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ORIGINAL RESEARCH PAPER

ANTIOXIDANT AND ANTIBACTERIAL PROPERTIES OF CAPSAICINE MICROEMULSIONS†

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The aim of this study was to prepare capsaicin microemulsions and to assess their antioxidant and antibacterial properties. Pseudoternare phase diagrams were made and were highlighted O/W/S/CoS weight ratios corresponding to microemulsion states. The oil phase (O) was soybean oil and for the aqueous phase (W) was used a mixture of water and glycerol in a ratio of 4:1 (wt/wt). As a surfactant (S) was used Tween 40 and cosurfactant (CoS) was ethanol in the mass ratio S:CoS = 2:1. Viscosimetric and conductometric analyses revealed the transition state of the O/W, W/O microemulsions and bicontinuous structures. The antioxidant properties of the capsaicin microemulsions were assayed based on the capacity to counteract DPPH (2,2-diphenyl-1-picrylhydrazyl) radical. The scavenging capacity of the crude capsaicin was IC_{50} (DPPH) = 2.63±0.34 and for capsaicin microemulsions was IC_{50} (DPPH) = 5.26±0.28, lower than the value BHT (IC_{50} = 9.21±0.36μg·mL^{-1}) ($p<0.5$). Antibacterial activity of capsaicin and capsaicin microemulsions were evaluated using Kirby-Bauer disk diffusion susceptibility tests against three common bacteria: *Staphylococcus aureus*, *Salmonela enterica*, and *Escherichia coli*.

Keywords: microemulsions, capsaicin, antioxidant activity, antibacterial activity

Introduction

Capsaicin is a natural compound that can be found in the fruit of *Capsicum Annuum* L (*Capsicum frutescens*) from the *Capsicum* species, *Solanacee* family

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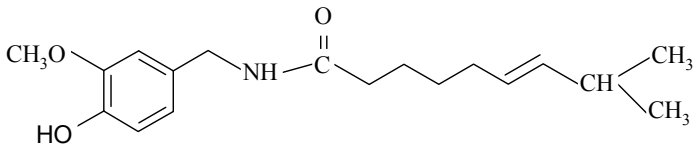
(Cordell and Araujo, 1993). From a chemical point of view, capsaicin is an alkyl vanillylamide (capsaicinoid) with the chemical formula $C_{18}H_{27}NO_3$ and the molecular mass M = 305. The scientific name according to IUPAC is: trans-8-methyl-N-vanillyl-6-nonenamide and it corresponds to the structure formula below:



The *cis* capsaicin isomer is called *civamide* and it is a compound of synthesis (Monsereenusorn et al., 1982). In the hot pepper fruit, along with capsaicin, there can be found other components from the capsaicinoids class: dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin. Out of these, capsaicin is the component with the most irritating action which gives the sensation of hot. The research on capsaicin revealed a high variety of actions with applications in different areas such as: food industry, pharmacy, medicine etc [3-5](Barceloux, 2008; M□zsik et al., 2009; Hayes et al., 1981).

In food industry capsaicin is one of the most important spices used since ancient times. Capsaicin proved to be a compound with a large bioactivity with manifested actions over the cardiovascular, respiratory and nervous systems and with a good antimicrobial activity against *Staphylococcus aureus*, *Salmonella typhimurium*, *Bacillus cereus*, *Listeria monocitogene*, *Helicobacter pylori* (Donnerer et al., 1990).

The low solubility of capsaicinoids in water raises high difficulties in the preparation of some formulas for introducing capsaicin in the food systems. The technical literature mentions some techniques of capsaicin incorporation within a matrix such as: molecular inclusion (cyclodextrins), micellar solubilisation, emulsification (simple or multiple emulsions), microemulsification, liposomes (Gutiérez et al., 2008; Friberg et al., 2004; Dickinson, 2009).

The present paper focuses on the solubilisation of capsaicin in a microemulsion type ternary system. Microemulsions are colloidal systems, thermodynamically stable, optically isotropic (transparent), with lower viscosity in comparison with emulsions. They are obtained by blending the two phases (hydrophilic and lipophilic) in the presence of a blend of surfactants and cosurfactants. The dimension of the drops is lower than 150 nm and for this reason they are sometimes mistaken with nanoemulsions which are thermodynamically unstable systems (Garti and Aserim, 2006). Capsaicin microemulsions were tested regarding the antioxidant and antimicrobial activity with the purpose of exploring their use at the preparation of some meat products.

Materials and methods

Materials

The capsaicin 99% was purchased from INRES France and the soybean oil was purchased from Supremia SRL, Romania. The 2,2-diphenyl-2-picrylhydrazyl (DPPH) and the butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich, Germany. All the other chemicals and solvents used were of analytical grade.

Microorganism

The indicator microorganisms used for testing the antimicrobial activity were the following: *Staphylococcus aureus*, *Salmonella* enterica and *Escherichia coli*. These strains were provided by the Microorganisms Collection of Microbiological Analysis Laboratory from "Sf. Andrei Hospital", Galati.

Designing the pseudoternary phase diagrams

In designing the pseudoternary phase diagrams it was used the water titration method, at room temperature, based on a protocol used by Hathout et al. (Hathout et al., 2010), adapted for the conditions and the goal of our experiments. The phase diagrams were designed with the mass ratio Span 80/ethylic alcohol (Surfactant:Cosurfactant) = 1:1. For each phase diagram different mass ratios were used – Soybean Oil/(S/CoS) = 1:9; 2:8; 3:7; 4:6; 5:5; 6:4; 7:3; 8:2; 9:1. The O/S/CoS blends were titrated with distilled water under mild stirring. After each water volume added, there was a waiting period of 5 minutes for establishing the steady state, followed by the visual and microscopic observation of the sample. The aspect of the samples was noted in the diagram with regard to transparency and microscopic homogeneity, knowing that the transparent samples correspond to microemulsions while the muddy ones correspond to emulsions.

Preparation of W/O microemulsions with capsaicin

The corresponding quantity of capsaicin was introduced in a known quantity of O/S/CoS blend and was sonicated until complete dissolution. Next, the corresponding quantity of water was added following the determined W/O/S/CoS ratio and stirred with the electromagnetic stirrer for 10 minutes at room temperature.

Our experiments made use of soybean oil and a hydroalcoholic solution of capsaicin. The surfactant was Span 80 and the Co-surfactant was the ethylic alcohol. A microemulsion type ternary system was prepared with the following composition: Water/Soybean Oil/Span 80:Ethanol/capsaicin = 33:61:2:2:2.

Measuring the electrical conductivity and the pH

The electrical conductivity was measured at 25 °C with a multiparameter Consort 868 apparatus, provided with a SK10B conductometric electrode that has the constant of the cell of 0.975 ± 0.16 % cm^{-1}. The error limit of the conductometric measurements was of ± 0.02 µS cm^{-1}. The pH was determined with the same apparatus using the pH electrode (HI 1131B) at 25 ± 0.5 °C with an error of ± 0.02. In the preparation process of microemulsions the pure water used had a content of 0.01 M NaCl. All measurements were carried out in triplicate.

Measuring the dynamic viscosity

The viscosity of the samples was measured at 25 °C with a Brookfield DV-III rheometer using a cone-plate system of 60 mm diameter and 1 degree angle. The shear rate was of 10 – 20 s^{-1}.

Antioxidant activity

The 2, 2-diphenyl-2-pcrylhydrazyl (DPPH) was used as generating substance of free radicals in order to determine the radical scavenging activity of capsaicin and capsaicin microemulsions (Stoilova et al., 2007). Different quantities of capasaicin and capsaicin microemulsions were added in a hydroalcoholic solution water:ethanol = 2:1 (w/w) that was homogenized through ultrasonication for 1 minute. There were obtained solutions with concentrations between 1 and 50 µg/mL capsaicin. 1 mL sample in different concentrations of capsaicin was added on 2 mL DPPH ethanolic solution (5 mg/mL) and was strongly stirred. The blends were kept in darkness for 60 minutes, after which the absorbance was measured at 515 nm. The antiradical activity (AA%) was computed with the following relation:

$$AA\% = 100 - [(A_{sample} - A_{empty\ sample}) / A_{control}] \times 100 \quad (1)$$

where: A_{sample} is the absorbance of the sample with DPPH; $A_{empty\ sample}$ is the absorbance of the sample without DPPH (2 mL ethanol + 1 mL sample with capsaicin); $A_{control}$ is the control absorbance of a blend formed from 2 mL DPPH solution + 1 mL ethanol.

Also, it was computed the IC_{50} parameter which represents the capsaicin concentration needed for the quench 50% of initial DPPH radicals under the experimental conditions. As reference substances were used BHT.

Antimicrobial activity

Antimicrobial activity of capsaicin and capsaicin microemulsions were evaluated using Kirby-Bauer disk diffusion susceptibility tests against three common bacteria: *Staphylococcus aureus*, *Salmonela*, and *Escherichia coli*. (Oussalah et al., 2007). Suspensions of tested microorganisms were poured in specific culture

agar medium (MEA, respectively PCA) tempered at 42°C and then homogenized in the sterile Petri dishes. After solidification wells of 5 mm size made in the agar medium and were loaded with 200 µL of hydroalcoholic solution of capsaicin and capsaicin microemulsion. The plates inoculated with bacteria were incubated at 37°C for 48h and at 25°C for 96h for yeast and moulds respectively (Oussualah et., 2006). After incubation, the formation of clear inhibition zone around the well indicated the antimicrobial activity. The inhibitory effect was assessed by measuring the diameter of the inhibition zone around the well.

Statistical analysis

The Design Expert (Version 8.0.7.1; Stat-Ease Inc., Minneapolis, Minnesota) for Windows XP was used for statistical analysis. Conventional statistical methods were used to calculate means and standard deviations. The results of antioxidant properties were analysed using one-way analysis of variance ANOVA and the differences were considered significant at $P < 0.05$. A correlation procedure was performed to evaluate IC_{50} parameters.

Results and discussion

Pseudoternary phase diagrams

The pseudoternary phase diagram (Fig.1) was designed to identify the concentrations of the components corresponding to the microemulsion state.

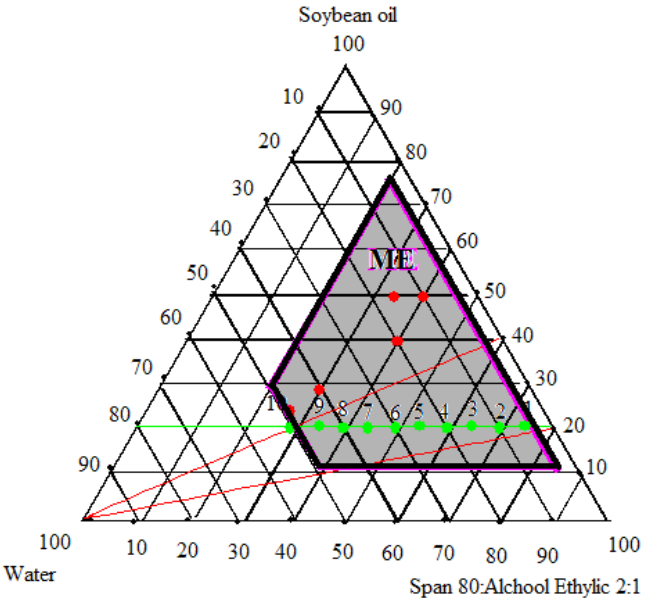


Figure 1. Pseudo-ternary phase diagram of microemulsion system made of soybean oil/span 80/water/ethanol/capsaicin

The analysis of the pseudoternary phase diagram (Fig.1) led to the conclusion that the surface corresponding to the microemulsion state represents 58.77 % ± 2.78 % out of the total surface of the diagram, which proves that the Water/Soybean Oil/(Span 80:ethaol 1:1) system favours the forming of microemulsions. The presence of the ethanol in the systems with non-ionic surfactants increases the solubility of the surfactant in the watery phase and improves the solubility of water in the oily phase. The ethanol molecules are distributed mainly between the watery layer and the region of the polar heads of the surfactant. Part of these molecules replaces the surfactant molecules in micelles, altering the interfacial tension and the flexibility of the interfacial film, and favouring the forming of microemulsions.

The analysis of the capsaicin microemulsions microstructure

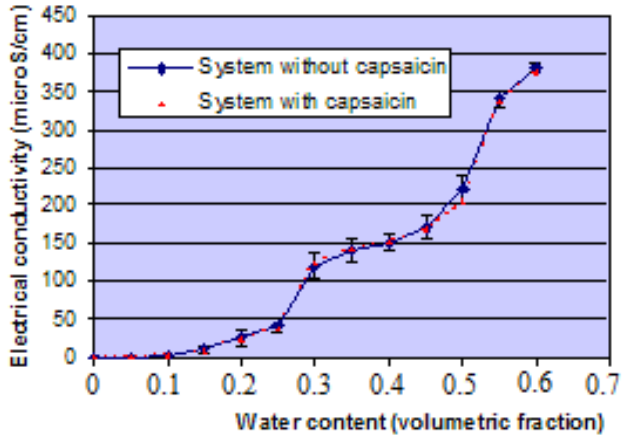
The structure of microemulsions influences the solubilisation capacity of compounds and determines the release rate of the active substances. For determining the internal structure of microemulsions different techniques are applied, such as: conductometry, viscosimetry, microscopy in polarized light, electronic microscopy (SEM, TEM), nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC) (Alani et al., 2009).

Electrical conductivity and the viscosity of the capsaicin microemulsions

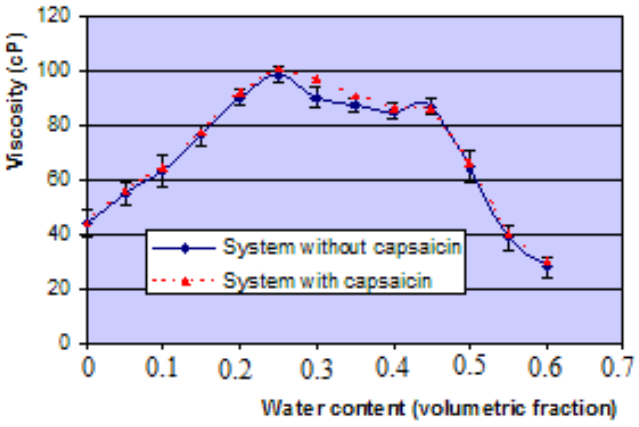
The electrical conductivity represents a conventional technique of investigation of phase transformation for emulsions and microemulsions. The present study followed the variation of the electrical conductivity with the water content of the Water/Soybean Oil/Span 80/Ethylic alcohol (S:CoS; 1:1) system, on the dilution line U40 without capsaicin and with 0.15 % capsaicin at 20 °C. A 0.01 M NaCl solution was used since the conductivity of the deionized water used for the preparation of microemulsions is very low.

The graphic in Figure 2 displays three inflexion points corresponding to some phase transitions. For a low content of water the electrical conductivity is low and it reflects the inferior mobility degree of water drops in oil. Beginning with the φ = 0.25 water volume fraction the electrical conductivity abruptly increases from 42.13 ± 9.8 $\mu S \cdot cm^{-1}$ to 120.57 ± 16.5 $\mu S \cdot cm^{-1}$ due to the percolation phenomenon. The value of the volume fraction where the increased electrical conductivity is registered is known as *percolation threshold* and it is noted with (φ_c). For values of the volume fraction lower than the percolation threshold, when W/O microemulsions are present in the system, the water drops are isolated from each other by the oily phase thus leading to lower electrical conductivity. As the water volume fraction increases around the percolation threshold, the water drops get closer and form associative structures known under the name of clusters. The number of the clusters rapidly increases over the percolation threshold. This phenomenon determines an increased electrical conductivity due to the Na^+ and Cl^- ions transportation from one drop of water to another, along the open water channels between drops during their collision. For the water volume fraction values between 0.35 and 0.45, the graphic displays a plateau to which it corresponds small increases of the electrical conductivity when increasing the water volume fraction. For these water concentrations bicontinuous structures are formed when the W/O

microemulsion coexists with the O/W one. In this state the curve of the interphasic surface is altered, the clusters collapse and the ions are less mobile. The second transition corresponds to the transfer of the bicontinuous structures into O/W microemulsions and it appears for volume fractions higher than 0.45. Thus, the electrical conductivity will increase from 171.56 ± 14.12 $\mu S \cdot cm^{-1}$ to 221.31 ± 8.32 $\mu S \cdot cm^{-1}$, at φ = 0.5. Adding water on the 0.5 volume fraction leads to muddy biphasic systems (classic O/W emulsions), with high electrical conductivity (380.41 ± 6.12 $\mu S \cdot cm^{-1}$). Microemulsions are systems whose flowing is of Newtonian type, for which the shear stress increases proportionally with the shear rate. In the present study measurements of dynamic viscosity were carried out in order to emphasize the dynamic properties of microemulsions. To this end, the variation of viscosity was followed with the increase of water content of the Water/Soybean Oil/Span 80/Ethylic alcohol (S:CoS; 1:1) system, on the U40 dilution line without and with 0.15 % capsaicin, at 20 °C .



(a)



(b)

Figure 2. Electrical conductivity (a) and viscosity (b) as a function of water percentage solubilized in the microemulsion system

For the Water/Soybean Oil/Span 80/Ethylic Alcohol (S:CoS; 1:1) system, to which water is added on the U40 dilution line, the gradual increase of viscosity, for water volume fraction values of φ = 0 – 0.25, from 43.67 ± 5.12 cP to 98.54 ± 3.15 cP proves that the dispersed phase is represented by the water drops corresponding to the W/O microemulsion. The growth of the dynamic viscosity with the water content for φ < 0.25 is a result of the attractive interactions between water drops and of the reorganization of the surfactant and cosurfactant molecules at the interface of W/O from the present microemulsions. The slight drop of the dynamic viscosity from 98.54 ± 3.15 cP to 85.12 ± 2.78 cP corresponding to the plateau between φ = 0.25 and φ = 0.45 indicates a transition of the system from the W/O microemulsion state to a bicontinuous structure. The value of the water volume fraction of φ = 0.25 corresponds to the percolation threshold of water. This value is correlated with the one obtained through electrical conductivity measurements. For a water volume fraction of φ = 0.45 the value of the viscosity reaches a new maximum at 86.89 ± 3.27 cP when the second transition takes place, namely from the bicontinuous structure to the O/W microemulsion state. The abrupt decrease of the dynamic viscosity for a water volume fraction over 0.45 indicates that water, which is the least viscous component of the microemulsion, becomes external phase and indicates the forming of the O/W emulsions.

Antioxidant activity. Recent studies carried out by Okada et al., (Okada et al., 2010) revealed that the antioxidant activity of capsaicin is owed to the phenolic OH group and depends on the nature of the solvent, and on the nature of the system in which the capsaicin is included, respectively.

The antiradical action of the crude capsaicin and capsaicin microemulsions was highlighted in the presence of DPPH as generator of radicals substance. The results were compared to the antiradical activity of the synthetic antioxidants (BHT) used in the preparation process of meat products. Thus, the IC_{50} values were computed for the crude capsaicin and capsaicin microemulsions and for the BHT, respectively, using the regression equations:

$Y_{Crude\ capsaicin} = 12.3856X + 17.4212$ ($R^2 = 0.9981$); $Y_{Capsaicin\ microemulsion} = 6.7436X + 14.5286$ ($R^2 = 0.9986$); $Y_{BHT} = 2.7707X + 23.0570$ ($R^2 = 0.9965$). The both values of $IC_{50crude\ Capsaicin} = 2.63 \pm 0.28 \mu g \cdot mL^{-1}$ and $IC_{50Capsaicin\ microemulsion} = 5.26 \pm 0.28 \mu g \cdot mL^{-1}$ were smaller than the value BHT ($IC_{50BHT} = 9.72 \pm 0.36 \mu g \cdot mL^{-1}$) ($p < 0.5$). This shows that capsaicin has antioxidant activity greater than BHT.

Antimicrobial activity. Capsaicin has been reported to inhibit an extensive spectrum of microorganisms (Lopez-Carrillo et al., 2003).

Antimicrobial activity of crude capsaicin and capsaicin microemulsions against reference strains was presented in Table 1.

Results show that the pure capsaicin manifests a higher antimicrobial activity than the capsaicin microemulsions since in hydroalcoholic solution the capsaicin molecules are more exposed to bacteria than in the case of W/O microemulsions.

Both pure capsaicin and capsaicin microemulsions manifest antimicrobial activity with different inhibition degrees towards the three microorganisms tested. The

highest inhibition degree is manifested towards *Staphylococcus aureus* and twice less towards *Escherichia coli.*

Table 1. Antimicrobial activity of crude capsaicin and capsaicin microemulsion

Sample	*Staphylococcus aureus*		*Salmonela*		*Escherichia coli*	
	Diameter of inhibition zone (mm)	**RI***	**Diameter of inhibition zone (mm)**	**RI***	**Diameter of inhibition zone (mm)**	**RI***
Crude capsaicin	18±0.12	3.0	10±0.17	1.67	9±0.06	1.5
Capsaicin microemulsion	14±0.08	2.33	7±0.07	1.16	7±0.05	1.16

*IR: Inhibition ratio: the ratio between the diameter of the inhibition zone and washer of filter paper diameter

Conclusions

Our research was focused on the solubilisation conditions of capsaicin. To this end, capsaicin was solubilized in a microemulsion type system consisting of Water/Soybean Oil/Span 80/Ethanol/Capsaicin in the mass ratio = 33:61:2:2:2 with the purpose of using it in the preparation of some meat products. The blending ratios were established on the basis of the pseudoternary phase diagram. The microemulsions microstructure was studied through conductometric and viscosimetric measurements emphasizing the phase transformations: microemulsions W/O → bicontinuous structures → microemulsions O/W. The antioxidant activity was determined, demonstrating that both pure capsaicin and capsaicin microemulsions manifest higher inhibitory capacity than the synthetic antioxidant BHT. The antimicrobial tests revealed that both the pure capsaicin and capsaicin microemulsions are active towards *Staphylococcus aureus*, *Salmonella* and *Escherichia coli*. The premix obtained by solubilizing capsaicin in the quaternary system Water/Soybean Oil/Span 80/Ethanol/Capsaicin in the mass ratio = 33:61:2:2:2 could be used in the preparation of some meat products.

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