TOWARDS THE SUBSTITUTION OF FOOD ADDITIVES THROUGH SUPERCRITICAL TECHNOLOGY. EXTRACTION AND CHEMICAL CHARACTERIZATION OF VEGETAL EXTRACTS

J.A. Mendiola[1], F. J. Señoráns[2], G. Reglero[2], A. Cifuentes[1], A. Capodicasa[3], F. Nazzaro[4], A. Sada[4], E. Ibáñez[1*]

[1]Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid
[2]Sección Departamental Ciencias de la Alimentación, Universidad Autónoma de Madrid, 29049 Madrid
[3] Bioma Agro Ecology CO, Switzerland
[4]CNR - Istituto di Scienze dell'Alimentazione (ISA), Avellino, Italy
[*]Correspondence to: elena@ifi.csic.es

1.- Abstract

Food additives play an important role in today's complex food supply, nevertheless, they represent one of the most misunderstood topic in food safety that raises consumer's concern. Food additives are intentionally added to food in order to produce a desired positive effect, although their level has to be maintained within regulated limits. At present, there is an enormous interest in the development of novel class of food-additives based on substances extracted from natural sources by using environmentally friendly extraction processes, such as supercritical fluid extraction (SFE).

In the present work, several supercritical conditions have been tested to obtain natural food ingredients from natural sources such as plants, fruits, etc, with the scope of substituting some currently used chemicals, which are subject to regulation restriction and might be harmful for human health. The extracts have been fully characterized from the chemical and biochemical point of view, and attempts have been made to understand their mechanism of action. Therefore, several chromatographic methods have been developed based on gas chromatography coupled to mass spectrometry (GC-MS) and high performance liquid chromatography coupled with diode-array detection (HPLC-DAD) to study the complex profile of the obtained extracts. New analytical methods for profiling the compounds responsible of the antioxidant activity have been developed allowing the simultaneous determination of water soluble vitamins (ascorbic acid, thiamine, folic, pyridoxine, nicotinamide, cobalamine), fat soluble vitamins (α-tocopherol, retinol acetate, cholecalciferol), phenolic compounds (phenolic acids, cinnamic acids, flavanones, isoflavones, anthocyanins), carotenoids (β-carotene and lutein) and chlorophylls in a single run.

2.- Introduction

Food additives play an important role in today's complex food supply. Food controls focuses mainly on chemical additives, which are very often present, even if only in minor, or trace amounts. They are intentionally added to food in order to produce a desired positive effect, although their level has to be maintained within regulated limits. The present work is focused in the development of novel class of food-additives based on substances extracted from natural sources by using environmentally friendly extraction processes, such as supercritical fluid extraction (SFE).

SFE is getting a renewed attention in the food industry as a clean technology for ingredient/additives manufacturing. SFE has been recognized as one of the new technologies able to produce higher quality natural ingredients, such as food aromas, colorants, antioxidants and even antimicrobial. SFE has shown some advantages compared to traditional extraction methods using organic solvents or steam distillation to extract different compounds from natural sources. For instance, the potential to process natural products at mild temperatures under chemically inert conditions, using CO_2 as an extraction

fluid also results in a low environmental impact. Also, due to these mild conditions, the functional, sensorial and nutritional properties of the products are kept unaltered. Using CO_2, SFE meets most of the requirements of the Montreal Protocol for solvents that do not contribute to ozone depletion. In addition, when using CO_2 as an extracting solvent the extraction selectivity can be varied through adjustment of the pressure and/or temperature.

An important challenge in the food industry when dealing with natural additives is to know their exact chemical composition and to be able to correlate it with their biological activity. Therefore, in the present work, a full characterization has been undertaken from the chemical and biochemical point of view to be able to understand their mechanism of action.

3.- Experimental

3.1.- Supercritical Fluid Extraction

In a first step, dried raw materials (*Spirulina pacifica*, *Citrus Compositum*, *Raphanus niger, Malpighia punicifolia, Rosmarinus officinalis, Propolis, Medicago composite, Carica papaya*) were extracted using near supercritical conditions with CO_2 .

Extraction conditions ranged between 68 and 75 atm and 37 and 45 ºC for each raw material. Ten conditions were evaluated as indicated in Fig. 1. The novelty of this approach is the extraction rationale which is the opposite of the traditional extraction processes.

Instead of heating the extraction system in order to shorten the extraction time, the process is carried out at room temperature using a rising pressure on the extraction liquid that interact with the solid matrix. Extraction at low temperatures is a relevant issue since it is possible to avoid a thermal stress on thermo-labile substances.

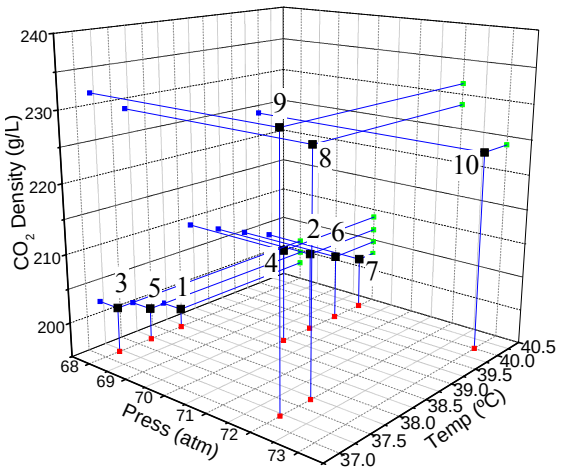

Fig 1.- Extraction conditions used.

3.2.- Chemical characterization

The extracts were analyzed using a previously developed method [1]. An Agilent-6890N GC system with a programmed split/splitless injector coupled to an Agilent-5973N quadrupole mass spectrometer (Agilent, Palo Alto, CA, USA) was employed. The system was controlled by means of an Agilent MSD Chemstation software. The column used in the GC was a 30 m x 0.25 mm i.d. fused silica capillary column coated with a 0.25 µm layer of SE-54 (HP-5MS, Agilent, Palo Alto, CA, USA). The injection was carried out at 250 ºC in split mode (ratio 1:20). The volume of sample injected was 1 µl.

Extracts were injected at a concentration 5 mg/ml. Helium was the carrier gas (7 psi). The oven temperature was programmed as follows: 40 ºC as initial temperature (maintained for 2 minutes) to 150 ºC in 24 minutes 5ºC/min, and from 150 ºC to a final temperature of 300 ºC at 15 ºC/min.
A solvent delay of 4 min was selected before analyzing the compounds reaching de MS. Compounds were tentatively identified by mass spectrometry in SCAN mode using a mass interval ranging from 35 to 450 m/z. Their spectra were compared with those in a mass spectrometry library (Wiley), with data found in the literature and with standards when available.
A new HPLC method was developed in order to quantify vitamins present in extracts [2]. By using this method, water soluble vitamins (ascorbic acid, thiamine, folic, pyridoxine, nicotinamide, cobalamine), fat soluble vitamins (α-tocopherol, retinol acetate, cholecalciferol), phenolic compounds (phenolic acids, cinnamic acids, flavanones, isoflavones, antocyanins), carotenoids (β-carotene and lutein) and chlorophylls were separated in a single run. Briefly, an ACE-100Å C_{18} (150 mm × 4.6 mm, 3µm particle size) was used combining isocratic and linear gradient elution with a mobile phase consisting of 0.010% trifluoroacetic acid (solvent A) and methanol (solvent B) at the flow rate 0.7 ml/min. The gradient profile (A:B) started at 95:5 and was constant in the first 4 min, then linearly changed up to 2:98 during the next 6 min, then it was constant in the next 7 min, increased up to 0:100 in 2 min, then constant until a total analysis time of 40 min and finally linearly increased up to 95:5 to reach initial conditions. The most suitable detection wavelength for simultaneous vitamin-polyphenol determination was 280 nm.

3.3.- Functional characterization

The antioxidant activity was evaluated using two methods: the DPPH radical scavenging test [3] and the total phenolics measurement using Folin reagent [4].
The total phenolics method is based on the original F-C method developed in 1927. The measurements were carried out in an absorbance plate-reader, using Elisa 96 well plates of 250 µl. The method consists of mixing 200 µl of Na_2CO_3 (2% w/v in water) with 10µl of extract; after 3 minutes of reaction, 5µl of Folin reagent was added. After 30 min of incubation the absorbance was measured at 700 nm (the longest wavelength that could be used in our multiwell absorbance reader, Tecan Sunrise, Switzerland).
The antioxidant activity of the extracts was also measured by DPPH radical scavenging method. This method is based on the previously developed by Brand-Williams et al [3] adapted to use 96 microwell plates. The method consist on measuring the change on absorbance occurred at 517 nm by mixing 195 µl of DPPH• solution (23,5 mg/L in ethanol) with 5 µl of extract. Since reaction time depend on the extract, data was collected every 15 min for 5 hours until. The stabilization time occurred when a plateau was reached, i.e. the end of the reaction for each sample.

3.4.- Statistical Analysis

In order to find the main components of the extracts that contribute to its antioxidant activity, previous data related with antioxidant activity and vitamin-phenolic HPLC profiles, were submitted to statistical analysis using: PLS (partial least squares), MLR (Forward Stepwise Multiple Linear Regression) and Neuronal Networks models. All the statistical analysis was done using Statistica Software (Statsoft, USA)

4.- Results

In terms of yield, the best results were obtained in the extraction of acerola (*Malpighia punicifolia*), specially using 39.2 ºC and 70 atm; on the other hand the material that gave the lowest yields was *Spirulina*.

In the chemical analysis of extracts several compounds with antioxidant and antimicrobial activity were found by using GC-MS namely: free fatty acids (hexadecanoic, linoleic…) in most of the samples, totarolone and isothiocyanates in *Raphanus* (radish), furane derivatives in propolis, neophytadiene in *Medicago* (alfalfa) and Spirulina, 1-H-indolic derivatives in *Spirulina* and oxygenated terpenes and ferruginol in rosemary.

When the antioxidant activity of vegetable extracts was measured, three levels of activity were found in the DPPH radical scavenging test: low (citrus, propolis and radish), mean (*Spirulina*, alfalfa and papaya) and highly antioxidant (rosemary). Otherwise the activity of the extracts shows the same trend when plotting antioxidant activity (EC_{50}) vs total polyphenolic content, as can be seen in Fig 2.

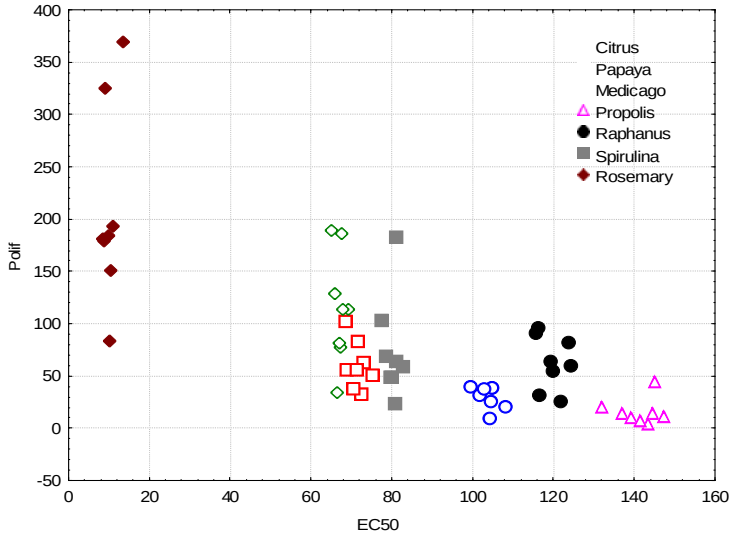

Fig. 2.- Radical scavenging activity (EC50 mg/g) vs total polyphenolic content (mg gallic acid equivalents/g extract)

As for the chemical characterization by using the new method of RP-HPLC-DAD [2], in general terms, three zones were easily differentiated in the chromatograms: water soluble vitamins (2-10 min), phenolic compounds (10-20 min) and, finally, fat-soluble vitamins (20-40 min).

In the vitamins analysis performed, the main remark was the absence of fat-soluble vitamins, only a small amount of α-tocopherol could be detected in some extracts of Rosemary. The main vitamin found in the extracts was thiamine, except for malpighia extract, where ascorbic was the most important compound.

The HPLC analysis of extracts revealed the presence of carnosic acid and its derivatives in rosemary extract. The medium activity of *Spirulina*, alfalfa and papaya can be attributed to the presence of carotenoids and phenolic compounds and their synergistic effects. The DPPH radical scavenging reaction media was not compatible with acerola extracts, but the different statistical analysis performed (PLS, MLR and Neuronal) predicted an antioxidant activity similar (R^2 among 0.86 and 0.98) to Spirulina mainly by the presence of ascorbic acid and polyphenols in acerola extracts.

Also, many flavonoids were detected in extracts from propolis, medicago, rosemary and fruits and most of them have been related to the antioxidant and antimicrobial activity of the alcoholic and supercritical extracts [5, 6-8, 9-12], even against *Clostridium botulinum* growth [13-16]. Specifically, antimicrobial activity in propolis has been associated to the presence of cinnamic acids such as caffeic, ferulic and cumaric acids [17] while a flavonoid, medicarpin, has been described as the responsible of the antimicrobial activity of *Medicago* [12]. Some flavonoids have shown even antiviral activity, i.e. quercetin, a flavonol aglycone, found in a wide number of fruits, has shown antiviral activities against *Herpes simplex* virus type 1, para-influenza virus type 3, and polio virus type 1 both in the *in vivo* and *in vitro* studies [16, 18, 19].

5.- Conclusions

The results presented in this work showed the possibility of using vegetal extracts obtained using near-critical fluids as food preservative agents for the food industry as, for example, an alternative to nitrites and nitrates due to their antioxidant and antimicrobial activities.

Acknowledgements:

This work has been financed by the European Union (STREP Project Food-CT-2006-023060, NoChemFood).

REFERENCES

1. Herrero, M., et al., Dunaliella salina microalga pressurized liquid extracts as potential antimicrobials. Journal of Food Protection, 2006. 69(10): p. 2471-2477.
2. Mendiola, J.A., et al., Profiling of different bioactive compounds in functional drinks by HPLC. Journal of Chromatography A, 2008. (In press).
3. Brand-Williams, W., M.E. Cuvelier, and C. Berset, Use of a free radical method to evaluate antioxidant activity. Lebensmittel-Wissenschaft und-Technologie, 1995. 28: p. 25-30.
4. Cuvelier, M.E., H. Richard, and C. Berset, Antioxidative activity and phenolic composition of pilot-plant and commercial extracts of sage and rosemary. Journal of the American Oil Chemists Society, 1996. 73(5): p. 645-652.
5. Scazzocchio, F., et al., Multifactorial aspects of antimicrobial activity of propolis. Microbiological Research, 2006. 161(4): p. 327-333.
6. Tripoli, E., et al., Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review. Food Chemistry, 2007. 104(2): p. 466-479.
7. Nowakowska, Z., A review of anti-infective and anti-inflammatory chalcones. European Journal of Medicinal Chemistry, 2007. 42(2): p. 125-137.
8. Friedman, M., Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas. Molecular Nutrition and Food Research, 2007. 51(1): p. 116-134.
9. Mokbel, M.S. and T. Suganuma, Antioxidant and antimicrobial activities of the methanol extracts from pummelo (Citrus grandis Osbeck) fruit albedo tissues. European Food Research and Technology, 2006. 224(1): p. 39-47.
10. Moreno, S., et al., Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. Free Radical Research, 2006. 40(2): p. 223-231.
11. Lule, S.U. and W. Xia, Food phenolics, pros and cons: A review. Food Reviews International, 2005. 21(4): p. 367-388.
12. Guo, L., R.A. Dixon, and N.L. Paiva, Conversion of vestitone to medicarpin in alfalfa (Medicago sativa L.) is catalyzed by two independent enzymes. Identification, purification, and characterization of vestitone reductase and 7,2'-dihydroxy-4'- methoxyisoflavanol dehydratase. Journal of Biological Chemistry, 1994. 269(35): p. 22372-22378.

13. Reddy, N.R., M.D. Pierson, and R.V. Lechowich, Inhibition of Clostridium botulinum by antioxidants, phenols, and related compounds. Applied and Environmental Microbiology, 1982. 43(4): p. 835-839.
14. Pierson, M.D. and N.R. Reddy, Inhibition of Clostridium botulinum by Antioxidants and Related Phenolic Compounds in Comminuted Pork. Journal of Food Science, 1982. 47(6): p. 1926–1929.
15. Cushnie, T.P.T. and A.J. Lamb, Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents, 2005. 26(5): p. 343-356.
16. Formica, J.V. and W. Regelson, Review of the biology of quercetin and related bioflavonoids. Food and Chemical Toxicology, 1995. 33(12): p. 1061-1080.
17. da Silva, J.F.M., et al., Correlation analysis between phenolic levels of Brazilian propolis extracts and their antimicrobial and antioxidant activities. Food Chemistry, 2006. 99(3): p. 431-435.
18. Bae, E.A., et al., In vitro inhibitory effect of some flavonoids on rotavirus infectivity. Biological and Pharmaceutical Bulletin, 2000. 23(9): p. 1122-1124.
19. Kaul, T.N., E. Middleton Jr, and P.L. Ogra, Antiviral effect of flavonoids on human viruses. Journal of Medical Virology, 1985. 15(1): p. 71-79.