The Annals of the University Dunarea de Jos of Galati
Fascicle VI – Food Technology 36(2) 81-91

ORIGINAL RESEARCH PAPER

ENRICHED ANTIOXIDANT ACTIVITY OF PEAR JUICE BY SUPPLEMENTATION WITH OREGANO AND WILD THYME EXTRACTS

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Received on 21st November 2012
Revised on 20th December 2012

In the last years, it has been noticed an increased interest in natural antioxidants and the use of these compounds in the field of new polyphenol-rich drinks. Antioxidants are compounds responsible for free radical scavenging in the body. They protect the organism from oxidative modification of cells and tissues. The aim of this study was to improve the antioxidant capacity of a pear juice, as well as its compounds stability during 12 days of storage. The stabilization of phenols and their delivery in pear juice was possible thanks to microemulsions obtained by mixing different ratios of oil, surfactant and cosurfactant. The characterization of microemulsions was performed using pseudoternary phase diagrams and which have highlighted reports of mixing A / W / S / CoS corresponding states of microemulsion. The obtained microemulsions have stability and provides good stabilization of linoleic acid and walnuit oil, up to 70% w/w water. Through conductometric analysis we have highlighted the transition states of micro W/O, O/W bicontinuous structures. The pear juice with plant extracts had higher antioxidant values than the pear juice with synthetic antioxidants. Thus the juice with oregano improved the antioxidant capacity approximately 1.45 times compared to the juice with BHA and approximately 1.67 times compared to the juice with BHT.
These results are promising in order to replace the synthetic antioxidants with natural antioxidants.

Keywords: pressurized liquid extraction, oregano, wild thyme, microemulsion, BHA, BHT, natural antioxidants, pear juice

Introduction

Nowadays, the concept of chemical preservetives in food is very controversial. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly used in the food industry due to their effectiveness and low price. Nevertheless, the toxicity of these compounds drew attention to the their side effects on the human body and possible carcinogenic

properties (Williams *et al.*, 1999). Modern consumer options have been redirected to natural alternatives, healthier and more diversified in terms of chemical composition and functional properties. Therefore, in the last years it has been observed an increasing interest in obtaining healthy food, functional food because it can provide additional physiological benefit, other than energy and nutrition (Goldberg, 1996). Based on this definition, currently the attribute "functional" means food that induces beneficial effects for one or more physiological functions, increases wellness and/or reduces the risk of certain diseases (Guarneri and Azpiroz, 2005). Frequently, functional foods are obtained from traditional foods enriched with an ingredient able to provide or promote a beneficial action for human health (Herrero *et. al*., 2006). These are the so-called functional ingredients. Among the functional food ingredients, phenolics and polyphenols are the most desirable food bioactives due to their antioxidant capacity (Higdon and Frei, 2003), antimicrobial activity (Daglia, 2012) and role in reducing certain types of cancer (Clere *et al*, 2011) and cardiovascular diseases (Visioli, 2011). Unfortunately, many of these compounds are poorly soluble in water, showing a low solubility in the gastrointestinal tract and very low bioavailability (McClements and Li, 2010).

Therefore, recent researches have focused particularly on developing new methods of solubilization of bioactive compounds and their intake in some systems to ensure a good delivery to all metabolic processes (McClements and Li, 2010).
Some of the most important systems for solubilization and delivery of bioactive compounds are microemulsions. Using the microemulsion vehicles, water insoluble and oil-soluble components from different plant extracts can be co-solubilized in order to attain synergistic effect for a specific therapeutic goal (Kogan and Garti, 2006). Microemulsions, on the other hand, are thermodynamically stable, transparent isotropic solutions with particle sizes ranging from 5 to 100 nm, and arise from the spontaneous self-assembly of the hydrophobic or hydrophilic parts of surfactant molecules (Flanagan and Singh, 2006). Microemulsion systems are recognized for their numerous advantages: thermordynamic stability, simple technology of preparation, low viscosity and considerable potential for solubilizing a variety of poorly soluble compounds (Spernath *et al*., 2002; Kriegel *et al*., 2009; Flanagan. *et al*., 2009).

The aim of this study was to investigate the potential of microemulsion systems for solubilizing and delivery of bioactive compounds from extracts of oregano (*Origanum vulgare* L.) and wild thyme (*Thymus serpyllum*) obtained by pressurized liquid extraction (PLE). The solubility of in several vegetable oils in order to identify which oil can be used for further formation of microemulsions. The most common oil phases used to prepare soft drinks are flavor oils, such as lemon and orange oils (Ziani *et al*., 2012). To prepare microemulsions there have been used several proportions of oil: surfactant, co-solvent. Enhanceing solubilization of bioactive compounds in O/W microemulsions is also believed to enhance their bioavailability and to maximize their absorption in human tissues. After obtaining microemulsions rich in bioactive compounds, they were used for the addition of natural pear juice to improve the antioxidant properties and the

results were compared to those obtained using two synthetic antioxidants: butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT).

Materials and methods

Two different plants, belonging to three botanical families which are commonly grown in Romania, were chosen for this study: oregano (*Origanum vulgare*) and wild thyme (*Thymus serpyllum*). The plant samples were obtained from a local herbalist's shop (Galati, Romania) and dried using a traditional method.

The reagents: 1,1 diphenyl 2-picrylhydrazyl (DPPH) (95 % purity), Folin-Ciocalteu and sodium carbonate (Na_2CO_3) and antioxidant standards, gallic acid and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (Steinheim, Germany). The surfactant Tween 40, Tween 80 were purchased from Sigma Aldrich, and SPAN 80 was purchased from Fluka (Switzerland). Linoleic acid was purchased from BDH Chemicals Ltd (Poole, England). Synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were supplied by KUK (Bucharest, Romania).

Dimethyl sulfoxide (DMSO), 99.9 % purity was purchased from Fluka (Switzerland).

Pressurized liquid extraction (PLE)

Extractions were performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller. Ultrapure water was used as solvent. At the beginning of the day, the solvent was sonicated for 10 min. Extractions were carried out at 100 °C whereas the static extraction time was 20 min. An extraction cell heat-up step was carried out for a given time prior to any extraction. An instrumentally-preset warming-up time of 5 min was also accomplished before the static extraction period. One gram of oregano or wild thyme material was packed into 11 mL stainless steel extraction cells after mixed with 2 g of sea sand. Extraction method was performed according to a procedure previously described (Herrero *et al.*, 2004).

Once extractions were finished, the solvent was removed. The water extracts were lyophilized using a freeze-dryer (Labconco Corporation, Missouri, USA).

The stability of phenolic compounds

The oregano and wild thyme extracts obtained by PLE extraction at 100 °C, were dissolved in DMSO solution 20% (v/v). The pH was adjusted to 5.0, 7.0 and 9.0 with HCl 1N and NaOH 1N. These samples were stored at 5 °C (dark) and at 20 °C (light and dark) during 12 days and the total phenolic content was measured every 3 days. The phenolic content of PLE extracts was estimated as gallic acid equivalents (GAE), expressed as mg gallic acid/g d.m. (dry matter) according to the Folin-Ciocalteau assay (Koşar *et al.*, 2005). The total volume of reaction mixture was miniaturized to 10 mL. Briefly, 0.1 ml sample (5 mg/ml) and 6 ml ultrapure water were mixed, to which 0.5 ml undiluted Folin-Ciocalteu reagent was subsequently added. After 1 min, 1.5 ml of 20% (w/v) Na_2CO_3 were added and the volume was made up to 10 ml with water. The samples were incubated for 2 h at 25 °C in the darkness. The absorbance was measured at 760 nm in a spectrophotometer Jenway 6300. A standard curve with serial gallic acid solutions

(0.031 – 2 mg/mL) was used for calibration. Data were presented as the average of duplicate analyses.

Construction of pseudo-ternary phase diagrams

The pseudo-ternary diagrams were constructed by mixing different ratio of components. Thus, the oily phase was obtained by mixing oil (walnut oil, linoleic) with ethanol in 2:1 mass ratio. The oregano and wild thyme extracts were dispersed in oil. Oily phase was mixed with surfactant Span 80 (HLB=4.3), in different mass ratio: 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1. These mixtures were titrated with aqueous phase containing water and glycerol in the mass ratio 4:1. Samples were gently shaken and classified by visual and microscopic analysis to observe the samples which remained transparent to be considered microemulsions.

Preparation of water-in-oil microemulsions (W/O) containing plant extracts

Depending on the diagram layout phase were prepared and studied two groups of microemulsions, one of them has used walnut oil and other has used linoleic acid. The content of plant extracts in microemulsions was different: 0.08% (w/w), 0.12% (w/w), 0.24% (w/w). The surfactant/cosurfactant mass ratio was 3:1 (w/w) for each sample. To prepare microemulsions with walnut oil, the percentage of surfactant remained constant at 30% and the content of aqueous phase varied from 0% to 50% every 5% (w/w). The linoleic acid microemulsions were prepared maintaining constant the surfactant at 40% (w/w) and the content of aqueous phase varied from 0% to 20% every 5% (w/w). Mixtures were prepared by sonication for 3 minutes at an amplitude of 30% and a frecuency of 20 kHz. All samples were stored at room temperature for 24 h to establish steady state before analysis.

Conductivity and pH measurement

Electrical conductivity and pH were measured at 25 °C with Multi-parameter analyzer C 868 (Consort), with conductivity electrode SK10B with a cell constant of 0.11 cm^{-1}, and a pH electrode SP10B.

Additives for pear juice to improve functionality

The pear juice was prepared using a Philips HR2744 juicer. In order to improve the antioxidant capacity were added 0.1 ml microemulsion with oregano and wild thyme extract to 5 ml of pear juice. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were added at a concentration of 100 ppm. These juices were stored at 5 °C and 20 °C for 12 days and the antioxidant capacity was measured by DPPH method, every 3 days.

Determination of antioxidant capacity

The antioxidant activity of all the obtained juices was estimated using the DPPH radical scavenging assay. Briefly, a solution was prepared dissolving 23.5 mg of DPPH in 100 mL of methanol. This stock solution was further diluted 1:10 with methanol. Both solutions were stored at 4 °C until use. Thus, 0.1 ml of juice were added to 3.9 ml of DPPH diluted solution to complete the final reaction medium (4 mL). After 1 h at room temperature the absorbance was measured at 516 nm in a spectrophotometer Jenway 6300. The results were expressed as µmol trolox equivalents (TE)/L juice. Measurements were done, at least, by triplicate.

Results and discussion

Stability analysis of wild thyme extracts under different storage conditions

Generally plant extracts are soluble in polar solvents and therefore methanol, ethanol, dimethyl sulfoxide (DMSO), propanol, acetone, ethyl acetate, are the most common solvents used for the solubilization of plant extracts. The drawbacks of using these solvents are representing their toxicity and therefore can not be added to foods. Thus, the use of non-toxic solvents and solvent mixtures such as vegetable oils or microemulsions could be beneficial to solubilize the plant extracts and also for adding to foods.

Another important issue is the stability of plant extracts when these extracts are solubilized in certain solvents or oils. Since de final objective of this study is the addition of plant extracts to food and to improve the antioxidant capacity, the stability of these extracts (dissolved in DMSO) at different temperatures and different pH values was studied.

As can be obverved in figures 1, 2 and 3, the polyphenols compounds are stable at pH 5 and lose effiency at pH 9. Thus for selection of a food product for the addition of the oregano and wild thyme extracts it is important to have considered these issues and it is ideal to choose a food product with an acid pH.

As it can be observed in Figures 1, 2 and 3, the content of phenols varied when the samples were kept at 20 °C. Moreover, the influence of light on the stability of phenols and antioxidant capacity was also reported by other authors (Cvetkovic and Markovic, 2008).

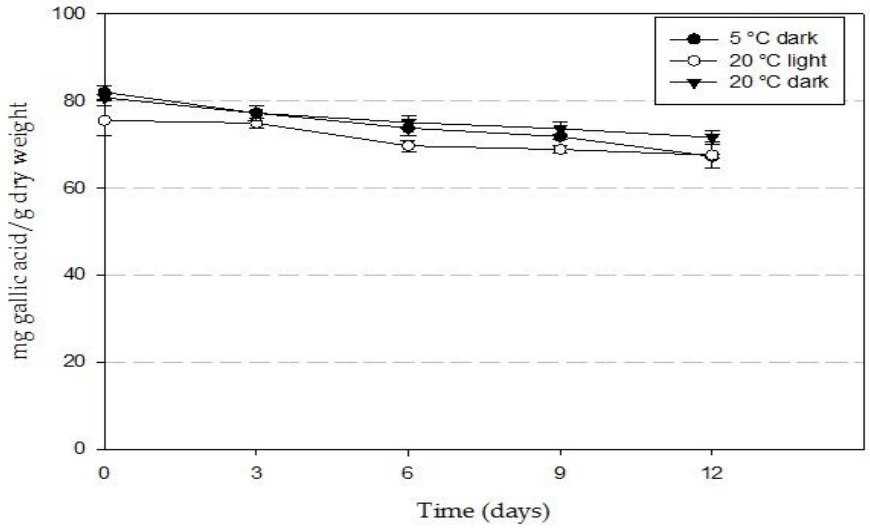

Figure 1. Stability of wild thyme extract at pH 5 correlated with temperature and the presence/absence of light radiation

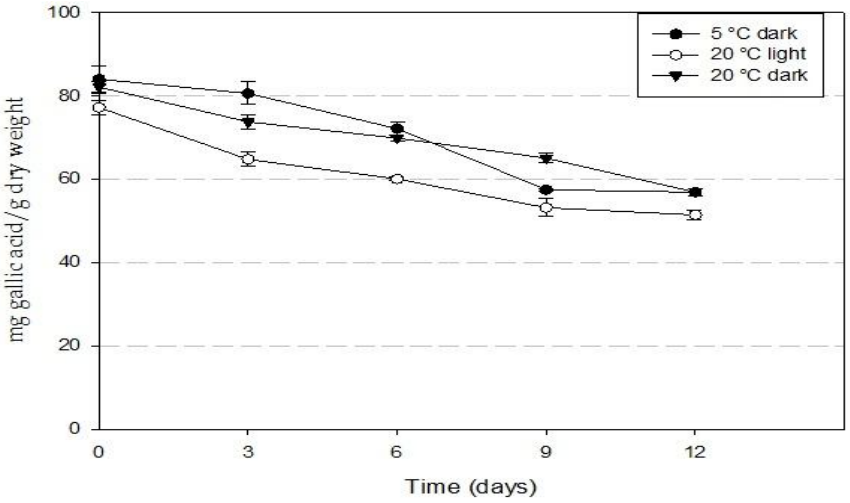

Figure 2. Stability of wild thyme extract at pH 7 correlated with temperature and the presence/absence of light radiation

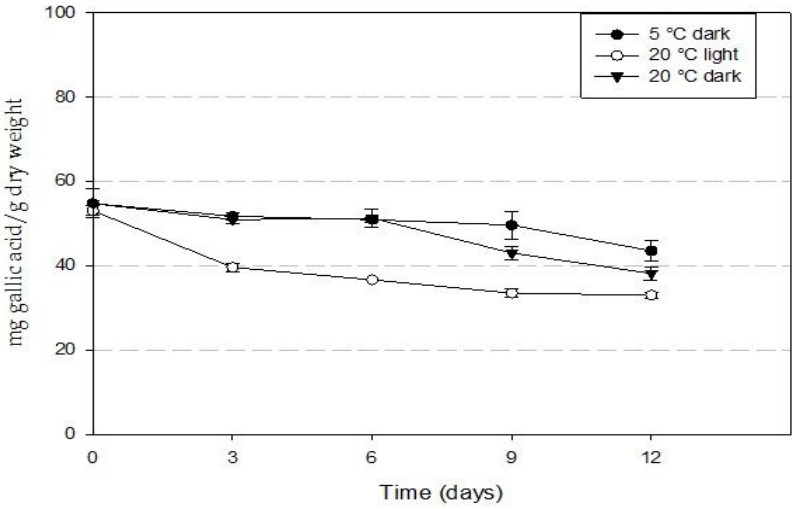

Figure 3. Stability of wild thyme extract at pH 9 correlated with temperature and the presence/absence of light radiation

The study of phase diagrams

The phase diagrams were used to determine the concentration of components and to establish the field of microemulsion. In microemulsion system, the cosurfactants are designed to reduce the interfacial tension in addition to surfactants. They are adsorbed at the O/W interface and modify the interfacial strength of the flexible membrane forming microemulsions. In our study, the linoleic acid is more efficient in forming microemulsions, as it can be observed in the phase diagrams, the field of microemulsion is higher when using linoleic acid than walnut oil. The

analysis of samples corresponding to the line AB (Figure 4) indicates that the first 10 samples, obtained by adding water every 5% (w/w) are homogeneous, transparent, colored yellow/orange.

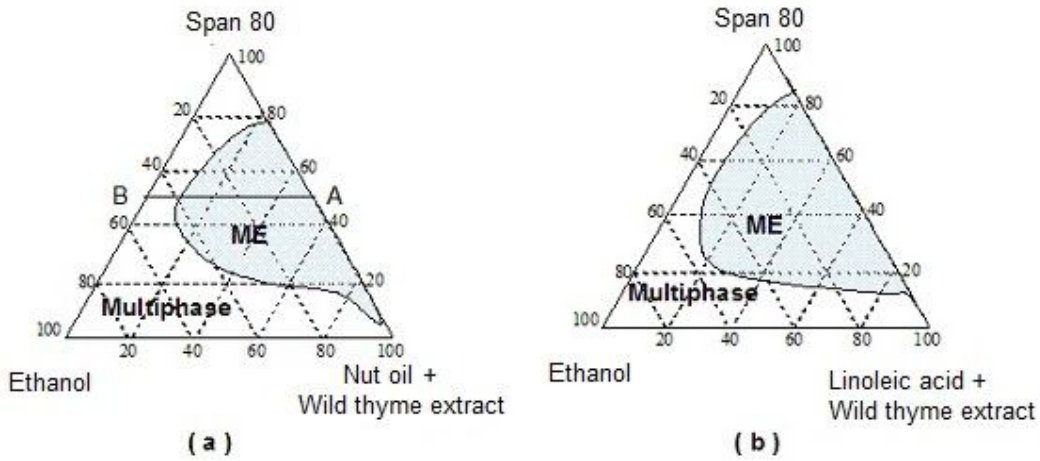

Figure 4 Pseudo-ternary diagrams of microemulsions systems: (a) ethanol/nut oil – wild thyme extract/Span 80, (b) ethanol/linoleic acid- wild thyme extract/Span 80
ME-microemulsion

Electric conductivity

Absence of ions in the samples analyzed has led to very low values of electrical conductivity. This research has however revealed variations of electrical conductivity by increased water content. At low water content less than 0-10% wt/wt the conductivity values are about 1.15 µS/cm, followed by a slight increase to 30% (w/w) water and a faster growth after this value (Figure 5).

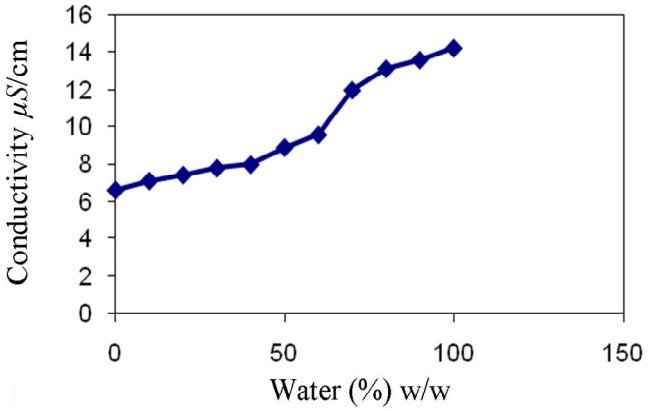

Figure 5. Variation of electrical conductivity with water content of microemulsion with plant extract and linoleic acid

These variations, which correspond to viscosity variations, can be due to water micro-emulsions transition O/W, with low values of conductivity in microemulsions O/W, which increase the conductivity of aqueous continuous phase.

The antioxidant capacity of pear juice with additives

The final objective of this study was to increase the antioxidant capacity of a pear juice supplemented with plant extracts. A microemulsion containing 26% linoleic acid: 20% Span 80: 14% ethanol and 40% water was used to dissolve the dried extrats. The content of oregano and wild thyme extracts in microemulsions was different: 0.24% (w/w). The analysis was performed bz comparing with a smaple of juice supplemented with synthetic antioxidants BHA and BHT (100 ppm). As blank, fresh pear juice obtained with a Philips HR2744 Juicer was used.

The antioxidant capacity of this juice was determined by DPPH method.

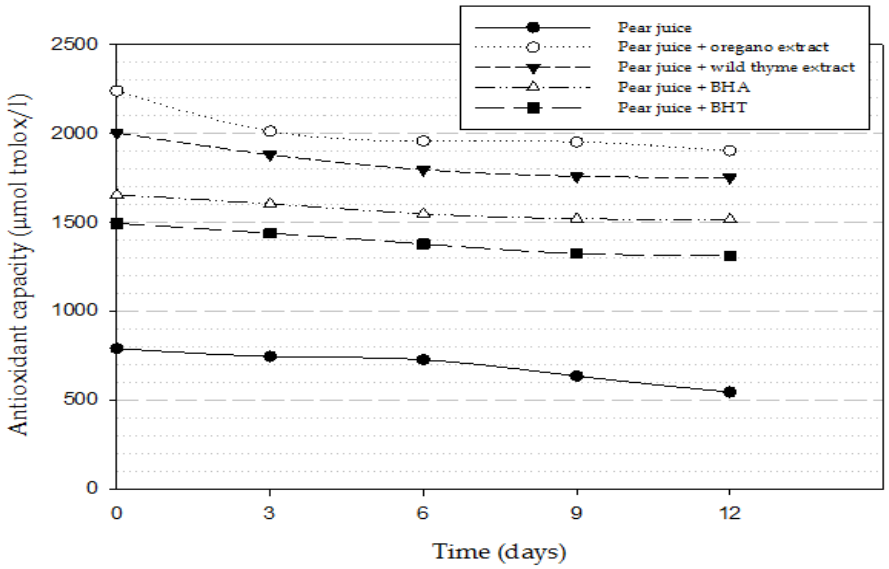

Figure 6. Antioxidant capacity of pear juice (control) and pear juices supplemented with antioxidants, at 5 °C for 12 days

As shown in Figure 6 after storage at 5 °C for 12 days, oregano and wild thyme extracts improved antioxidant capacity of pear juice approximately 3.42 times for oregano extract and approximately 3.15 times for wild thyme extract. An increased antioxidant content in juice enriched with dried extracts was also reported by several authors (Mastrodi Salgado *et. al*. 2012; Celiktas *et al.*, 2010).

Comparing pear juice supplemented with oregano and wild thyme extracts to pear juice supplemented with BHA and BHT, it is observed that oregano extract improved the antioxidant capacity of pear juice approximately 1.25 times higher than pear juice supplemented with BHA and approximately 1.44 times higher than pear juice supplemented with BHT.

The wild thyme extract improved antioxidant capacity of pear juice approximately 1.15 times compared to pear juice supplemented with BHA and approximatively 1.33 than pear juice supplemented with BHT.
Concerning the pear juice kept at 20°C, the same trend as for storage at 5°C was observed (Figure 7). Thus, the juice with oregano extract improved the antioxidant capacity of approximately 5.75 times, while the juice with wild thyme extract approximately of 4.62 times compared to pear juice (control sample).

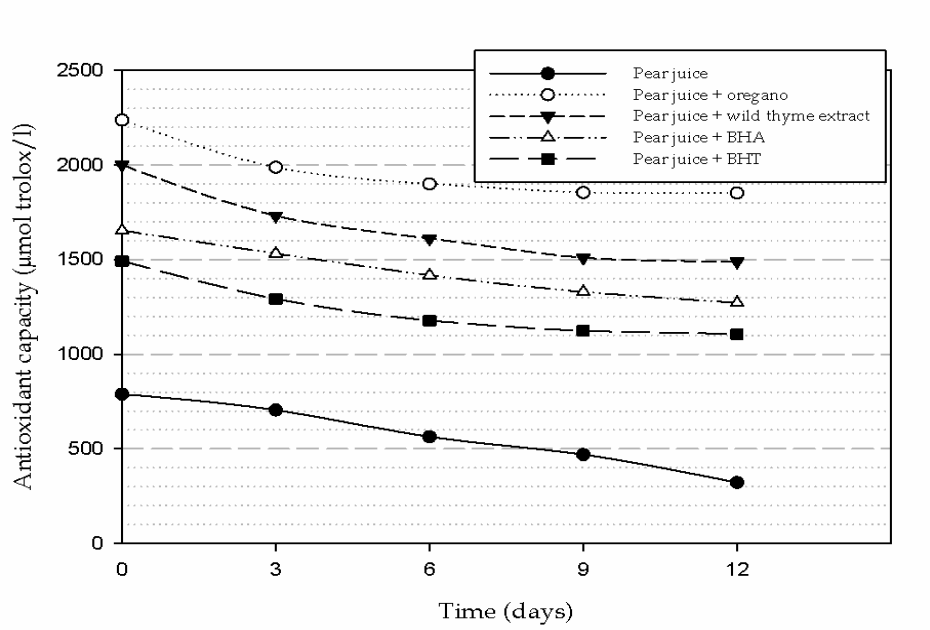

Figure 7. Antioxidant capacity of pear juice (control) and pear juices supplemented with antioxidants, at 20°C for 12 days

Comparing the antioxidant capacity of pear juice with oregano and wild thyme extracts with those with synthetic antioxidants (BHA and BHT) it can be observed that the plant extracts at low concentrations (1 mg/ml) can improve the functional quality of these juices. Thus the juice with oregano improved the antioxidant capacity of approximately 1.45 times compared to juice with BHA and of approximately 1.67 times compared to juice with BHT.
These results are in accordance with other studies which demonstrated that the natural antioxidants improved the antioxidant capacity at least at the same values as the values of synthetic antioxidants (Naveena *et al.*, 2008).

Conclusions

In this study was investigated the stability of phenols from oregano and wild thyme extracts, obtained by pressurized liquid extraction (PLE), under various conditions of pH values, temperature and presence or absence of light. All these factors

influence differently the antioxidant capacity of plant extracts. The organic solvents could be successfully replaced by microemulsions to solubilize the plant extracts. These microemulsions provide good realease and stabilization of phenols from plant extracts. For conductometric analysis, the transition states of micro W/O, O/W bicontinuous structures have been highlighted.

Finally, the oregano and wild thyme extracts improved the antioxidant capacity of pear juice higher than synthetic antioxidants which are compounds with possibly negative effects on consumer's health.

Acknowledgements

This work has benefited from financial support through the 2010 POSDRU/89/1.5/S/52432 project, *Organizing the national interest postdoctoral school of applied biotechnologies with impact on Romanian bioeconomy*, project co-financed by the European Social Fund through the Sectoral Operational Programme Human Resources Development 2007–2013.

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