# Short Term Scientific Mission Report – COST Action FP1405 ActInPak

Investigation of the Oxygen Barrier Properties of Polyethylene Films Coated with Green Tea Extract Loaded Liposomes

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# COLOPHON

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# Introduction

Active antioxidant packaging have received considerable attention as one of the most promising alternatives to the traditional packaging since it facilitate to reduce the oxidation of the food products (one of the main causes of the food spoilage) by antioxidant materials incorporated into or coated onto food packaging materials (Lopez-De-Dicastillo, Gomez-Estaca, Catala, Gavara, & Hernandez-Munoz, 2012). In this regard, several studies have reported demonstrating the release of antioxidants from packaging films (Camo, Beltran, & Roncales, 2008; Camo, Lores, Djenane, Beltran, & Roncales, 2011; Lopez-De-Dicastillo et al., 2012; Torres-Arreola, Soto-Valdez, Peralta, Cardenas-Lopez, & Ezquerra-Brauer, 2007).

In recent years, green tea (Camellia sinensis) has become as one of the most important and commonly consumed herb due to health benefits associated with its high catechin content including protec- tion against cancer and cardiovascular diseases, anti-inflammatory, antiarthritic, antibacterial, antiangiogenic, antioxidative, antiviral, neuroprotective, and cholesterol-lowering effects (Chacko, Thambi, Kuttan, & Nishigaki, 2010; Hosseini, Gorjian, Rasouli, & Shirali, 2015; Labbé, Têtu, Trudel, & Bazinet, 2008). The major chemical components of the green tea leaf that pro- vide all these health benefits are polyphenols. Main polyphenols in green tea include gallic acid, quercetin, kaempferol,myricetin, and their glycosides but the major part of polyphenols is composed of different forms of catechin including (−)-epicatechin (EC), (−)- epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin-3-gallate (EGCG) (Babu & Liu, 2008).

In food applications, green tea polyphenols have been suggested as food additives to enhance the antioxidant properties and to extend the shelf life of foods by acting as free radical scavengers to terminate the radical chain reactions that occur during the oxidation of triglycerides (Siripatrawan & Noipha, 2012). However, poor stability of green tea polyphenols limits the application of green tea in food products although it exhibits even higher antioxidant properties than α-tocopherol, hydroxyanisolebutylated or hydroxytoluenebutylated (Kailaku, Mulyawanti, & Alamsyah, 2014; Rashidinejad, Birch, Sun-Waterhouse, & Everett, 2014). Thus, entrapping green tea into liposomes could bear out to overcome limitations of green tea polyphenols due to the fact that liposomes are considered as a promising delivery system for phenolic compounds (Gibis, Vogt, & Weiss, 2012).

In this study, it is desired to observe the oxygen barrier properties of green tea extract loaded liposomes when they are utilized as food packaging films. It was hypothesized this system will not only protect the food from oxygen directly through the packaging film but also through the possible release of the green tea from the films to the food. To check this hypothesis modified DPPH method (Vapour-Phase DPPH Method) that designed by Prof. Yildirim and his co-workers at ZHAW was performed for green tea extract loaded liposomes.

# Materials & Methods

## Materials

Green tea extract had a content of 36% polyphenol was purchased by Spring Valley, Bentonville, Arkansas, USA. Soy lecithin, Lipoid S75 with 70% phosphatidylcholine was provided by Lipoid GmbH, Ludwigshafen, Germany. Ultra-pure grade lysozyme was obtained from Biomatik Corporation (Wilmington, DE, USA). Whey protein isolate was obtained from Bipro, Hard Line Nutrition, Kavi Food Co. (Istanbul, Turkey). Gum arabic, chitosan (medium molecular weight, viscosity=200-800 cP in 1% acetic acid solution, 75-85% degree of deacetylation, 2,2-diphenyl-1-picrylhydrazyl, and methanol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

## Preparation of Green Tea Extract Solution

The extract solution was prepared by dissolving 1% green tea extract (w/v) in distilled water around neutral pH (pH:6.5), stirring for 30 minutes at 300 rpm and filtering using a folded cellulose filter paper. Distilled water was obtained using 0.2 µS/cm purity mpMinipure Dest system (mpMinipure Ultrapure Water Systems, Ankara, Turkey).

## Preparation of Primary Green Tea Extract Loaded Liposomes

In order to obtain homogenous unilamellar vesicles with small particle diameter (40-50 nm) two-step homogenization process was carried out. 1 % (w/v) soy lecithin was added into the extract solution and the obtained coarse dispersion was first blended using an UltraTurrax (WiseTis Homogenizer, Witeg Labortechnik GmbH, Germany) at 20,000 rpm for 2 minutes and then repeatedly (five times) passed through high pressure homogenizer (Nano Disperser – NLM 100,

South Korea) at homogenization pressure of $13x10^7$ Pascal. The homogenization chamber was cooled during homogenization with ice to prevent the degradation green tea extract polyphenols.

### Preparation of Secondary Green Tea Extract Loaded Liposomes

1 % (w/v) biopolymer stock solutions were prepared by dissolving chitosan in acetate buffer and lysozyme, whey protein and gum arabic in distilled water. Biopolymer solutions were stirred for 24 h at 400 rpm to ensure complete dissolution. Biopolymer solutions in the optimum amount (which is characterized before) were added dropwise to 10 ml primary liposomes under continuous stirring (700 rpm, 2 min) to avoid flocculation depletion and potential cross-linking caused by the lacking of the coating materials (Madrigal-Carballo et al., 2010).

### Preparation of Eugenol Loaded Liposomes by Sonication

1% and 5% (w/v) soy lecithin was added into eugenol solution (10% and 20% v/v) and the mixture was blended using an UltraTurrax (Polytron, PT 2500 E, Kinematica, Switzerland) at 20,000 rpm for 2 minutes and then sonicated for 5 min at 100% amplitude and 1 cycle by Digitana UP 200H tip sonicator (Digitana, Switzerland).

### Preparation of Eugenol Loaded Liposomes in Ethanol

1% and 5% (w/v) soy lecithin was added into 10 % v/v eugenol-ethanol solution and the mixture was blended using an UltraTurrax (Polytron, PT 2500 E, Kinematica, Switzerland) at 20,000 rpm for 2 minutes.

### Preparation of Liposomal OPP Films

The liposomes were coated to OPP packaging material by with ZAA 2300 automatic film applicator (Zehntner GmbH Testing Instruments, Switzerland) with different rod size (11.43 and 20.57 µm). After the films were allowed to dry at room temperature for 2 hours they were stored in plastic bag before the antioxidant activity measurements.

### The Vapour Phase DPPH Method

The antioxidant properties of the film were investigated through the vapour phase by Vapour Phase - DPPH method (VP-DPPH) in tightly closed petri dishes (Eppendorf cell culture dishes, 60 mm).

The filter paper of 55 mm diameter containing 250 µL of 1% green tea solution on filter paper were stuck by double sticky tape to the inside of the lid of the petri dish. 2.5 mL of 0.1 mM DPPH methanol solution was placed onto the bottom of plate (non-direct contact with filter paper or coating). 250 µL of methanol on filter paper was used as negative controls. Petri dishes were tightly closed by placing a rubber ring between the lid and the bottom plate, sealed with ParafilmM® and kept in linear shaker (80 rpm) at room temperature in the dark. After a certain incubation time (1h, 5h and 3 days) 240 µL of sample were pipetted into sample well and the absorbance of DPPH solution at 517 nm was measured by using a plate reader (Synergy HT, BioTek Instruments, Switzerland). The scavenging of the stable DPPH radical was calculated by using the following formula:

$$I = \frac{A_B - A_0}{A_0} * 100\ \%$$

where: $A_0$ is the absorbance of the initial DPPH solution, and $A_B$ is the absorbance of DPPH solution with a sample.

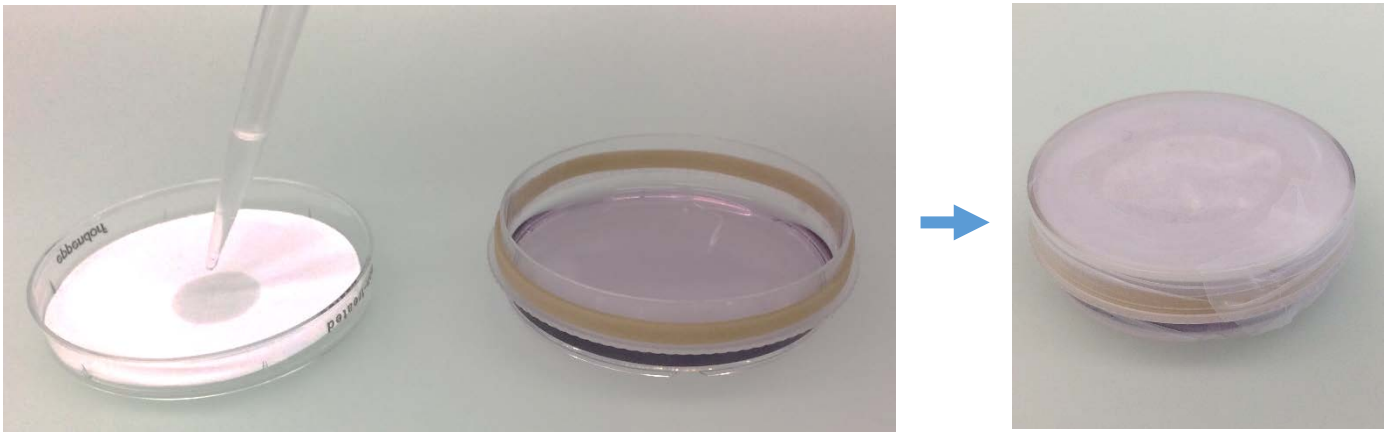

**Figure 1**. The images from the Vapour Phase DPPH Method Experiment

## Direct Contact DPPH Assay I

The antioxidant properties of films consist of cellulose diacetate and green tea were assessed by measuring the disappearance of the purple color of methanol solution of the DPPH (2-2-diphenyl-1-picrylhydrazyl). 7.5 mg of the film were added in to 5 ml of 0.1 mM DPPH (0.04 mg/ml) solution, the beakers were closed by Parafilm, and kept under stirring (300 rpm) in the dark for 30 min. Methanol in DPPHH solution was used as a blank. After 1 h incubation time the absorbance was measured by spectrophotometer. The inhibition of DPPH was calculated by using following formula:

$$I = \frac{A_B - A_0}{A_0} * 100\ \%$$

where: $A_0$ is the absorbance of the initial DPPH solution, and $A_B$ is the absorbance of DPPH solution with a sample.

## Direct Contact DPPH Assay II

The radical scavenging activity of the films was determined with DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical, according to method by Byun, Kim, and Whiteside (2010), with slight modifications. Approximately 100 mg of film samples were cut into small pieces and vortexed with 3 mL ethanol for 3 min. The mixture was left to stand for 3 h, at 25 °C, and then vigorously vortexed for another 3 min. One aliquot (100 µL) of the diluted ethanol solution (1/10) was mixed with 3.9 mL of 0.1 mM DPPH in methanol. The mixture was vigorously stirred and kept in the dark at room temperature for 1 h. Absorbance was measured at 517 nm using a spectrophotometer. DPPH radical scavenging activity was calculated by the following equation:

$$I = \frac{A_B - A_0}{A_0} * 100\ \%$$

where: $A_0$ is the absorbance of the initial DPPH solution, and $A_B$ is the absorbance of DPPH solution with a sample.

# Results & Discussion

## 1. The Vapour Phase DPPH Method Results for Green Tea Extract Solution

First of all, the Vapour Phase DPPH Method was performed to 1% green tea extract solution in order to check whether there is the release of the green tea solution from the films. As a preliminary experiment, the samples was firstly inserted to the filter papers instead of OPP films for the Vapour Phase DPPH Method. Antioxidant activity of 30 % eugenol solution by Vapour Phase DPPH Method was also observed to ensure the method was done correctly. Different incubation time was chosen in order to determine the optimum incubation time for green tea extract sample. As illustrated in Table 1., the % inhibition results of 30 % eugenol solution was between 84 and 90 % for different incubation time. The decrease in the inhibition with increase in incubation time could be explained by that the eugenol in the petri dishes might volatilize to the outside. For this reason, closing the petri dishes with a rubber ring and parafilm was done carefully. Unfortunately, according to the inhibition percentage of the green tea extract solution results the release of the green tea to the DPPH solution in the petri dishes could not be observed. As a result, it was concluded that green tea extract is not suitable sample for the Vapour Phase DPPH Method since it is not volatile as essential oils. Thus, two different Direct DPPH Method was performed for green tea extract solution and liposome containing green tea extract.

**Table 1.** % Inhibition results of 1% green tea extract solution and 30% eugenol solution by Vapour Phase DPPH Method

| | % Inhibiton | |
|---|---|---|
| **Incubation Time** | **1 % Green Tea Extract Solution** | **30% Eugenol Solution** |
| 1h | -9.29 | 90.36 |
| 5h | -13.38 | 89.59 |
| 3 days | -53.44 | 84.48 |

## 2. Direct DPPH Methods Results for Green Tea Extract Solution & Liposomes

According to Direct Method I results (as illustrated in Table 2.), it might be stated the reasonable inhibition result could not be obtained for 1% green tea extract solution and liposome containing 1% green tea extract. The possible reason could be that the extraction of the phenolic compounds from the films was not achieved by using solvent. The low inhibition percentage of the samples was investigated by Direct Method II (including extraction step with ethanol). The low inhibition percentage could be explained by the low concentration of the green tea extract. Due to the lack of green tea extract with same lot number the experiment could not performed for high green tea extract concentration samples. It was concluded that antioxidant packages with high antioxidant activity could not be obtained by using green tea extract in low concentration.

**Table 2.** % Inhibition results of 1% green tea extract solution and green tea extract loaded liposome by two Direct DPPH Method

| | % Inhibition | |
|---|---|---|
| **Method Type** | **1 % Green Tea Extract Solution** | **Green Tea Extract (1%) Loaded Liposome** |
| Direct Method I | -5.16 | -5.37 |
| Direct Method II | 5.69 | 2.07 |

## 3. The Vapour Phase DPPH Method Results for Eugenol Loaded Liposomes

5 different types of eugenol loaded liposomes (changing lecithin and eugenol concentration, and liposome preparation method) were prepared and the first trial for Vapor Phase DPPH Method was performed adding liposomes to filter paper. For convenience, the samples were labelled as A, B, C, D and E and the description of the letter was explained in Table 3. In Figure 2. it was shown the image of the eugenol loaded liposomes. The turbidity of the system implied that the liposomes have high particle size when the ultrasonication was used to form liposomes. Previous studies indicate that the droplet size of the systems prepared by ultrasonication was larger compared to other homogenization techniques. For instance, in the study of Jafari, He, & Bhandari, 2007 it was shown the emulsion with smaller emulsion droplet size was produced by microfluidization compared to ultrasonication.

**Table 3.** The description of the samples labeled as A, B, C, D, E

| Letter | Description |
|---|---|
| **Control** | 10 % Eugenol Solution |
| **A** | Eugenol (10%) Loaded Liposomes (5% lecithin) |
| **B** | Eugenol (20%) Loaded Liposomes (5% lecithin) |
| **C** | Eugenol (10%) Loaded Liposomes (5% lecithin) (Adding eugenol after liposome formation) |
| **D** | Eugenol (10%) Loaded Liposomes (1% lecithin) |
| **E** | Eugenol (10%) Loaded Liposomes (1% lecithin) (Adding eugenol after liposome formation) |

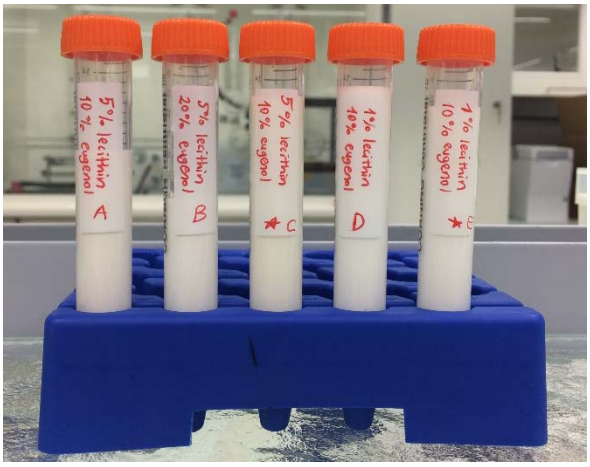


**Figure 2.** Image of the eugenol loaded liposomes with different parameters. * means that eugenol was added to empty liposomes.

After samples were transferred into filter paper (taped at the top of petri dishes) it was incubated for 1 hour at 80 rpm at room temperature in the dark. The release of the antioxidant of the DPPH solution that is placed at the bottom part of the petri dishes was investigated by measuring absorbance at 517 nm. The absorbance results were converted to inhibition percentage and shown in Table 4. As can be seen in Table 4, high inhibition percentage of eugenol from liposomes to the DPPH solution was investigated even for 1 h incubation time. Since there is no significant difference in incubation percentage between 1 h and 5 h incubation time it was decided to continue the experiments with 1 h incubation time. As expected, the high release of eugenol from liposomes

to DPPH solution was examined due to the fact that eugenol is volatile compound with high antioxidative properties.

**Table 4.** % Inhibition results of eugenol loaded liposomes with different parameters by Vapour Phase DPPH Method (using filter paper)

| | Incubation Time | |
|---|---|---|
| | **1h** | **5h** |
| **Control** | 89.54 | - |
| **A** | 88.56 | 89.63 |
| **B** | 88.15 | 88.63 |
| **C** | 89.68 | - |
| **D** | 90.26 | - |
| **E** | 88.99 | - |

In Figure 3., it can be seen the image of microplate for the A, B, C, D, E samples after 1 h incubation time with several replicates. This assay is based on the measurement of the reducing ability of antioxidants toward DPPH radical, through electron spin resonance (EPR) or by measurinf the decrease of its absorbance. DPPH is a stable radical in solution and appears purple colour absorbing at 515-520 nm. During the experiment, DPPH accepts a hydrogen (H) atom from the scavenger molecule (i.e. antioxidant) resulting into reduction of DPPH to DPPH2, the purple colour changes to yellow with concomitant decrease in the absorbance between 515 and 520 nm (Mishra, Ojha, & Chaudhury, 2012).

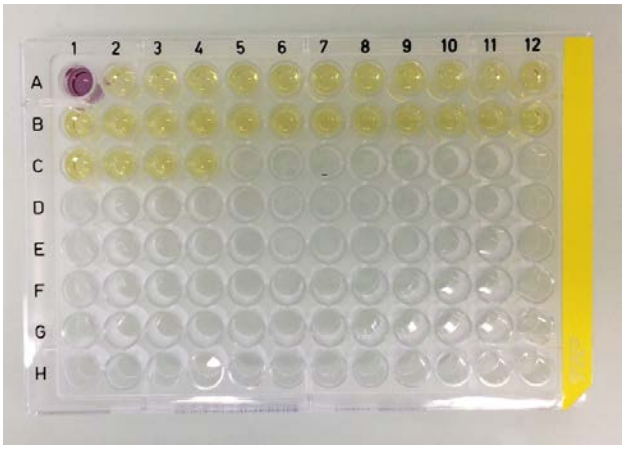


**Figure 3.** Image of the microplate containing the eugenol loaded liposomes after 1 h incubation time

After first trials were performed by adding the liposomes into filter paper, the liposomes were coated to OPP films by coating machine with road (11.43 µm). Following the drying of the OPP films for 2 h they are allowed to wait in the plastic bag for the experiments. The images of coated OPP films with eugenol loaded liposomes were illustrated in Fig. 3. As can be seen, non-homogenous coating of the liposomes was observed.

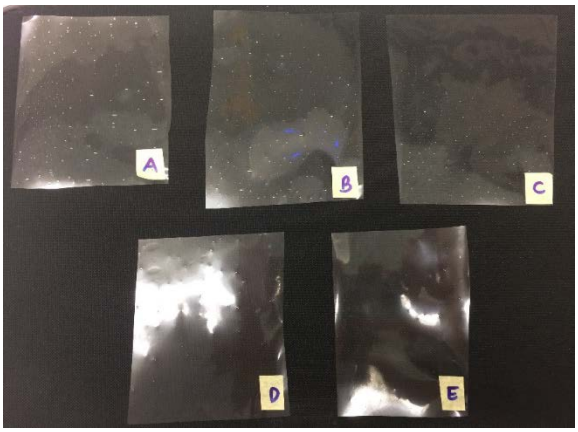


**Figure 3.** Image of the OPP films coated with eugenol loaded liposomes

The concentration of the liposomes on the OPP film after coating was calculated (from initial and final weight of the films) and reported in Table 5. For the Vapour Phase DPPH experiment, the OPP film coated with liposomes were cut as circle shape with 55 mm diameter and taped at the top of petri dishes. After incubation for 1 hour at 80 rpm at room temperature in the dark the absorbance was recorded by spectrometer. The inhibition percentage of the OPP films containing liposomes was shown in Table 6. From the inhibition percentage results, it can be concluded the inhibition decreased compared to addition of the liposomes to filter paper. This shows the coating of the eugenol coated liposomes to OPP films leads to 45-55 % reduction for A, B, C samples and 78-83% reduction for D and E samples. Lower inhibition percentage of D and E samples compared to A, B and C samples could be explained lower lecithin concentration in the liposomes. It was investigated the positive effect of lecithin concentration in the coating of the liposomes to OPP films. Moreover, it was investigated the higher inhibition percentage was observed for the liposome has 20% eugenol and 5 % lecithin concentration. As compared with liposome with 10% eugenol and 5% lecithin it was seen 8% difference. From this results, it was concluded increase in the eugenol concentration has positive effect on inhibition percentage.

**Table 5.** Concentration of eugenol loaded liposomes on the OPP films

| | Concentration (g/m2) |
|---|---|
| **A** | 0.825 |
| **B** | 0.921 |
| **C** | 0.758 |
| **D** | 0.858 |
| **E** | 0.971 |

**Table 6.** % Inhibition results of eugenol loaded liposomes with different parameters by Vapour Phase DPPH Method (in the OPP films)

| | % Inhibition |
|---|---|
| | **1h Incubation Time** |
| **A** | 40.39 |
| **B** | 48.35 |
| **C** | 41.89 |
| **D** | 19.11 |
| **E** | 14.50 |

## Conclusion

During the STSM in Switzerland, the cooperation between Middle East Technical University, Department of Food Engineering and Zurich University of Applied Science in the Department Life Science and Management was fortified. In the project, it was checked whether there is release from coated OPP Film with green tea extract loaded liposomes or not. Unfortunately, the release of green tea extract loaded liposome could not be investigated by Vapour Phase DPPH Method. For this purpose, new system (eugenol loaded liposomes) was prepared with different conditions and the release of the antioxidant from the liposomes was observed. It is planned that the results obtained from project was used in one of the conference participation by representing Cost Action successfully.

## Acknowledgement

I’m grateful to my advisor Dr. Mecit Oztop for his very useful advices and his time. I want to deeply acknowledge Prof. Selcuk Yildirim and his co-workers at ZHAW for this collaboration and for guiding me during the STSM. Lastly, I want to thank COST action management for giving me this possibility to initiate this collaboration.

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