5-8 dm^3/day with an estimated recovery *ca* 70-80 wt. %)

Grape pomace mass/ volume ethanol = 0, 33 kg/ dm^3

Grape pomace mass /day = 9 kg /day

Co-solvent (feed extract) / CO_2 wt. /wt. (%) = 10

Products

Concentrated extract volume ratio (concentration factor) = 5 vol. %

Concentrated extract / day = 4, 32 dm^3/day

Dry extract / day = 0,108 kg/ day

3. - Economic balance.

3.1 Pilot plant costs. Total costs, operating and investment costs. Benefits

Total capital investment cost has been estimated employing the simplified method (Lang's), with a time of amortisation of 10 years considered. Operating costs were estimated taking into account the energy consumption associated to the compression and pre-treatment of the feed streams, as well as the spry-drying of final product. In order to estimate the annual production and energy costs,

The feasibility of the process lies in the potential ability of the scCO2 to concentrate an alcoholic solution of grape extracts and, at the same time, produce a selective extraction of the phenolic compounds where impurities such as waxes, chlorophylls, etc., have been removed. This way, the quality of the final product is enhanced presenting a higher antioxidants capacity and/or polyphenols content (wt.%). Table 2 presents some characteristic data of both the raw and the refined extracts obtained in the process. As seen, the dry extract content is significantly improved due to the concentration process and as well as the total polyphenol content (weight of polyphenol/weight of total solids) a total operation time *ca* 300 days/year and 24 h /day were considered.

Table. Characterization of the raw and purified polyphenol extracts

	Raw extract	Purified extract
Dry extract [g/mL]	0,006	0,020
AOA [mg/mL]	2000	12500
Total polyphenol content [wt.%]	33,3	62,5

The SCF process permits to produce a final dried extract with a higher content in polyphenols (wt. % solid), and so increase the added value of the final product allowing for a higher price in the market (the is key point for the economic feasibility of the process, following figure).

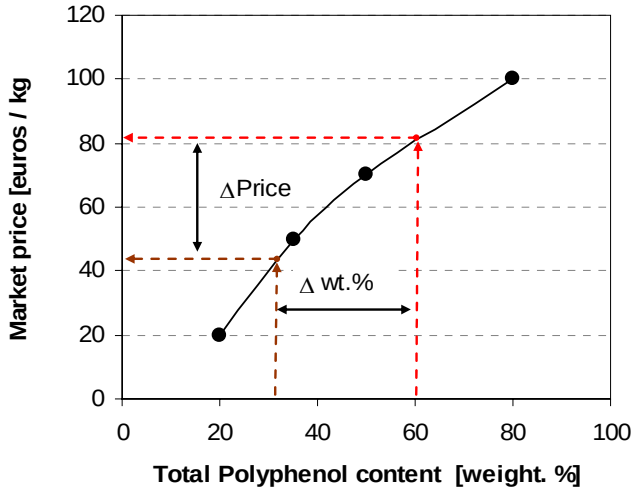


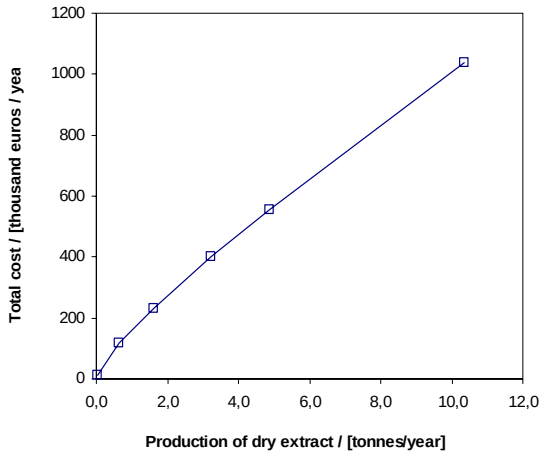
Figure 1. Market price of the spray-dried product vs. scale factor of the plant

3.2. Process scale-up. Market price.

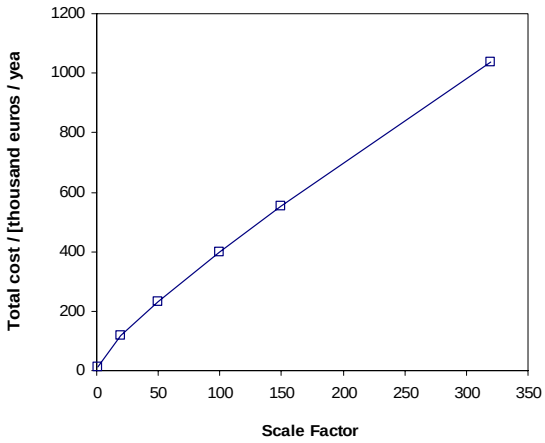
In general, pilot-scale plants -designed just for research aims- are not profitable since their small capacity do not permit to take advantage of the economy of scale and the net incomes of the process are often negative.

In order to carry out a more realistic study of the economy of the process it is necessary to consider the potential benefits associated to the scale-up. As the scale factor is higher the manufacturing costs per unit of product decrease and, for a certain value, the net benefits of the process reach a cero value and start to be positive instead of negative.

As seen in figure 2a-2d, net benefits decreases as the scale is increases until reach a minimum value, from which, the slope of the curve becomes positive. At a certain value of scale (*ca* 300-350) the net benefit becomes cero, so that gross incomes and total costs are equalized, (these total cost include also amortization of the equipment and plant facilities).



a)



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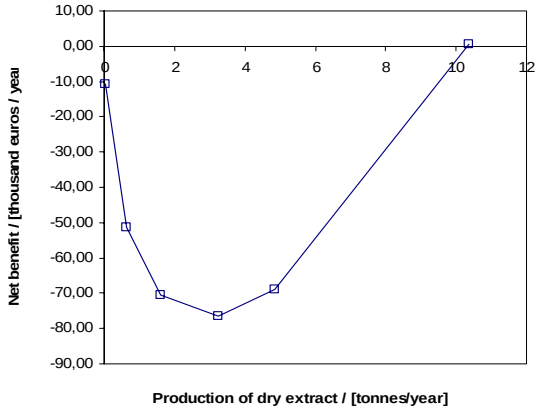
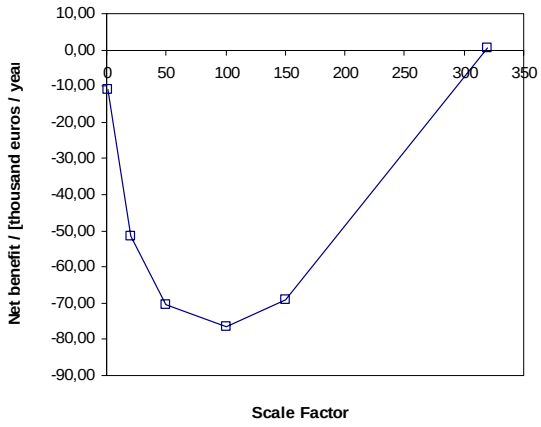


c)



d)

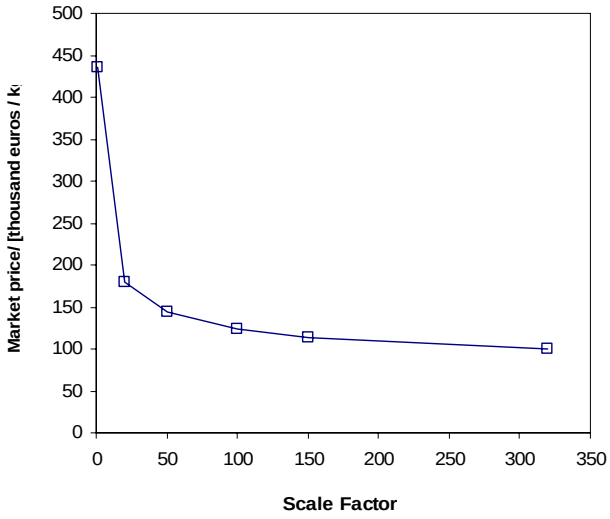
Total cost and net benefit in terms of the process scale-up. 2a) Total cost vs. dry plant capacity (tonnes dry extract/ year); 2b) Total cost vs. scale factor; 2c) Net benefit vs. dry plant capacity (tonnes dry extract/ year); 2d) Net benefit vs. scale factor

Table. Costs and benefits vs. scale factor (amortisation time: 10 years, market price of dried extract: 100 euros/kg)

Scale factor	Capital costs [thousand euros]	Operating Costs [thousand euros]	Total cost [thousand euros]	Net benefit [thousand euros]
1	12	1,8	14	-10,87
20	80	36,6	116	-51,37
50	141	91,4	233	-70,56
100	218	182,9	401	-76,58
150	281	274,3	555	-68,90
320	451	585,3	1036	0,6

The price of the dried extract in the market exhibits a significant variability depending on the quality of the final product, (polyphenol content, AOA). In principle, for high polyphenol-content extracts, (ca 80 wt.%), the initial market price could be set at 70-100 euros/ kg. Nevertheless, the final market price could rise up to 100-150 euros/kg depending on the demand of the market for new high grade applications.

As seen in next figure, for a scale factor = 1 (the current pilot plant in continuous mode operation), the fixed market price to run the process without costs would be set over 400 euros/kg. However, with a scale-up factor over 50, the minimum market price to run the process with a net benefit approximated to zero could be set around 150 euros/kg

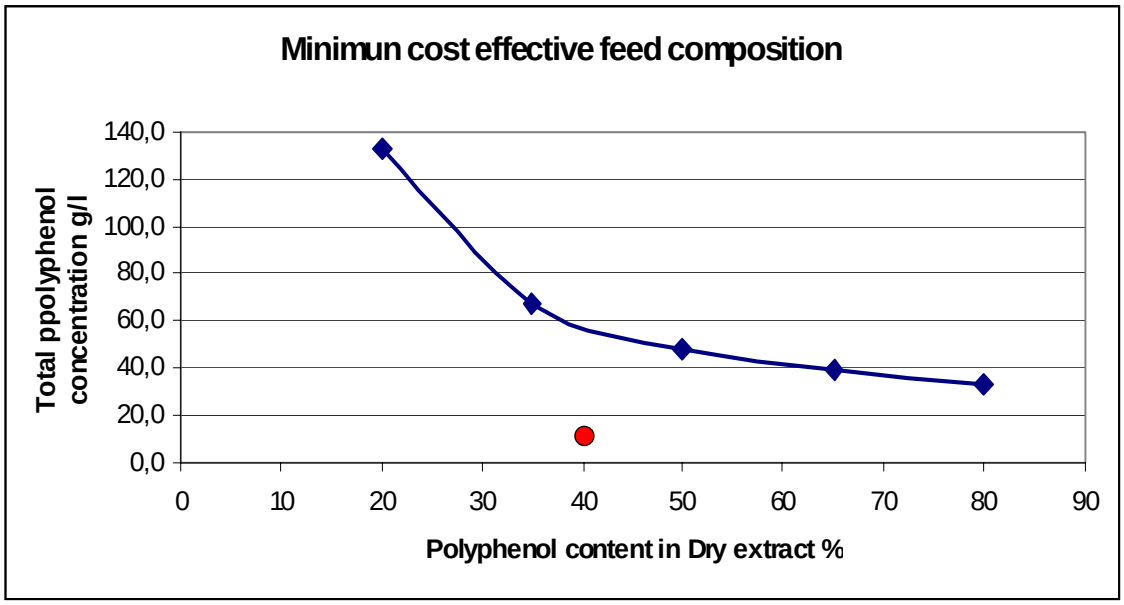


Market price of the spray-dried product required to meet net benefit =0

3.3. Concluding remarks

In general terms, it can be concluded that the process is feasible from a technical point of view, but relevant remarks should be done on the quality of raw material study since profitability of the process will strongly determined by this factor.

Next figure shows relation between the composition of feed solution (expressed in polyphenols concetration) and the total content of polyphenol in a final theoretical to be cost effective for the extranat process.



Relation to be cost effective concentration

Those values of concentration laying under the curve will not be profitable, since quality and content of polyphenol are not high enough to perform a cost effective operation. In this sense, the red point shows the medium composition of polyphenols solution obtained when performing the extraction with ethanol. It is clear that, although the process is able to produce a profitable product, the initial material to work with (vegetal waste) is not able to supply the required quality to perform the extranat process.

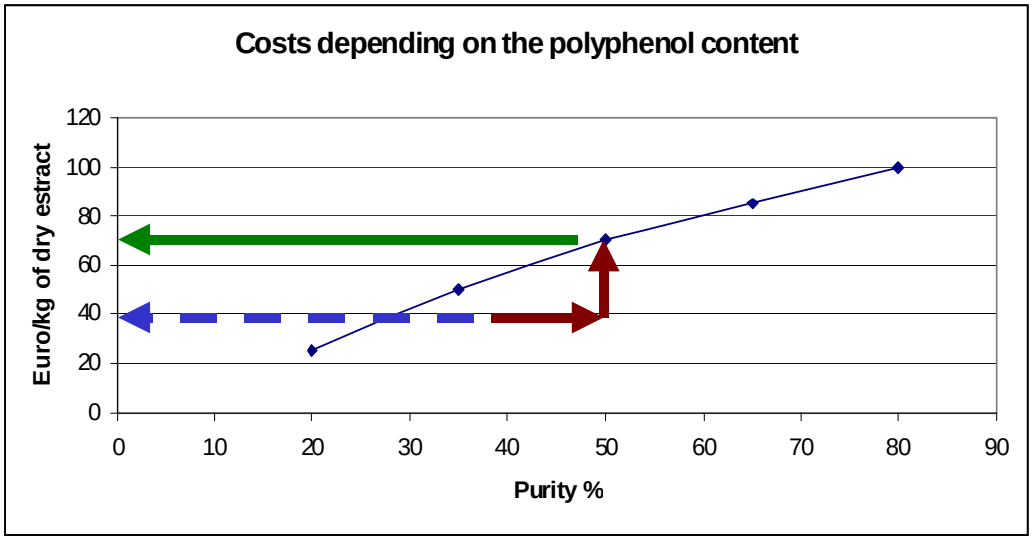
This should be considered along the scale-up factors, since lower scale plant could be obtained if it is intended to work with better raw materials.

Process scale-up and/or a higher market price according to the added-value of the final product should be target in order to tight the costs per unit of product and balance the net benefits of the manufacturing process. The technical-economic study of the process revealed that:

- The technology developed in this process permit to obtain a refined concentrated extract with a significant content in polyphenols, (ca 60-80 wt. % per total dry extract). The market price of the final product is, initially estimated at 70-100 euros/ kg. Nevertheless, the content in polyphenols of the final extract could be improved depending on the quality of the original the raw material.
- From an economic point of view, the capacity of the plant should be increased to balance the economics of the process. A scale-up factor around 300, (ca 10 tonnes of dry extract /year) should be applied in order to run the process without a negative economic balance. This level production could be excessive and saturate the market.
- Another point of view to increase the profitability of the plant is to reconsider the market price of the final product. Preliminary costs estimations predict that the

market price for the dried extract should be fixed at 100-150 euros/kg to make the process economically viable, so that the capacity of the scaled-up plant could be set at 50-100 (1,5-3,0 tonnes/year).

Although the pervious considerations, it should be noted that the most striking effect of the process is that the quality of initial extract is fairly improved since not valuable compounds are removed during extranat process, and so the final value of product obtained is higher than traditional methods of extraction.



Improvement of final cost through extranat process

Task 7.3 Dissemination of the results.

The main objective of this action was to execute different dissemination actions about the progress of the project. In this sense, several actions were taken. This task was programmed, following the work plan in the Technical annex, to start in month 18, but as requested in the general rules of FP6 projects, some efforts were dedicated to make the general public aware of the EU funded projects. These efforts were placed during the first period in order to advance in the dissemination activities as soon as possible. During the second period, the dissemination of the poject continued.

A presentation on the actions is commented:

CONGRESSES

- **Poster**. "V International Symposium on High Pressure Process Technology and Chemical Engineering", 24-27 th of June 2007, Segovia, Spain). The content of the poster is commented in the abstract book of this congress. (A general view of this poster is showed in the figure)

- **Publication.** "XXVIII Congresso Nazionale **SIP 2006"**, 19-22th of September **2006,** Pavia, Italy. "Antioxidant and Antiproliferative Effect of Grape Waste Extracts Obtained Using the Supercritical Fluid Extraction (SFE) technology".

Other additional **disemination actions included in the extranat project were:**

- Feria Health Ingredients (HI) Europe, Frankfurt, noviembre 2006.
- VITAFOODS – GINEBRA, may 07.
- Functional Foods, Patrocinio Danone. Madrid, june 07.
- Legislación alimentaria de Health Claims. Madrid, june 07.
- Feria Health Ingredients (HI) Europe, Frankfurt, noviembre 2006.
- VITAFOODS – GINEBRA, mayo 07.
- Functional Foods, Patrocinio Danone. Madrid, junio 07.
- Legislación alimentaria de Health Claims. Madrid, junio 07.

REF. APLI-28

SUPERCRITICAL EXTRACTION-CONCENTRATION PROCESS OF PHENOLIC ANTIOXIDANTS IN A CONTINUOUS PILOT PLANT

Gutiérrez F. J. (*), Antolín J. M., Piñero R., Gatón. P., Ruiz M., Porcu M. C [2]., Antolín G

Chemical Process Laboratory- Centro de Automatización, Robótica y Tecnología de la Información y de la Fabricación (CARTIF)
Parque Tecnológico de Boecillo, 205, 47151-Boecillo (Valladolid), SPAIN; Phone: +34 983 54650 Fax: +34 983 546521; e-mail: fragut@cartif.es ; http://www.cartif.es

[2] Porto Conte Ricerche S.r.l – Tramariglio 07041, Alghero (SS)- ITALY; Phone: +39 079998400 Fax +39 079996567; http:// www.portocontericerche.it

V INTERNATIONAL SYMPOSIUM ON HIGH PRESSURE PROCESS TECHNOLOGY AND CHEMICAL ENGINEERING 24-27 th of June, Segovia (Spain)

1 SUMMARY

The present work aims to develop an effective process for the recovery of selective concentrated extracts of antioxidant compounds from vegetable and fruit waste material using the scCO2 technology. Phenolic antioxidants such as gallic acid or epicatechin were obtained as key components from grape waste material. The solid matrix was first exhausted by a solid-liquid extraction performed at 30-60 °C and ambient pressure using ethanol as extractive solvent. The extract was subsequently concentrated in a pilot plant by a continuous scCO2-assisted process which allowed for a selective separation of the phenolic compounds present in the grape pomace. The aimed compounds were quantified by HPLC, and the antioxidant capacity of the final extract was measured by two standard methods: DPPH and Folin-Ciocalteu. The results showed that the extracts of red grape pomace were rich in polyphenolic compounds with an antioxidant activity significantly improved after the scCO2 treatment. A quantitative separation of antioxidant compounds (polyphenols) and pro-oxidant compounds (chlorophylls) was achieved in the extractor and separator units respectively. In addition, the effect of the water content in the initial raw material was investigated.

2 EXPERIMENTAL & MATERIALS

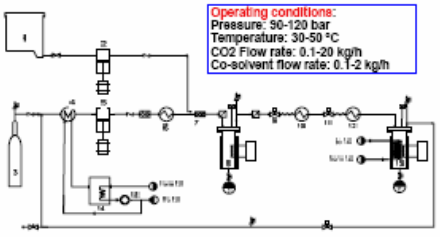


Figure1.- Process flow diagram: (1) Cosolvent storage tank; (2) Cosolvent high pressure pump; (3) CO2 cylinder; (4) CO2 chiller (double-tube); (5) CO2 high pressure pump; (6) Heat exchanger; (7) Mixer; (8) Extractor; (9) Electro-pneumatic valve (1st expansion); (10) Heat exchanger; (11) Electro-pneumatic valve (2nd expansion); (12) Heat exchanger; (13) Separator; (14) Cooling unit; (15) cooling fluid pump

Figure 2.- Extractor

3 RESULTS AND DISCUSSION

• Experimental Results

Chlorophyll was obtained in the separator unit but not antocianinns were found. (Fig.3)

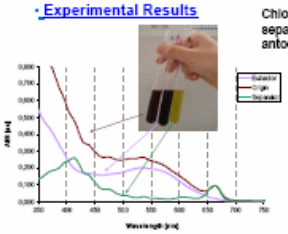


Figure 3.- Spectra: origin, separator and extractor samples

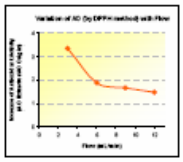


Figure 4.- Variation of AO (by DPPH method) vs. flow rate

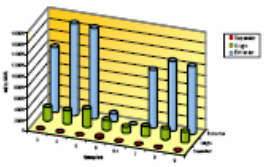


Figure 5.- Polyphenols content by Folin. Ciocalteu method

The antioxidant activity (AOA) of the concentrated extract decreases as the cosolvent-CO2 flow rate ratio is increased (Fig. 4)

The AOA enhances regarding the original extract feed, (Fig 5). A significant amount of water is required to retain the polyphenols compounds in the extract unit, (Sample 6 is free water)

• Extraction-concentration process

A scheme of the of the block diagram of the process is presented in figure 6. The process is characterised by two steps: 1) A conventional solid-liquid extraction of red grape pomace residue was performed with an aqueous ethanol solution; and 2) a sc-CO2-assisted process for the concentration of the natural products of interest extracted in the previous stage.

The sc-CO2 extraction process aims two goals:

1st.- Concentrate the polyphenolic original solution and remove the organic cosolvent by means of the CO2

2nd.- Achieve a selective separation of the antioxidant compounds (polyphenols) – retained in the extractor unit- and the less polar compounds, (oil, fat, chlorophyll) which are drag with the ethanol-CO2 stream

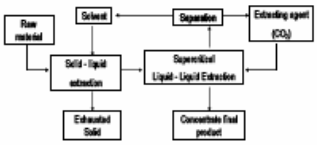


Figure 6.- Block diagram of the integrated extraction-concentration process

4 CONCLUSIONS

- The sc CO2 assisted process was employed successfully in order to concentrate and purify hydroalcoholic extract of polyphenols from grape pomace residue, (ca. 5-6 concentration factor)
- Ethanol is separated quantitatively and recovered in a second separation unit (80-90 % vol) along with non polar present in the original extract (e.g. chlorophyll, oil, etc)

FUTURE WORK

- ✓ Study of the thermodynamics and fluid phase equilibria of the extraction process
- ✓ Optimization and Economical study
- ✓ Screening study with different raw materials

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[1] Alonso, A.M., Guillén, D.A., Barroso, C.G., Puertas, B., García, A., "Determination of antioxidant activity of wine byproducts and its correlation with polyphenolic content" J. Agric. Food Chem. 50, (2002), 5832-5836.
[2] Negro C., Tommasi A., Miceli A., "Phenolic compounds and antioxidant activity from red grape marc extracts" Bioresource Technology, 87 (2003), 41-44
[3] Palma, M., Taylor, L.T., "Extraction of polyphenolic compounds from grape seeds with near critical carbon dioxide" J. Chromatogr. A, 983, (1999), 117-126
[4] Monge, R., Ruiz, R., Berli an, S., Cabezas, J.L., "Extraction of natural complex phenols and tannins from grape seeds by using supercritical mixtures of carbon dioxide and alcohol", J. Agric. Food. Chem. 48, (2000) 3465-3472

Acknowledgements: EU Project EXTRANAT (ref COOP-CT-2004-512550-EXTRANAT)

As it was expected, a **promotional CD-ROM** was produced containing information on the results of the project, as well as information of the SME's of the consortium.

The changes suffered by the Consortium during the first year running (see Evolution of the Consortium), caused that the amount of money appointed to this task was immobilized for a long time. These facts made the consortium to decide that the different bids will be searched and the subcontract awarded in the second year of the project. Therefore, during the second period of the Porject, a subcontract was foreseen to help in this tasks, a minor part of the innovation activities aimed to the dissemination beyond the consortium and study of the standardisation and regulatory aspects that influence the exploitation of the results. These are Innovation activities for the project as a whole but it was considered that the most suitable approach was to allocate the budget for this potential sub-contracting (18000 €) to a given partner rather than sharing its costs among the whole consortium. In order to have this dissemination strongly coordinated with the exploitation of the results and ensure that use of the pilot plant as demonstrator is adequate it has been envisaged to allocate the costs of the contract to ACOMSA, afterwards substituted by MATARROMERA (where it implemented the plant).

Project Presentation:

One of the objectives of the European Commission 6th Framework Programme is to improve the dialogue between science and society. This objective can only be reached if the efforts done by the European Commission and the Participants in this program, along with the results reached, are properly made available to the general public.

In order to respond to this particular interest of the European Commission about dissemination of research activities beyond the academic ambit and the interested industries, this project has taken several actions. These actions are gathered and explained in the following pages:

Actions agreed:

During the first six months, and mainly in the first two consortium meetings (kick-off and 6th month), the participants agreed on the first actions to be taken in order to fulfil the contract regarding the dissemination of the project. The first action agreed was to write a project summary that could be published in several media. It was also decided that it would be useful to have a graphical identification of the project, a logo. This two disseminating elements were first deployed by the Co-ordinator and then amended by all the partners, some directly in the meetings and some via e-mail.

The project Summary, including the Logo, was sent to the services that the European Commission has made available to ease the dissemination actions. It was sent to Cordis wire and Cordis Express.

It was also agreed by the consortium to start the development of the website of the project. This website has two different parts: one public and one private. The first is being used as a tool to make the project objectives, and in the future, the achievements available to any person interested in the issues related to Extranat.

The private part of the project will only be available to the members of the consortium. This tool is used as a tool of communication between partners, and therefore it makes the administrative and management activities easier.

Description of the actions.

The project Summary was sent to Cordis Wire and immediately published. Cordis wire is an e-mail system alert from the EU about innovation and research news. It has also been sent to Cordis Express: A weekly briefing on what's new in European research and innovation. The service is designed to highlight the most significant recent developments, as reported in Cordis, the European Commission's Research and Development Information Service.

The **Project Summary** has been translated to the different languages of the partners in order to ease acceptance in publications that use different languages from English.

Another action has been the development of the **website of the project**. There has been reserved a space in the web from the Co-ordinator assistant, Idetra. Its URL address is: http://www.idetra.com/extranat/. In this website several areas can be found, under two main zones, the public and the private.

In the public zone, the main departments are:

-Home: where the project Summary and the Logo can be found.

-Partners: A list of the partners with their contact addresses and their logo.

-Documents: where public documents can be shared with the public.

-Recommended links: Links to the partner's websites and other interesting webs related to the project.

Public part of the web

In order to access the private side of the website, a user name and a password have to be provided. If those are introduced correctly, the permitted user will have access to:

-The same departments of the public zones, but this time with the right to edit and change some of the data, for example, add new links, correct the address of the partners or add a new public document.

-The Agenda of the project, where the meetings and other important events can be located.

-Private sharing of documents. It works the same way as the public part but it is restricted to signed users.

In the main page of the website the logo of the European Commission and the Text "Funded by the Commission of the European Commission" can be found, complying thus with the legal provisions for Publicity of the funded projects.

Links to this website have been also added in the websites of the partners. This task is taking some time, given that most of this websites are not managed by the partners but by some external agents.

During the development of the Project, **another web** page was also implemented by MATARROMERA. The following links were defined to present information:

http://www.matarromera.es/es/imasd/extranat.php. An example of developed wed page is presented in the next figure:

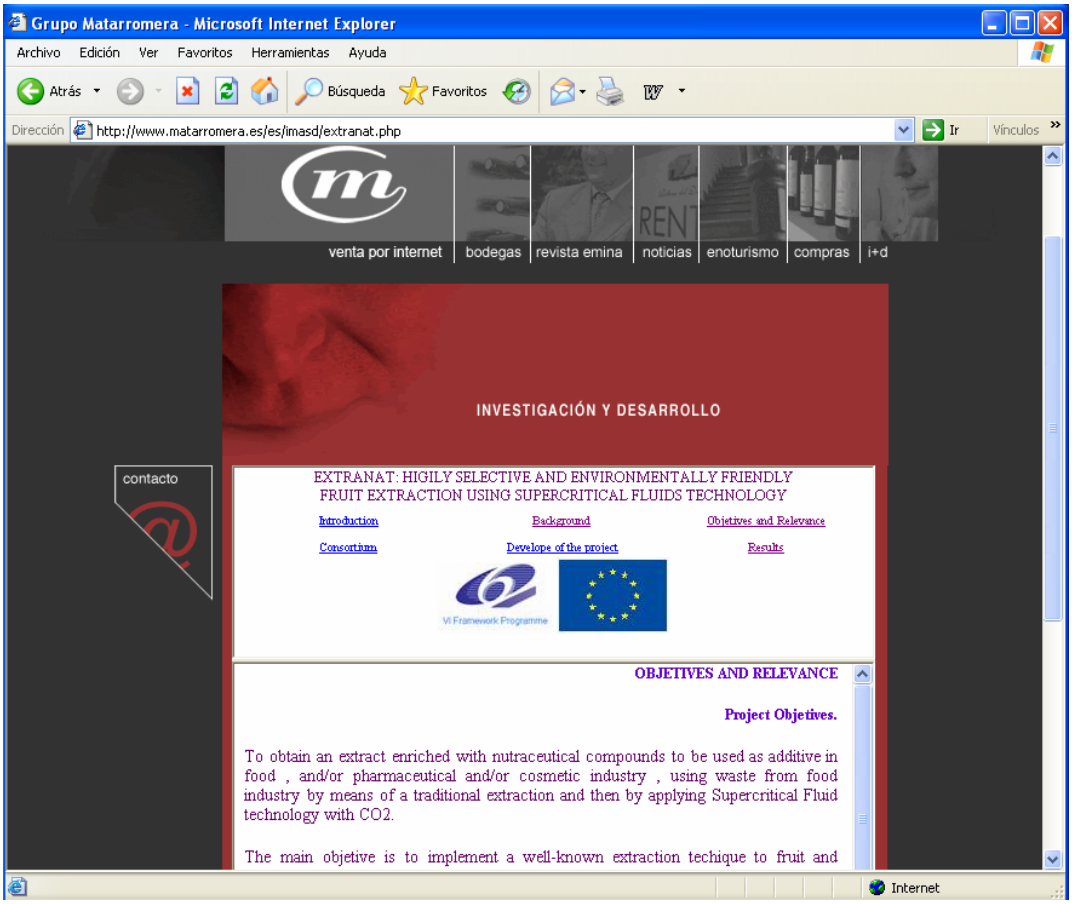


Figure: Example of the aspect of the web page developed for the extranat project

Results.

Several **electronic publications** have made reference to Extranat:

Aquí Europa
(http://www.aquieuropa.com/portada.asp?tipo=2&buscar=1&tema=&palabras=extranat&fechaFija=7&ultimas=5),

Bulletins-electroniques

http://www.bulletins-electroniques.com/actualites/31620.htm

Evirocentre

http://www.envirocentre.ie/news.asp?id=31&cid=1940

Finanzamenti per l'innovazione , la ricerca e lo sviluppo tecnologico
http://first.aster.it/news/show_news.php?ID=12739

Innovatech
http://www.innovatech.be/archives/details.php?type=actualites&actualite_id=833&cerdt_Session=064d4ca2764328d1e4a1e8fc58fe5f08

Castilla la Mancha Innovación
http://www.clminnovacion.com/actualidad/noticias/articulo.htm?REG=4442

Observatorio Tecnológico de la Industria Agroalimentaria de la Comunidad de Madrid
http://www.observatorio-alimentario.org/proyectos/detalle.php?numero=146

Innowatch(Cordis):
http://project.idetra.com/component/option,com_contact/Itemid,25/catid,70/

Observatorio Tecnológico de la Industria Agroalimentaria de la Communidad de Madrid:
http://www.observatorioalimentario.org/

Eurociecette:

http://www.euroricette.it/notizia.asp?idn=2702

FIRST:

http://first.aster.it/news/show_news.php?ID=12739&PRN=1

EMS 4 SMEs:

http://www.envirocentre.ie/news.asp?id=31&cid=1940

FP6 eBulletin:

http://www.apre.it/nuovo-sito/PMI/schedeProgetto/Rural-NewsLetter/newsletter_10.pdf

Bulletin d'information du Bureau Europe d'Innovalis Aquitaine:

http://www.innovalis-aquitaine.org/Images/Upload/doc/europe/BULL_136.pdf

Bulletins-electroniques.com:

http://www.bulletins-electroniques.com/actualites/031/31620.htm

Cordis Focus (December 2005, Issue Number 261, ISSN 1022):
ftp://ftp.cordis.europa.eu/pub/focus/docs/261_en.pdf

Notizie dallo Sportello Apre:
http://images.to.camcom.it/f/InnovazTecnologica/no/notizieapredicembre2005.pdf

The **Project Logo** is being used in all the official documents of the project and it will be used also on all the rest of the dissemination tasks.

The **Extranat webpage** has created a good working space for the partners, making much easier the sharing of documents, information and other resources.

Deviations from the project workprogramme

As explained before many factors have affected the development of the work within this workpackage, ranging from technical to problems in the consortium magement.

The Technical delay, mainly in the construction of the pilot plant, has caused to enlarge task 7.1, until the production cost analysis could be done.

The subcontracting of the dissemination tasks did not have an impact on the development of task 7.3 since this was started by September 2006.

List of Deliverables and Milestones

Deliverable no.	Deliverable Name	WP no.	Date due	Actual delivery date	Lead contractor
1	Project Presentation	7	September 2005	November 2005	ALDIVIA
5	Draft of the "Plan for use and dissemination of knowledge"	7	April 2006	June 2006	CARTIF
9	List of estimated costs of SFE of the selected compounds.	7	Month 27 (June '07)	Month 27 (June '07)	ALDIVIA
12	Final version of the Plan for the use and dissemination of knowledge.	7	Month 29 (August '07)	Month 29 (August '07)	ALDIVIA

Milestone no.	Milestone Name	WP no.	Date due	Actual delivery date	Lead contractor
5	Definition of User Needs and Exploitation Protocol	7	Month 24 (March '07)	Month 24 (March '07)	CARTIF
6	Protection of know-how	7	Month 29 (August '07)	Month 29 (August '07)	CARTIF

Section 3 Consortium Management

Workpackage 8: Project Management

The main objective of this workpackage is to ensure the achievements of the general and technical objectives of the project, within the constraints of the time schedule and budget, by planning, organising and controlling the integrated effort of the whole consortium.

The management work can be divided into:

Administrative

It comprises all efforts made to assure the fulfilment of the EC requirements for this kind of projects. Among these tasks, the writing of reports, the exchange of information among partners and between the Consortium and the European Commission should be remarked.

Technical

Under this work, all activity oriented to the scientific/technical work itself: how the technical progress will be monitored and assessed, the exchange of technical information amongst partners, the generation and submission of deliverables, etc are included.

Strategic

It comprises any issue related with the final objectives of the project to assure the success of the whole project looking forward the consecution of the expected results both in terms of quality, costs and time, and further dissemination and exploitation of these.

The length of this workpackage, as well as its starting and final point are related to the nature of the work comprised in it. It is clear that it has to be active all along the project, since all tasks have to be coordinated with others. Therefore its starting point is the same as the starting point of the project.

During the project the good level of cooperation and coordination described in previous reports has continued, and in some aspects has been improved. Although these good aspects have facilitated the coordination of the different tasks, several problems have arisen, and therefore many efforts have been located to solve them. The following list tries to summarize the efforts done:

Signature of the Consortium Agreement:

Although this happened before the starting date of the project, it is referred here as an important issue for future decisions, and agreements. This agreement was reached and signed after the withdrawal of the former coordinator, registered in the first amendment to the contract (March 2005).

This Agreement had to be updated after the withdrawal of two of the partners after the starting date of the Project (ACOMSA and AROMATIC). These changes were communicated in the second amendment to the contract sent in December 2005. Due to the delay on the approval of this second amendment.

Due to the decision of the enterprise Instituto Biochimico Pavese Pharma, S.p.A. according to which said enterprise is leaving the Consortium formed in order to carry out the Project EXTRANAT, the same Consortium decided its substitution by other Spanish SME. The third amendment took place in March 2007.

Kick-off meeting

The kick off meeting was held in April 29th at the coordinator facilities (Valladolid, Spain). During this meeting the partners presented their company profile along with the reasons to join the project and their possible contributions.

During this meeting there were also some explanations about:

- The objectives of the project and the general way of achieving them.
- Management procedures.
- Initial actions to get the project really running.

This meeting was the real starting point of the project, since the partners were able to evaluate the potential of the consortium and the ambitious of the objectives.

Communication among partners.

The communication among partners has been done mainly by electronic means. It has been very fluent, and it has facilitated some decisions taking processes. In order to achieve even better levels of information interchange, in the web page of the project, there has been incorporated a space to upload and download documents.

6th Month Meeting:

On September 27th, the 6th month meeting was held in Brussels. This location was chosen to facilitate the attendance of the partners from North and Eastern Europe. During this meeting the development of the workpackages and the achievement of the first conclusions explained in the 6 month progress report were studied by the partners.

Dissemination tasks were also brought to this meeting. It was decided to publish the project summary previously agreed in the European Commission tools, and its delivery to specific publications of the food sector. The logo of the project was also presented in this meeting.

Mid-Term meeting

On May 5th the mid-Term meeting was held at the facilities of the University of Pavia in Milano. During this meeting a review of the technical progress of the project was done as well as a review of the foreseen technical activities. All the partners that attended this meeting agreed to continue with the research line presented by the RTD's since the results seemed encouraging.

Regarding the Innovation activities, it was agreed to increase the efforts to achieve a correct dissemination of the project results. It was in this meeting where the consensus on the Draft of the Plan for the use and dissemination of knowledge was reached.

18 Month Meeting

In November, the 18 month meeting was held in Valladolid, at the coordinator facilities. During this meeting the development of the work-packages and other issues regarding the general objectives achieved during this period of time.

This meeting was also used to make the presentation of the pilot plant to the partners and a little demonstration about this project and others that are being developed by Cartif.

Final Meeting

In July 27th, the Final Meeting took place in Valladolid at MATARROMERA´s facilities. During this meeting, the major achievements gathered during the whole project were presented.

Exploitation of the results was commented. Some management Issues were explained regarding the closure of the project.

Other Technical meetings

During the whole project several other meetings were done in order to coordinate actions where more than one member was involved. During these meetings specific issues where treated. It is especially noteworthy the continuous contact between the two RTDs in order to follow similar research lines.

Most of these meetings were held by the coordinator and the conclusions reported to the rest of the partners.

Distribution of the Community's contribution.

Following the instructions from the Consortium Agreement, the distribution of the funds provided by the European commission was carried out. This distribution suffered several delays caused mainly by the evolution of the Consortium.

Evolution of the Consortium.

The Consortium has suffered several changes. The first took place even before the starting date of the project. The coordinator at that moment, a Spanish SME called Crop Ibérica, had to withdraw from the project. This withdrawal, caused by the change of the strategic goals of the company, made that one of the RTD performers identified in the proposal, CARTIF, had to assume the coordination. These changes were adopted by the commission in the Amendment to the contract Nº 1.

Once the project had already started and all the compromises signed two of the SME´s, Acomsa and Aromatic, showed their will to leave the consortium. Although big efforts from the partners were done to maintain them in the project, they took a firm decision in August 2005.

In order to solve this new challenge, the consortium started the search for new members that would not reduce and even increase its capacity to develop the project. In one case it was easy to find a company with an adequate profile to substitute Acomsa. Bodega Matarromera was first approached in September, and from the first moment they expressed their big interest in the project.

Aromatic was harder to be substituted. After searching possible members in the tools provided by the European Commission and approaching some candidates from associations and other meeting points, no feasible new partner was found. The solution came from within the consortium. Two of the SME´s already involved in the project, Aldivia and Gradiens, that played a similar role as Aromatic, agreed to assume the tasks firstly assigned to Aromatic.

These changes, withdrawal of ACOMSA and AROMATIC and the entry of MATARROMERA, were firstly agreed with the project officer and then reflexed on the second amendment to the contract submitted in December 6th 2005. In this amendment it was also communicated the change of legal status of the coordinator given that CARTIF became a foundation in September 2005

With this approval, the final funds were distributed and the costs justification for this first period was done.

In July 2006 the Mid term reports were submitted along with the first cost certification forms from the different partners. These reports were prepared by the co-ordinator but with the logical

collaboration from the rest of the partners.

In August 2006, the Review Report from the Administrative Officer of the European Commission was received. This report registers the good development of the project until that moment, but points out some concern about the technical delays reported.

It is noteworthy, anyway, that the report review received talks several times about the high quality reporting. This aspect was received from the whole consortium as recognition to their work.

The money transfer corresponding to the 2nd payment was received in September 2006 and immediately distributed among the partners.

After the first year reports were submitted to the European Commission, one of the partner, Istituto Bichimico Pavese, expressed its intention to withdraw from the project. Few months ago, IBP changed its managing structure, and now it is part of a bigger group. The new management team has decided that Extranat and in general any investment in R&D is out of their interest fields. Therefore they expressed their intention to leave the consortium.

Since a Cost certification had already been submitted, claiming the costs of the first year, an audit certificate was pointed as mandatory by the EC.

As soon as this withdrawal request was received, a search for the new member started. An Spanish SME was found with a similar profile as IBP. Its name is EXXENTIA, a company with expertise in obtaining vegetal extracts with applications in the food and pharmacological markets. The entering SME, EXXENTIA, does not differ from Pavese very much. Both are SMEs. The latter is a pharmaceutical company for the production of medicinal and also for the pharmaceutical containers. On the other hand, EXXENTIA is a manufacturer of dry extracts from selected medicinal plants, fulfilling the needs of costumers in the dietetic pharmaceutical, nutritional and cosmetic markets. Therefore, the leaving of Pavese did not seriously affect the availability of pharmaceutical know-how, since EXXENTIA provided its expertise in this field. This third amendment took place in March 2007.

Performance of the Consortium

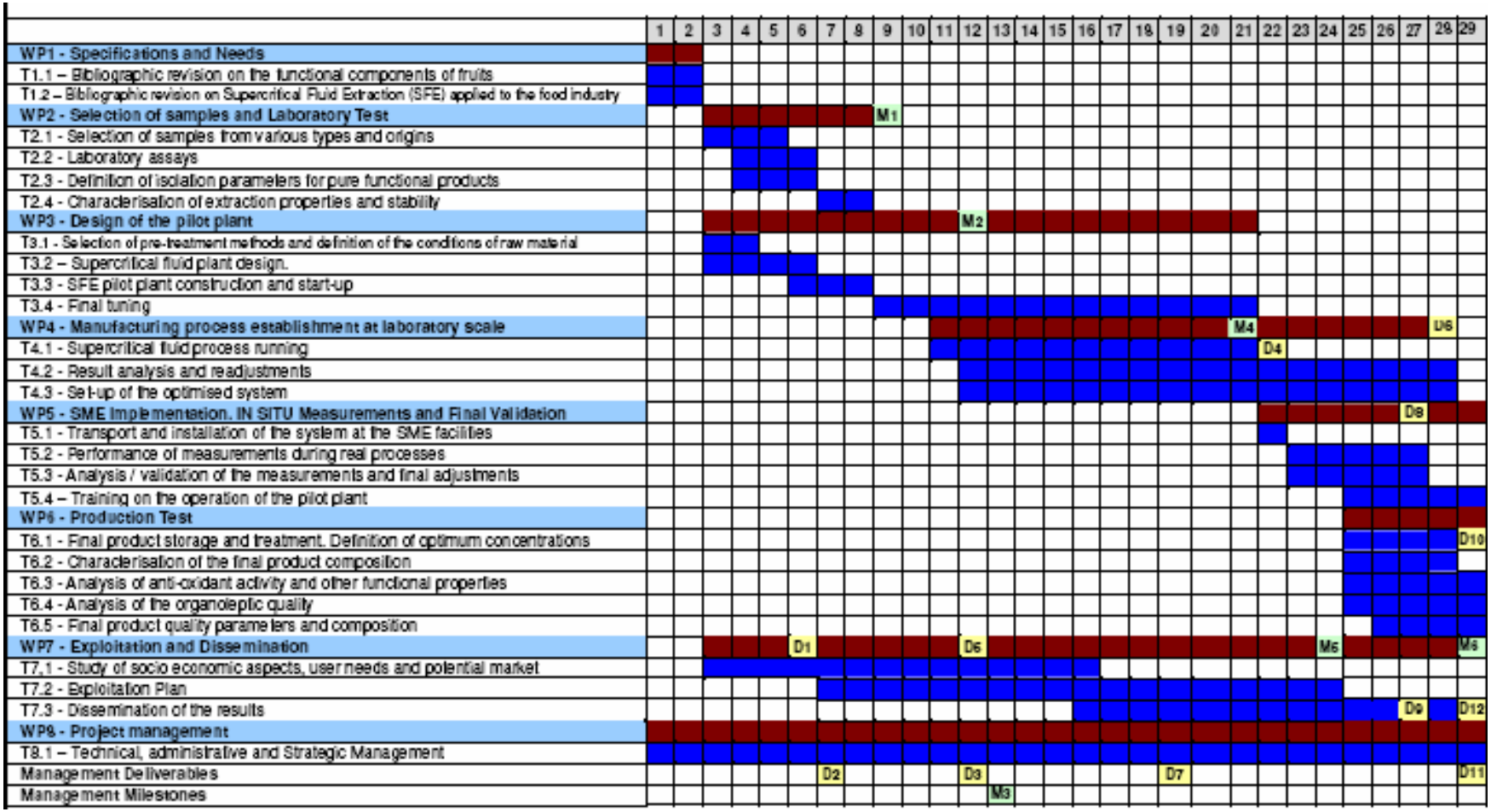
The consortium has shown a clear motivation to develop the project. A marked collaborative framework has been established, with an active participation of all partners in which individual tasks are performed in accordance with the common goal. Open and frequent discussions have been held to adopt common focus and methodologies, and to analyse the results.

In particular, it is remarkable the effort of all partners to compensate the negative effects of the withdrawal of the three members, searching for alternative solutions. In several cases increasing the working rate to attempt to counterbalance the delays has been done.

Due to some technical problems regarding the building of the pilot plant, the last phases of the project had to be carried out two months after the foreseen period. For this reason the Technical Annex had to be adapted to the new situation. An extension of duration was also modified to 29 months from the start date in June 2007.

The final workprogramme is like follows:

Work Plan and Timetable:



List of Deliverables and Milestones

Deliverable no.	Deliverable Name	WP no.	Date due	Actual delivery date	Lead contractor
2	Six Month Progress Report	8	September 2005	November 2005	CARTIF
3	Midterm Report	8	April 2006	June 2006	CARTIF
7	18TH Month Progress Report	8	Month 19 (October 06)	Month 19 (October 06)	CARTIF
11	Final Report	8	Month 29 (September 07)	November 07	CARTIF

Milestone no.	Milestone Name	WP no.	Date due	Actual delivery date	Lead contractor
3	Mid-Term Report + Draft of the "Plan for the use and Dissemination of Knowledge"	8	September 2005	November 2005	CARTIF

Section 4.- Other Issues

Ethical Issues

In the Extranat project there are no sensitive issues associated to ethical aspects that could be prohibited or specially ruled.

SME Contributions and relations with the RTD

The achievements arisen from the Extranat Project will mean potential benefits to the different SMEs involved. Within the consortium are represented the different market areas that could be interested in the developments carried out in Extranat. There are two vegetable producers that would benefit of the great revalorization of their wastes, COPAISAN and MATARROMERA. There is a company with importance in the building of production plants that may include supercritical Fluid Extraction, ENVIPLAN. ALDIVIA has expertise in the use of antioxidant compounds in the cosmetic industry. The compounds yielded from the Extranat Process will have some applications in the food additive industry and in this market sector GRADIENS will apply their expertise. The pharmacological applications for the antioxidant compounds were studied by EXXENTIA.

The other industry participating in Extranat is HELIOS. This big company has joined with a two fold interest. On one hand as a canned vegetable producer it will also see how some of its wastes develop a high revalorisation, and in the other if a food additive application is found, Helios may commercially exploit that result.

During the whole project the main efforts have been technical and therefore the biggest work charges were appointed to the RTD performer. Anyhow, these technical developments were in every moment guided from the group of SME towards exploitable results that may turn in benefits for the enterprises.

During the first workpackage, the SMEs decided which would be the vegetable wastes targeted for the project taking into account those that may be more interesting for them. During the bibliographic study in the SFE applications, those SME with expertise in this technique also added their opinions and resources to CARTIF. This RTD was the one in charge of making the bibliographic reports.

In the second workpackage only those SMEs that may produce the wastes for the process were involved, COPAISAN, MATARROMERA and HELIOS. The decision to choose the raw materials to be used was taken by these enterprises along with the two RTDs.

During the development of the third workpackage, the most involved enterprises were those with expertise on the Supercritical fluid extraction technique, Enviplan and Gradiens. In these tasks only CARTIF was involved from the RTDs. The design of the pilot plant, and the different parts comprised, was done by CARTIF and overseen by these two SMEs.

In workpackage 4 Cartif was the Lead Contractor. Enviplan and Gradiens also collaborated in the Manufacturing process establishment at laboratory scale.

Cartif lead workpackage 5, regarding the SME Implementation – In situ measurements and final validation. Enviplan, Gradiens and Matarromera participated in this tasks.

The Production test, workpackage 6, was lead by UNIPV who coordinate the SMEs' work testing the final products.

The activities that form part of the workpackage 7 are mainly Innovation activities. These activities are closer to the market, and commercial applications and relationships with the outside of the consortium, and have been leaded and done in its majority by the industrial partners.

The Management of the consortium has seen a great collaborative ambient between SMEs and RTDs. The important decisions that had to be taken, both in technical and administrative aspects, were taken by consensus. The technical developments lead to some choices presented by the RTDs in different meetings and the final choices were made by the SMEs. The coordinator also took into account the opinions of the rest of the partners before taking any action.

ANNEX

DELIVERABLE 5

"PLAN FOR THE USE AND DISSEMINATION OF KNOWLEDGE"

Introduction.-

The EXTRANAT project has as main objective: "to implement a well-known extraction technique to fruit and natural extract wastes in order to achieve the following strategic objectives: Extraction of high added value functional compounds from fruit waste Reduction of fruit elimination costs.Recycling of solvent-free fruit waste to protect the environment" According to the rules for CRAFT Programme, "Small and Medium Enterprises (SME)" contractors (COPAISAN, MATARROMERA, ENVIPLAN, GRADIENS, ALDIVIA, PAVESE), shall gain the joint property of the possible patents and any other intellectual property right arising from work carried out under the project. The owners of knowledge that are capable of industrial and commercial application are obliged to assure an adequate protection within the applicable law, the contract and the consortium agreement, as well as with consideration of the legitimate interests of the concerned partners. The "other enterprises" participants (HELIOS) will have access to the results at the level of their exploitation. Within the frame of the project, it is intended to protect some of the following results by the SMEs participants:

-Protocol to extract antioxidant compounds from carrot and grapes

-Design of the pilot plant

-Evaluation of anti-oxidant capacity

Each SME participant will guarantee that other participants within EXTRANAT are granted mutual access to "pre-existing know-how" and "knowledge" resulting from research activities, as necessary for carrying out the said activities of the project and/or for the use of the resulting knowledge ("use" is the direct or indirect use of knowledge within either research activities or for the development, creation and marketing of a product or process or for creation or supply of a service). This will be done in the form of licences or use agreements to which a given national law will apply. Access rights in order to carry out the project shall be granted royalty-free. Access rights in order to use knowledge shall be granted under equitable and non-discriminatory conditions. Any participant with limitations or restrictions regarding this issue should be informed its partners before the sign of the Consortium Agreement. RTD performers (CARTIF, UNIPV) will only have access to pre-existing know-how of the project partners in order to carry out their own work. They have no knowledge of their own which they can use, and therefore no access rights to pre-existing know-how for use purposes. The Exploitation Committee (EXC) will study, and if adequate will allow RTD performers to publish the knowledge they will generate within EXTRANAT. However, they have to assure the

protection of the knowledge beforehand. The Commission has to be informed about the publication in written form and in advance. It has the right to object. Access rights will be granted to a third party, if EXC consider it as adequate. In this case, the licence agreement will define the purpose of such third party access rights and guarantee that they will use it in accordance with the aim provided for (the agreement will be rescinded if the access right is used for another purpose). In any case, this will be possible provided it does not restrict the obligatory access rights which may be requested by partners.

The Consortium Agreement establishes the relationship among SMEs relating to the intellectual property rights, exploitation and dissemination of the EXTRANAT results, taking into account the possible patents and marketing products. In this agreement, the following basic lines are considered:

- Scope: exploitable results. The agreement will be applied to any exploitable outcome at the end of each task, work-package or project.

- Property of the results: this will be shared by all the SME contractors. The percentage of property of each SME will be in relation to its contribution to the whole project (from the final costs statements approved by the EC) and work load. Costs from protection of IPR will be shared by SMEs in this same proportion. If any of the partners demands exclusivity, conditions related to IPR must be discussed. Moreover, the better conditions of a partner in order to exploit a particular result must be considered as a key factor as well –in order to assure the appropriate exploitation and dissemination. In this case, the agreement must explicitly determine the compensation from that partner to the rest of the consortium.

- Deadline for confirmation: Each SME partner should confirm its interest in the exploitation of the results within three months after the end of the project. If no SME claim for IPR, it will have to inform the EC and the other partners (RTD performers). In such a case, SME partners will offer, free of charge, the possibility of patent to the rest of the parts.

- Rights cession to third parties: a formal proposal and a letter of commitment should be presented by the third part in order to be approved by the consortium.

- Conditions for the dissemination: In this case, both a formal proposal and letter of compromiso from the third party must be presented to the Consortium. Any kind of information, data, documentation, report, deliverable, etc resulting from the scientific-technical work performed for the project is, in all circumstances, under protection within the Consortium. Therefore, dissemination by any of the RTD performers (or any other partner) will not be permitted without the positive decision of the Exploitation Committee (EXC). Dissemination strategy will be responsibility of the Exploitation Committee (EXC). Although deliverables have been already classified as confidential, public or restricted, this classification will depend on its final decision as a last resort.

The particular interest of each SME partner in the dissemination and exploitation of project' results are stated below:

COPAISAN: Its intention is the exploitation of the results mainly as an end-user, to include the procedure in its production process, so its wastes are of higher value.

MATARROMERA: This winery has a big interest to extract high added value products from a waste that is already being produce.

ENVIPLAN: The main interest of this company in the Project relies on the increment of knowledge about supercritical fluid extraction applications and on the design of the plant.

GRADIENS: It is interested in the application of the results in its business, developing a new marketable natural product to its list of products.

ALDIVIA: The product yielded by the process developed in the Extranat Project could be marketed directly by Aldivia to cosmetic producers.

PAVESE: The products extracted from the vegetable wastes may be applied as certain active principles in the pharmacology industry, sector in which this SME is very active.

Section 1.- Exploitable knowledge and its use Exploitable

Exploitable Knowledge (description)	**Exploitable product (s) or measure (s)**	**Sector (s) of application**	Timetable for commercial use	Patents or other IPR protection (potential patents)	**Owner** & Other Partner (s) involved (potential Owner)
1. Procedure to extract an interest compound from grape wastes.	Protocol	Cosmetic and food Sector.	2009 2008	Potential patent.	SMEs
2. Procedure to extract an interest compound from carrot wastes.	Protocol	Cosmetic and food Sector	2009 2008	Potential patent.	SMEs
3. Knowledge of the Supercritical fluid extraction applied to vegetables.		Food	2009 2008	Know-How	RTDs
4. Design of the pilot plant	SFE Pilot plant	Food	2009 2008	Potential Patent	SMEs
5. Knowledge of the evaluation of anti-oxidant capacity		Cosmetic and Food	2009 2008	Know-How	RTDs

Brief Description of each exploitable knowledge

1 & 2.- Procedure to extract an interest compound from grape or carrot

wastes. The main yield of the project will be a procedure on how to obtain a product suitable to be used as a component in cosmetics or food additives from different wastes. These procedures will incluye every necessary data required to obtain the product, from the preparation of the samples, to the extraction parameters.

3. Knowledge of the Supercritical fluid extraction applied to vegetables

This result will only consist on a deeper knowledge on how Supercritical Fluid Extraction can be applied to the vegetable sector. It will probably allow the RTD performers to apply this knowledge in new processes and procedures.

4. Design of the pilot plant

The design of the pilot plant for the new application of SFE sought in the project may incluye some innovations that can result in the patent of the pilot plant. This pilot plant will be the one of the main deliverables of Extranat, and the intellectual property of its design will be owned by the SMEs, Further their interest on the exploitation of this result will be studied and the necessaryagreements will be taken following the EC IPR rules, and the previous agreements gathered on the Contract and on the Consortium Agreement.

5. Knowledge of the evaluation of anti-oxidant capacity

In order to evaluate the efficiency of the extraction and the interest of the extract, a method to evaluate the anti-oxidant capacity will be developed and validated between the RTD performers of the project, always taking into account commercial indications from the rest of the participants. This method will use several already known methods and therefore the result will not be patentable, it will only mean a deeper knowledge of this kind of methods.

Sector 2.-Dissemination of Knowledge

Planned/actual Dates	Type	Type of audience	Countries addressed	Size of audience	Partner responsible/involved
Since September 2005	Extranat Website	General Public / Partners	Global	1000's	All the partners.
Since September 2005	Publication of summaries of Extranat Project in several publications	Food Professionals	Global	1000's	All Partners
Extranat Logo	Specific Logotype designed for the Extranat Project	General public / Contractors	Global	1000's	All the partners
Month Six	Project Presentation (Deliverable 1)	Professional of the food sector	Global	1000's	CARTIF
Along the project	Conferences, Congress and presentations	Professional of the food sector	Europe	500-1000's	All SME´s
Month 20	Promotional CD-ROM.	General Public	Global	1000's	All the partners
Along the project	To include a link to Extranat website in the partners website.	Free	Global	11	All the partners
Along the project	The partners will publicize the project in local publications	Free	Countries of the partners	1000´s	All partners
Project Finished	Scientific Publications	Food Researchers	Global	1000´s	RTD´s

Brief Description of each Dissemination Activity

1.- Extranat Website

The development of the website of the project was done at the first stages of the project. A space has been reserved in the web from the Co-ordinator assistant, Idetra. Its URL address is: http://www.idetra.com/extranat/. In this website several areas can be found, under two main zones, the public and the private. In the public zone, the main departments are:

- -Home: where the project Summary and the

Logo can be found.

- -Partners: A list of the partners with their contact addresses and their logo.

- -Documents: where public documents can be shared with the public.

- -Recommended links: Links to the partner's websites and other interesting webs related to the project.

In order to access the private side of the website, a user name and a password have to be provided. If those are introduced correctly, the permitted user will have access to:

- -The same departments of the public zones, but this time with the right to edit and change some of the data, for example, add new links, correct the address of the partners or add a new public document.

- -The Agenda of the project, where the meetings and other important events can be located.

- - Private sharing of documents. It works the same way as the public part but it is restricted to signed users.

- In the main page of the website the logo of the European Commission and theText "Funded by the Commission of the European Commission" can be found,complying thus with the legal provisions for Publicity of the funded projects.

Links to this website have been also added in the websites of the partners. This task is taking some time, given that most of this websites are not managed by the partners but by some external agents.

2.- Publication of summaries of Extranat Project in several publications

The project Summary has been sent to Cordis Wire and immediately published. Cordis wire is an e-mail system alert from the EU about innovation and research news. It has also been sent to Cordis Express: A weekly briefing on what's new in European research and innovation. The service is designed to highlight the most significant recent developments, as reported in Cordis, the European Commission's Research and Development Information Service.

The Project Summary has been translated to the different languages of the partners in order to ease possible acceptance in publications that use different languages from English.

Several electronic publications have made reference to Extranat: Aquí Europa (http://www.aquieuropa.com/portada.asp?tipo=2&buscar=1&tema=&palabras=extranat&fechaFija=7&ultimas=5), Bulletins-electroniques (http://www.bulletinselectroniques.com/actualites/31620.htm), Evirocentre (http://www.envirocentre.ie/news.asp?id=31&cid=1940), Finanzamenti per l'innovazione , la ricerca e lo sviluppo tecnologico (http://first.aster.it/news/show_news.php?ID=12739), Info Veille Biotech (http://www.info-veille-biotech.com/index.php?lettre=79), Innovatech

(http://www.innovatech.be/archives/details.php?type=actualites&actualite_id=833&cerdt_Session=064d4ca2764328d1e4a1e8fc58fe5f08), Castilla la Mancha Innovación (http://www.clminnovacion.com/actualidad/noticias/articulo.htm?REG=4442), Observatorio Tecnológico de la Industria Agroalimentaria de la Comunidad de Madrid (http://www.observatorioalimentario. org/proyectos/detalle.php?numero=146). It is foreseen to send the Executive Public Summary, attached to the Periodic Activity Report,

to these and other publications to continue with the public publicity of the project.

3.- Specific Logotype designed for the Extranat Project

The Project Logo was proposed during the six-month meeting and improved by the partners. It is available at the website and it is used in all the documents refered to the project. It represents a phase diagram showing the solid (grapes), liquid (tomatoes), gas (apricots) and supercritical (carrots) states and the triple and critical points.

4.- Project Presentation (Deliverable 1)

A public "Project Presentation", which corresponds to an important deliverable of the project, D1, enclosed in the Work-package 7, "Exploitation and Dissemination". This activity was programmed according the suggestion of the Commission, and it occurred in months 4-6 of the project. The publication of the report about the key issues, technical approach and expected achievements of EXTRANAT, in simple terms so that it was easy to be understood by any person, has been via World Wide Web. Two kinds of web sites will be useful in order to disseminate the report: the web site specifically designed for the project (EXTRANAT-Web Site, in its public section), and the particular web sites of the different partners. Nowadays, almost every corporation has its own web address, where the most relevant information about its activities is the public access. Thus, this is an outstanding way to promote the knowledge of the EXTRANAT activities. In addition a brief abstract about EXTRANAT was published in CORDIS. This is an information space devoted to European research and development (R&D) and innovation activities. The main aims of CORDIS are to facilitate participation in European research and innovation activities; to improve exploitation of research results with an emphasis on sectors crucial to Europe's competitiveness and to promote the diffusion of knowledge fostering the innovation performance of enterprises and the societal acceptance of new

technology. This project has been Publisher specifically in CORDIS EXPRESS and CORDIS WIRE in September 2006.

5.- Conferences, Congress and presentations

During the two years in which the project will be developed, the opportunities to present the project in congresses will be several. The participation will be agreed among the partners, and the material and information to be provided during this events will also be communicated to the rest of the consortium. It will be encourage to make reference to the project in those events usually attended by the partners.

6.- Promotional CD-ROM.

A promotional CD will be edited and produced when the results of the project and their applicability are defined. This CD will be used as a dissemination tool during the events described in the previous point. The possibility to distribute it by direct mailing will also be studied.

This CD will be part of the subcontracting envisaged for the dissemination tasks. As explained in other reports this subcontracting, firstly programmed for the first year, has suffered some delays and it will be executed during the first semester of the second year (April/September 2006)

7.- To include a link to Extranat website in the partners website.

During the whole duration of the project, a link to Extranat Website will be included, as far as possible, in each of the webpages of the partners.

8.- The partners will publicize the project in local publications

All partners will attempt to publicize the project in local newspapers, local journals, Chamber of Commerce news, etc. This action will allow people from the vicinity of the partners to know about the interest of its industrial societies in innovation investment, as well as in European participation.

From the point of view of citizens, in general, this usually has a very positive connotation, because it is a demonstration of confidence in the enterprise future and, hence, the interest in the consolidation of employment and richness for the area. This kind of actions carried out following the European Commission rules for Publicity and Dissemination will also have a positive impact on the knowledge that European citizens have on EU financing of innovative projects, and more widely, on how the European funds are invested, aiming to spread an optimistic idea of European collaboration.

9.- Scientific Publications

At the end, and maybe also during the project, it will be studied the possibility to publish some result generated within the Extranat in a Scientific-Technical Journal of the food and agricultural Sector. The Project Exploitation Committee (EXC), according to the rules established in the Annex I of the Contract (Technical annex) and the Consortium Agreement, will study, and if adequate Hill allow RTD performers to publish the knowledge they will generate within Extranat. However, it is necessary to assure the protection of the knowledge beforehand. The Commission must be informed about the publication in written form and in advance and it has the right to object.

DELIVERABLE 6

DEFINITION OF PROCEDURES AND WORKING METHODS

Working methods were defined as a part of the operation manual of the prototype. As far the new process could be divided in two stages (solid to liquid extraction with a GRAS solvent and liquid to liquid extraction with CO2) the procedures were fairly simplified. In this sense it can be found two procedures which are commented briefly to explain deliverable number 6.

Previous to the extraction it is required the determination of some basic parameters such as water content, fat content and initial antioxidant activity.

The first working method is devoted to the preparation of a liquid extract from the raw material, in this sense it is required to define the type of product to be obtained attending to the polarity. Polar compounds will be extracted with ethanol and/or hidroalcoholic solutions and non-polar compounds will be extracted with ethyl-acetate.

When the fat content

Defating

Three consecutive extractions with Hexane are been performed on sample X9020 in order to remove fat content. Initial mass sample: 150 g. Total hexane 300 ml. Temperature of extraction: 40º C.

The temperature used in this situation was set to avoid possible degradation of valuable compounds.

Rests of hexane in sample will be removed under vacuum conditions.

Extraction

Samples (defated X9020 and the rest) has been already extracted with Ethanol during 24 hours. Extraction were performed in a closed flask. The ratio sample solvent used was 83 g of material and 250 ml of ethanol.

Temperature was kept constant during extraction 40º C +/- 0.5. Direct contact with light was avoided during extraction covering the flask with a protective aluminium foil.

Finally, samples were filtered to remove solids using standard filtering paper (quality equivalent to whatman 1). Resulting liquid was stored in a closed bottle at –25º C until its extraction in the supercritical fluid plant

DELIVERABLE 8

SUPERCRITICAL FLUID

This report shows the final installation of SFE prototype at Matarromera's facilities. The prototype was transported from CARTIF facilities.

In order to adapt a suitable space in SME facilities it was studied different options. Safety considerations were observed in the selection of this space.

The required auxiliary services such as electricity and air were adapted to be supplied to the prototype. The information was collected to fullfill PED normative for R&D facilities.

The following images show the final disposition of the prototype ready to operate:

Figure 1: Image of prototype in its final disposition. The lay out was selected to make easier the operation.

Figure 2: Another perspective of the lay out where It can be observed the cooling unit disposition.

Figure 3: Vision of the prototype from the control point where the process is controlled.

DELIVERABLE 10

Final product quality and composition

According to the results of the WP6 it can be observed the following points:

- The use of Extranat process should consider the normative as a part of the definition of extraction conditions. This situation should be considered although the solvents intended to use would be considered GRAS. Some parameters, like the final content of solvent should be checked.

- The results show that the quantities used in the products are low. Then the main quality factors to be considered should be colour and antioxidant activity.

- The stabilisation of the product could affect to further uses and this should be considered.

- Finally quality parameters and composition details was obtained according to the requirements of enterprises and characteristics of obtained extracts.