National Technical University of Athens

Doctoral Thesis

Recovery, Characterization and Study of the Activity of Antioxidant Components of the plant Majorana syriaca

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Msc. Food Quality Management

Athens, 2011

«Η έγκριση της διδακτορικής διατριβής από την Ανωτάτη Σχολή Χημικών Μηχανικών του Ε.Μ.Π Πολυτεχνείου δεν υποδηλώνει αποδοχή των γνωμών του συγγραφέα. (Ν. 5343/1932, Άρθρο 202)»

Last soldier to die
in the battle

is
:
:
THE HOPE!

A Greek proverb

Η ελπίδα πεθαίνει τελευταία

To the patient soul, my father Saleh
To my mother Lilian,
To Nicola, Victor &Taghreed
and to all the poor

Acknowledgments

A great appreciation to my supervisor Dr. V. Oreopoulou, for her precious guidance and patience during the journey of my PhD, for directing my work and progress.

Big thanks to IKY (Organization of National scholarship) for granting me the scholarship, and for project Pithagoras for their financial support.

To my colleagues in the lab of Food Science and Technology, Dr. V. Iannou, Dr. E. Dermesonlouglou, Dr. E. Gogou, Dr. F. Tsironi, Dr. D. Tsimogiannis, Dr. C. Diamakou, V. Psimouli, R. Strati, Dr. G. Katsaros,Y. Papadaki,M. Tsevdou, D. Lembesi among others, I appreciate deeply their friendly welcoming me in the Lab., for the technical support and encouragement they offered. Thank you, also for Dr. P. Taoukis for collaboration in part of the experimental work. My sincere thanks to Dr. A. Detsi for introducing me to the laboratory of Food science and chemistry.

Thanks to Prof. C. Philippopoulos (laboratory of chemical processing engineering) for GC-MS analysis and H. Bardouki (Vioryl SA) for her help in LC-MS analysis.

Special thanks Dr. C. Tzia for her precious comments and Dr. O.Markopoulou from the three member committee for their advice and care about my thesis.

Blessings to the soul of my father resting in peace for his continuous sacrifice and love, and for the rest members of my supportive family.

To my closest friends for their unlimited support; Dani, Nona, Haydee, father Amir, father Michalis, Maria, Helen, Rita, Farid, Khaled, Vian, Amir, Paola and Lina. I am gratitude for my friends from Iraqi Christian community of Um-errahma, for their warm heart full of love, being my family in abroad. For Salah helping me in printing my thesis. For all my friends in the student campus, specially Evangelitsa, Lia, Stathis, Anthoula and Dimitra for the encouragement during difficult moments. I am specially gratitude to Dr. Yousef and Mr. Abdallah, for helping me financially, among many others.

Preface…

Majorana syriaca being widely consumed in middle-eastern diet, and originated from a group of herbs rich in natural antioxidants makes it a target of search to explore its antioxidant activity, that hasn't been investigated in details yet. Recently, there is an increasing demand for natural antioxidants being safer than synthetic ones, extending shelf life and delaying lipid oxidation in both processing and storage. To approach these concerns, the antioxidative effect of natural extracts from *M. syriaca* was intensively studied in various food systems, to investigate the contribution of *M. syriaca* to lipid stability under variable conditions.

As part of understanding the activity of the plant against oxidation it was important to analyze and characterize the components of plant extracts. The efficiency of the extraction of antioxidant components from a natural source depends on the solvents used for. Therefore, a main objective was to use solvents of different polarity in order to fractionate the antioxidants components of *M. syriaca* and determine the activity of each fraction. Additionally, the analysis of the components of each fraction was easier.

Antioxidants can be used to extend the shelf life of oils during storage. Accelerated storage test are most commonly used to predict their efficiency. However, the radical scavenging capacity is also used as a more rapid method to estimate the antioxidant activity. For this reason, both accelerated storage tests, using a common vegetable oil (corn oil) and radical scavenging test using a hydrogen acceptor radical (DPPH) were used to estimate the antioxidant potential of the extracts.

Food emulsions (such as salad creams, mayonnaise, coffee cream) are also lipid-based food products, that are subjected to deteriorative changes, mainly due to oxidation. Therefore, another objective was to evaluate the antioxidant potential of *M. syriaca* extracts in sample o/w emulsions.

High hydrostatic pressure (HHP) processing has been introduced as an alternative non-thermal technology. The effect of applying HHP processing to extend the shelf life of high fat-containing foods, is related to the effect of the treatment on the oxidative stability of the products. This study, examines the antioxidant effect of the ethyl acetate extract of *M. syriaca* in bulk corn oil and o/w emulsion after applying HHP. Beside that

the the effect of applying pressure amount of dissolved oxygen and droplet size of emulsions was studied.

Oil stability in deep-frying, being one of the most common food processes, was one of the goals of the study. The experiments were designed to evaluate the antioxidant effect of adding an extract from *M. syriaca* on oil deterioration during frying and storage of potato chips, focusing on oil oxidation in potato chips as a first remark previous to food explopitation.

Cookies are one of the lipid-based food systems that remain long time on shelves and consumed almost on daily basis (children, students, officers, etc.). Lipids are consideredan important constituent of their components, and it is highly required to find suitable natural antioxidants that can delay lipid oxidation. *M. syriaca* was employed in this study for this target, by examining its effect in inhibiting oil oxidation in cookies as well.

The present study was also undertaken to further evaluate the efficacy of *M. syriaca* extract in controlling the lipid oxidation and the microbial growth on minced chilled *Yellowfin* tuna. Several quality parameters such as sensory analysis, microbial growth, volatile compounds, and physical measurements were studied with respect to fish freshness evaluation, but neither of these tests discussed the freshness of tuna yellowfin fish regarding oxidation level. The antimicrobial and antioxidant effect of *M. syriaca* was under focus of this part of experimental work.

List of Abbreviations

1-EAc	one-step extraction by ethyl acetate
CSE	conventional soxhlet extraction
DPPH	1,1-diphenyl-2-picrylhydrazyl radical
D	diethyl ether
EAc	ethyl acetate
EC_{50}	antiradical efficiency
E	ethanol extract
HPLC	high performance liquid chromatography
HPLC-MS	high performance liquid chromatography coupled with mass spectroscopy
HPLC-DAD	high performance liquid chromatography coupled with diode array
HHP	high hydrostatic pressure
GC-MS	gas chromatography - mass spectrometry
M. syriaca	Majorana syriaca
OF	oxidation factor
P	petroleum ether
PUFAs	polyunsaturated fatty acids
PV	peroxide value
SC_{CO2}	supercritical extraction by carbon dioxide
TBARs	thiobarbituric acid reactive substances
d	days
h	hour
p	page

*Note: The numbering of tables and figures in each chapter is counted separately, and so tables or figures mentioned in the text refer to the same chapter, unless mentioned in the text otherwise.

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Περίληψη

Στην παρούσα διδακτορική διατριβή μελετήθηκε το φυτό "*Majorana syriaca*", το οποίο χρησιμοποιείται κυρίως ως καρύκευμα στη Μέση Ανατολή. Τα πειράματα σχεδιάστηκαν με βασικό σκοπό την ανάκτηση των αντιοξειδωτικών συστατικών του φυτού και την εκτίμηση της δράσης τους. Αγοράστηκαν αποξηραμένα φύλλα του φυτού από την τοπική αγορά της Βηθλεέμ, στην Παλαιστίνη, αλέστηκαν σε περιστροφικό μύλο με μαχαίρια που έφερε κόσκινο ανοίγαμτος 0.5 mm, και υποβλήθηκαν σε διαδοχικές εξαντλητικές εκχυλίσεις με τέσσερις διαλύτες αυξανόμενης: πετρελαϊκό αιθέρα (P), διαιθυλαιθέρα (D), οξικό αιθυλεστέρα (EAc), και αιθανόλη (E), για να διαχωριστούν τα συστατικά του φυτού ανάλογα με την πολικότητα. Ο πετρελαϊκός αιθέρας χρησιμοποιήθηκε για να εκχυλίσει τα συστατικά με χαμηλή πολικότητα, στα οποία συγκαταλέγονται τα αιθέρια έλαια του φυτού. Μετά την απομάκρυνση των αιθερίων ελαίων, ακολούθησε εκχύλιση με διαιθυλαιθέρα, ο οποίος είναι διαλύτης με χαμηλή πολικότητα, για την εκχύλιση αγλυκονών των φλαβονοειδών, ενώ ο οξικός αιθυλεστέρας, με μεσαία πολικότητα, χρησιμοποιήθηκε για την εκχύλιση των φλαβονοειδών με μεσαία πολικότητα, συμπεριλαμβανομένων και των γλυκοζιτών τους. Τέλος η αιθανόλη χρησιμοποιήθηκε για την παραλαβή των φαινολικών οξέων και των υπόλοιπων πολικών συστατικών.

Η συνολική απόδοση των εκχυλίσεων υπολογίσθηκε 23 %, σε ξηρή βάση, και η απόδοση με κάθε διαλύτη, βρέθηκε: πετρελαϊκός αιθέρας 9.04 %, διαιθυλαιθέρας 3.40 %, οξικός αιθυλεστέρας 4.10 %, και αιθανόλη 6.03 %.

Το εκχύλισμα P αναλύθηκε με GC-MS για ταυτοποίηση των συστατικών των αιθερίων ελαίων και έδειξε ότι έχει 43.89 % θυμόλη και 42.75 % καρβακρόλη. Τα εκχυλίσματα E, EAc και D αναλύθηκαν με HPLC-MS και HPLC-DAD. Τα αποτελέσματα έδειξαν ότι τα κύρια συστατικά που ταυτοποιήθηκαν στο D εκχύλισμα ήταν τα φλαβονοειδή, ταξιφολίνη και εριοδικτυόλη, και απιγενίνη στο EAc. Άλλα φλαβονοειδή που ανιχνεύθηκαν ήταν αγλυκόνες ή γλυκοζίτες φλαβονοειδών σύμφωνα με τα φάσματά τους και τα θραύσματα μάζας, αλλά δεν ήταν δυνατή η ταυτοποίηση των συγκεκριμένων ενώσεων. Επίσης βρέθηκε καρνοσόλη και ροσμαρινικό οξύ στα D και E εκχυλίσματα, αντίστοιχα.

Ακολούθησε, η μελέτη της αντιοξειδωτικής δράσης των εκχυλισμάτων. Αρχικά, για να βρεθεί αν τα εκχυλίσματα έχουν ικανότητα δέσμευσης ελευθέρων ριζών, που δημιουργούνται κατά την οξείδωση, εξετάστηκε η αντίδραση του κάθε εκχυλίσματος (σε διαφορετικές συγκεντρώσεις) με την ελεύθερηρίζα 2,2 διφαίνυλο πικρυλυδραζύλιο (DPPH), χρησιμοποιώντας δυο διαλύματα της ρίζας σε οξικό αιθυλεστέρα και σε μεθανόλη. Με βάση τα αποτελέσματα υπολογίστηκε η παράμετρος EC_{50}, η οποία εκφράζεται ως g εκχυλίσματος/kg DPPH ικανό να δεσμεύσει το 50 % της ελεύθερης ρίζας σε κάθε διαλύτη. Τα αποτελέσματα έδειξαν ότι τα εκχυλίσματα ακλούθησαν τη σειρά D > E > EAc > P. Το EC_{50} ήταν 1030, 1500, 2739, and 6000 g εκχύλισματος/kg DPPH για D, E, EAc, και P, αντίστοιχα σε διαλύτη οξικό αιθυλεστέρα. Σε διαλύτη μεθανόλη, η δράση των εκχυλισμάτων αυξάνεται και επομένων το EC_{50} μειώνεται και ανήλθε σε 1000, 1018, 1423 για D, E, EAc, αντίστοιχα, ενώ το εκχύλισμα P δεν διαλύεται σε μεθανόλη.

Στη συνέχεια, εξετάστηκαν τα εκχυλίσματα ως προς τη δράση τους σε αραβοσιτέλαιο προσθέτοντας δείγματα στο λάδι με ανάδευση ώστε η συγκέντρωση να ανέλθει σε 200, 500 και 1000 ppm, ενώ οι διαλύτες απομακρύνθηκαν με διαβίβαση αζώτου. Τα δείγματα με τα εκχυλίσματα υποβλήθηκαν σε συνθήκες επιταχυνόμενης οξείδωσης (70 ºC) σε κλίβανο με κυκλοφορία αέρα. Η αντιοξειδωτική δράση των εκχυλισμάτων εκτιμήθηκε με μέτρηση του αριθμού υπεροξειδίων (PV), και των συζυγών διενίων (CD) που αποτελούν πρωτογενή προϊόντα οξείδωσης. Τα αποτελέσματα έδειξαν ότι τα D και E εκχυλίσματα έχουν μεγαλύτερη προστασία, και ακολούθησαν τα EAc και P. Επίσης, η αύξηση της συγκέντρωσης από 200 σε 500 ppm, επιβραδύνει το σχηματισμό υπεροξειδίων, ενώ η αύξηση πάνω από 500 δεν έχει σημαντική επίδραση.

Στο δεύτερο μέρος των πειραμάτων, συνεχίστηκε η εργασία με βάση την προηγούμενη πειραματική εμπειρία. Πραγματοποιήθηκε απλοποιημένη εκχύλιση του φυτού *M. syriaca* με ένα διαλύτη, τον οξικό αιθυλεστέρα (1-EAc). Η απόδοση σε ξηρή βάση ήταν 12.6 %. Ακολούθησε η μελέτη της αντιοξειδωτικής δράσης του εκχυλίσματος. Εξετάστηκε η δέσμευση ελευθέρων ριζών του 1- EAc σε DPPH/ μεθανόλη και το EC_{50} ήταν 1000 g εκχύλισμα/kg DPPH. Στη συνέχεια, το εκχύλισμα 1- EAc εξετάστηκε ως προς τη δράση του σε αραβοσιτέλαιο, σε συγκεντρώσεις 200, 500 και 1000 ppm, στις συνθήκες επιταχυνόμενης οξείδωσης (70 °C). Η οξείδωση εκτιμήθηκε με μέτρηση των PV και CD.

Γενικά, η επιβράδυνση του PV από 1- EAc, βρέθηκε μικρότερη από τα εκχυλίσματα D, E και παρόμοια με τη δράση του εκχυλίσματος EAc από διαδοχικές εκχυλίσεις, ενώ η επιβράδυνση του CD ήταν παρόμοια με εκείνη από τα εκχυλίσματα από διαδοχικές εκχυλίσεις.

Στη συνέχεια, χρησιμοποιήθηκε το εκχύλισμα 1- EAc σε συστήματα λιπαρών τροφίμων, για να μελετηθεί η αντιοξειδωτική δράση σε διάφορες συνθήκες, αλλά και η αντιμικροβιακή του δράση. Αρχικά, το εκχύλισμα (200 ppm) χρησιμοποιήθηκε σε γαλάκτωμα 20 % λάδι σε νερό χρησιμοποιώντας Tween-20 ως γαλακτωματωποιητή, και τα δείγματα αφέθηκαν για οξείδωση σε υδατόλουτρο στους 40 °C και 60 °C. Η σταθερότητα των δειγμάτων εκτιμήθηκε μέσω του προσδιορισμού των CD, ως πρωτογενών προϊόντων οξείδωσης και των ενώσεων που αντιδρούν με θειοβαρβιτουρικό οξύ (TBARs), που αποτελούν δευτερογενή προϊόντα οξείδωσης και συγκεκριμένα μηλονική αλδεΰδη που προέρχεται από τη διάσπαση πρωτογενών προϊόντων. Τα αποτελέσματα έδειξαν ότι η αύξηση θερμοκρασίας προκαλεί αύξηση της οξείδωσης, ενώ η αντιοξειδωτική δράση του εκχυλίσματος διατηρείται και στις υψηλότερες θερμοκρασίες.

Στην επόμενη σειρά πειραμάτων, δοκιμάστηκαν δείγματα αραβοσιτελαίου και γαλακτώματος 20 % αραβοσιτελαίου σε νερό με, και χωρίς προσθήκη εκχυλίσματος 1-EAc από *M. syriaca* σε συγκέντρωση 200 ppm σε κατεργασία υπερυψηλής πίεσης (ΥΥΠ), η οποία αποτελεί μία καινοτόμο διεργασία συντήρησης των τροφίμων. Τα δείγματα τοποθετήθηκαν σε κυλινδρικό θάλαμο υπερυψηλής πίεσης και συμπιέστηκαν σε πιέσεις 200, και 650 MPa. Στη συνέχεια, όλα τα δείγματα, επεξεργασμένα και μη με ΥΥΠ, αποθηκεύτηκαν σε θάλαμο σταθερής θερμοκρασίας 70 °C, και μελετήθηκε η οξείδωση αυτών. Η οξείδωση εξετάστηκε με την μέτρηση CD και PV στο λάδι, και CD και TBARs σε γαλακτώματα, σε περίοδο δύο εβδομάδων. Τα αποτελέσματα των πειραμάτων οξείδωσης έδειξαν ότι τα έλαια και γαλακτώματα που υποβλήθηκαν σε επεξεργασία με ΥΥΠ παρουσίασαν ταχύτερο ρυθμό οξείδωσης, ο οποίος αυξανόταν με την

αύξηση της πίεσης από 200 σε 650 MPa. Αυτό οφείλεται στην αύξηση του διαλυμένου οξυγόνου με αύξηση της πίεσης. Συγκεκριμένα προσδιορίστηκε η περιεκτικότητα των δειγμάτων γαλακτώματος και ελαίου και βρέθηκε ότι η συγκέντρωση του διαλυμένου οξυγόνο αυξήθηκε από 0.014 mg/mL στα γαλακτώματα πριν την διεργασία ΥΥΠ, στο 0.029 mg/mL για γαλακτώματα που υποβλήθηκαν σε πίεση 650 MPa, και από 0.024 mg/mL στο λάδι πριν την διεργασία ΥΥΠ στα 0.029 mg/mL σε λάδι που υποβλήθηκε σε πίεση 650 MPa.

Σχετικά με την αντιοξειδωτική δράση του *M. syriaca,* τα αποτελέσματα έδειξαν ότι, ο εμπλουτισμός με 1-EAc στη συγκέντρωση 200 ppm προστάτεψε τα δείγματα που συμπιέστηκαν με ΥΥΠ σε 200 και 650 MPa έναντι της οξείδωσης των λιπαρών κατά την αποθήκευση σε συνθήκες επιταχυνόμενης οξείδωσης, μειώνοντας τα προϊόντα οξείδωσης κατά 35.8 % και 31.2 % στα γαλακτώματα για 200 και 650 MPa, αντίστοιχα, και στα 38.7 % και 33.7 % στο λάδι, για 200 και 650 MPa αντίστοιχα.

Το εκχύλισμα 1-EAc εξετάστηκε επίσης σε τρόφιμα που υφίστανται κατεργασία σε υψηλές θερμοκρασίες, όπως τηγάνισμα και ψήσιμο. Συγκεκριμένα χρησιμοποιήθηκε αραβοσιτέλαιο εμπλουτισμένο με το εκχύλισμα (500 ppm) σε τηγάνισμα τσιπς στους 185 °C, ενώ παρασκευάσθηκαν μπισκότα τύπου cookies με αραβοσιτέλαιο εμπλουτισμένο εμπλουτισμένο με το εκχύλισμα (500 ppm) τα οποία ψήθηκαν στους 160 °C.

Η δράση του εκχυλίσματος στο τηγάνισμα, εξετάσθηκε τόσο στη σταθερότητα του ελαίου τηγανίσματος όσο και στη σταθερότητα των προϊόντων κατά τη διάρκεια της αποθήκευσης. Η σταθερότητα του ελαίου κατά την διάρκεια του τηγανίσματος εξετάστηκε με τη μέτρηση των ολικών πολικών συστατικών, που αποτελούν τον πλέον αποδεκτό δείκτη για τα

έλαια τηγανίσματος όσο και με τη μέτρηση των CD, που παρουσιάζουν καλή συσχέτιση με τα πολικά συστατικά. Η αντιοξειδωτική δράση κατά την αποθήκευση των τσιπς εξετάστηκε με μέτρηση των CD και PV σε δείγματα που απομακρύνθηκαν από το τηγάνι μετά από 0 έως 16 h τηγανίσματος. Το εκχύλισμα του *M. syriaca* έδειξε μέτρια προστασία στο έλαιο τηγανίσματος, αλλά καλή δράση στα τσιπς, όπου μείωσε τα PVκαι CD από 41 έως 28 % και 49 έως 21 %, αντίστοιχα, με σύγκριση με τα δείγματα χωρίς εκχύλισμα. Η αντιοξειδωτική δράση κατά την αποθήκευση μειώθηκε στα δείγματα που είχαν απομακρυνθεί από το τηγάνι μετά από μεγαλύτερο διάστημα επαναλαμβανόμενων τηγανισμάτων, άλλα ήταν ακόμη σημαντική. Η μείωση της προστασίας μπορεί να οφείλεται σε απώλεια του αντιοξειδωτικού κατά την παρατεταμένη θέρμανση, αλλά και στη χειρότερη οξειδωτική κατάσταση του ελαίου που έχει απορροφηθεί στα τσιπς.

Τα μπισκότα τύπου cookies, στα οποία προστέθηκε το εκχύλισμα, μετά το ψήσιμο χωριστήκαν σε δύο παρτίδες, με συσκευασία και χωρίς, και υποβλήθηκαν σε συνθήκες επιταχυνόμενης οξείδωσης (70 ºC) σε κλίβανο με κυκλοφορία αέρα. Παράλληλα παρασκευάσθηκαν δείγματα αναφοράς και αποθηκεύτηκαν υπό τις ίδιες συνθήκες.

Τα αποτελέσματα έδειξαν ότι προσθήκη του εκχυλίσματος *M. Syriaca* στα μπισκότα οδήγησε σε μικρότερους ρυθμούς οξείδωσης όπως προέκυψε από τις τιμές CD και PV που μετρηθήκαν στο λάδι που εκχυλίστηκε από τα μπισκότα κατά την διάρκεια της αποθήκευσης. Το PV μειώθηκε κατά 25 % και το CD κατά 20 %. Η προστασία του εκχυλίσματος ήταν ίδια στα συσκευασμένα και μη συσκευασμένα δείγματα, ενώ η οξείδωση στα μη συσκευασμένα δείγματα ήταν ταχύτερη από τα συσκευασμένα αφού είχαν άμεση επαφή με τον αέρα.

Τέλος, μελετήθηκε η αντιοξειδωτική και αντιμικροβιακή δράση εκχυλίσματος *M. syriaca* σε τόνο (*Yellowfin tuna*). Τα δείγματα που παρασκευάστηκαν ήταν πολτοποιημένος τόνος αναμεμειγμένος με αραβοσιτέλαιο σε αναλογία ψαριού/λαδιού 3:1. Χρησιμοποιήθηκε μη εμπλουτισμένο αραβοσιτέλαιο για τα δείγματα αναφοράς και αραβοσιτέλαιο που περιείχε εκχύλισμα *M. Syriaca,* ώστε η τελική συγκέντρωση στο μίγμα να είναι 250, 500, 750, και 975 ppm, στα δείγματα ελέγχου. Τα δείγματα συντηρήθηκαν στους 0 ºC. Η αντιμικροβιακή δράση μετρήθηκε με προσδιορισμό της ολικής μικροβιακής χλωρίδας, των ψευδομονάδων, και των γαλακτικών βακτηρίων, και η αντιοξειδωτική δράση βάσει του προσδιορισμού των PV και TBARs του λιπαρού που εκχυλίστηκε από τα δείγματα. Τα αποτελέσματα έδειξαν προστασία έναντι της οξείδωσης για τα δείγματα του τόνου που εμπλουτίστηκαν με το εκχύλισμα, η οποία αυξανόταν με αύξηση της συγκέντρωσης.

Η χρήση του *M. syriaca* μείωσε επίσης τη μικροβιακή ανάπτυξη, με δράση αυξανόμενη με τη συγκέντρωση. Μεγαλύτερη ανάπτυξη στα δείγματα παρουσίασαν οι ψευδομονάδες, των οποίων η συγκέντρωση αυξήθηκε από 3.34-3.95 log CFU/g σε 8.0 log CFU/ g, εντός 10 ημερών, στα μη εμπλουτισμένα δείγματα. Η προσθήκη εκχυλίσματος 975 ppm μείωσε τη συγκέντρωση των ψευδομονάδων σε 5.5 log CFU/ g, δείχνοντας καλή αντιμικροβιακή δράση. Η ολική μικροβιακή χλωρίδα και τα γαλακτικά βακτήρια μειώθηκαν επίσης, και οι συγκεντρώσεις 750 και 975 ppm είχαν την καλύτερη αντιμικροβιακή δράση, χωρίς σημαντική διαφορά μεταξύ τους. Ο υπολογισμός της σταθεράς του ρυθμού της μικροβιακής ανάπτυξης (k) έδειξε ότι τα εμπλουτισμένα με εκχύλισμα δείγματα παρουσίασαν σημαντικά μικρότερες τιμές, οι οποίες γενικά μειώνονταν με αύξηση της συγκέντρωσης. Η μείωση ήταν 58 %, 43 % και 69 % πάνω από

συγκεντρώσεις 500, 750 και 975 ppm για τη σταθερά του ρυθμού ανάπτυξης των ψευδομονάδων, της ολικής μικροβιακής χλωρίδας και των γαλακτικών βακτηρίων, αντίστοιχα, ενώ περαίτέρω αύξηση της συγκέντρωσης δεν παρουσίασε σημαντική διαφορά.

Ως συνολικό συμπέρασμα από τα προηγούμενα αποτελέσματα, το φυτό *M. syriaca* έχει αντιοξειδωτική και αντιμικροβιακή δράση, και ένα απλό εκχύλισμα με οξικό αιθυλεστέρα (1-EAc) μπορεί να εφαρμοστεί σε διάφορα τρόφιμα για παράταση της διάρκειας ζωής

Summary

This study is employed on *Majorana Syriaca,* an important food-flavouring ingredient grown up in Palestine and middle east, commonly known as Za'atar.

The dried herb was brought from the local market of Bethlehem and used for a series of experiments studying intensively its antioxidative effect. In the first series of experiments, the dried leaves of *M. syriaca* were subjected to successive extractions, using a Soxhlet apparatus, with solvents of increasing polarity, specifically with petroleum ether (P), diethyl ether (D), ethyl acetate (EAc), and ethanol (E), in order to fractionate the components of the plant according to their polarity so as to investigate their activity and facilitate their identification. P was used to extract the less polar compounds including the constituents of the essential oil. The oil-free plant material was then treated with D, a low-polarity solvent for the extraction of low-polarity flavonoid aglycones, and EAc, a medium polarity solvent for the extraction of flavonoids with medium polarity. The extraction with E was performed to collect the residual, polar phenolics. The overall yield was 23% on dry basis, 9.04% obtained by P extraction, 3.40% by D, 4.10% by EAc, and 6.03% by E. The P extract was analyzed according to GS-MS analysis, and the main components were thymol (42.89 %) and carvacrol (42.75 %). The main

components of the other successive extracts (D, E, EAc) were detected according to analysis by HPLC-DAD and HPLC–MS. The D extract comprised taxifolin, eriodictyol, and carnosol, while the rosmarinic acid was found in both EAc and E extracts. Apigenin was also detected in the EAc extract, beside other peaks of flavones, flavone glucosides and flavanones.

Followingly, the activity of the extracts to scavenge the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$) was measured, and found that they followed the order D > E > EAc > P. The calculated EC_{50} values (i.e. the amount of dry extract that reduces DPPH concentration to half) were 1000, 1018, 1422, and 6000 g extract/kg DPPH for the D, E, EAc, and P extracts respectively. the antiradical efficiency in DPPH/methanol was 1000, 1018, 1423 for D, E and EAc respectively.

Moreover, the antioxidant activity in corn oil at concentrations of 200, 500 and 1000 ppm was monitored through peroxide value (PV) and conjugated dienes measurements (CD) at accelerated oxidation tests, and revealed that D and E extracts resulted in the highest protection followed by EAc and P extracts. Increasing the concentration from 200 to 500 ppm depressed peroxide formation, while a further increase had no effect, as shows values of CD (i.e in P =0.565± 0.052, 0.451± 0.126, 0.476± 0.011 for 200, 500 and 1000ppm).

In the second series of experiments, *M. syriaca* was subjected to a simplified, one step extraction by ethyl acetate (1-EAc), to recover the main antioxidant components that are soluble in oil. The yield of extraction was 12.6% on dry basis. The antiradical efficiency of the extract against DPPH was examined. The results revealed that EC_{50} value of 1000 g extract/kg DPPH. It also showed a recognizable antioxidant activity,

by reducing the oxidation level in bulk corn oil enriched with 200, 500 and 1000 ppm of the 1-EAc extract at accelerated oxidation tests. The antioxidant activity increased with concentration up to 500 ppm, while no significant difference was observed between 500 and 1000 ppm, as in the previous experiments. The depression of PV was generally lower than that obtained by D, E and EAc extracts of successive extractions, while the depression of CD was comparably close to extracts of successive extractions.

The oil enriched with the 1-EAc extract was applied in different food systems and variable conditions in order to extend the study of the antioxidant effect of the plant.

As a primary approach, the oil with 200 ppm of the 1-EAc extract was used in making 20% oil-in-water emulsions, stabilized with Tween 20, that were used in two main series of experiments: in the first series the emulsions were subjected to oxidation in a shaking water bath at 40°C and 60°C for two weeks. The oxidation tests in both experiments were monitored by measurement of CD and thiobarbituric reactive substances (TBARs). It was found that as temperature increased from 40°C to 60°C, oxidation was increased, but in both cases the enrichment of emulsion with extract improved the emulsion stability.

In the second series, the high hydrostatic pressure (HHP) treatment, used in the recent years as an alternative method in food processing, was employed. Bulk oil and oil-in-water emulsion were subjected to HHP treatments at 200 and 650 MPa so as to estimate the effect of the applied pressures on lipid oxidation during storage of the treated products and the antioxidant capacity of 1-EAc extract under these conditions. The HHP treated oil and emulsion

samples were stored under accelerated oxidation conditions (70 °C), and oxidation was monitored through PV and CD measurements for the oil, and CD and TBARs measurement for the emulsion. According to the results, the effect of pressure is already evident at the initial point just after pressurization and before applying samples to further accelerated oxidation. The initial CD value of non HHP-treated emulsion samples was 0.129±0.01 g/100 g oil while it was increased to values 0.194±0.01 and 0.346±0.02 g/100 g oil after HHP treatment at 200 and 650 MPa, respectively. Similarly, the CD value of non HHP-treated oil samples was 0.57± 0.01 g/100g and amounted to 0.61± 0.02 g/100g and 0.66± 0.03 g/100g after HHP treatment at 200 and 650 MPa, respectively. Similar differences were also observed in the other oxidation indexes, i.e. TBARs and PVs, and can be attributed to the increase of the concentration of dissolved oxygen caused by the HHP treatment (from 0.014 mg/mL in non-treated emulsion to 0.029 mg/mL in 650 MPa-treated emulsion and from 0.024 mg/mL in non-treated oil to 0.029 mg/mL in 650 MPa-treated oil). HHP treated samples presented higher oxidation rates during storage, especially as pressure increased from 200 MPa to 650 MPa. Concerning the antioxidative effect of *M. syriaca,* the results showed that, the addition of 1-EAc extract at 200 ppm decreased the level of oxidation during storage of the samples subjected to HHP treatment (200 and 650 MPa) and atmospheric conditions by (i.e CD of emulsions decreased by 35.8% and 31.2.0% for 200 650 ppm respectively, CD of oil was decreased by 38.7% and 33.7% for 200 and 650 ppm respectively).

Furthermore, antioxidant effectiveness of 1-EAc extract obtained from *M. syriaca* was evaluated in fried and baked food systems. More specifically, the extract was added at a concentration of 500 ppm to corn oil that was used for frying potato chips at 185 ˚C. The effect on the stability of frying the oil

was examined (through measurements of polar content and CD during frying, while the effect on the fried product was estimated through measurements of CD and PV during storage of potato chips. The protection of the frying oil by *M. syriaca* extract was moderate, however, a remarkable improvement of the oxidative stability of the fried chips was observed through the decrease of peroxide value (PV) and CD from 41 to 28% and from 49 to 21%, respectively, compared to samples with no additive. As frying proceeded, the antioxidant protection of the extract against the oxidation of the fried product during storage was less effective although it was still powerful. The decrease of protective action might be attributed to loss of antioxidant activity due to the prolonged application of high temperature, as well as to the oxidation status of the oil that is higher as frying proceeds.

Also, cookies were prepared by using corn oil enriched with 500 ppm of the extract. Samples of packaged and unpackaged cookies, were subjected to accelerated oxidation tests in oven at 70°C and oil stability was measured periodically.

The PV of oil extracted from cookies cookies decreased by 25 % and the CD content by 20%. No difference in the protection between packaged unpackaged samples were observed, although unpackaged samples showed higher oxidation rates, as expected due to contact with air.

The final approach was testing the antioxidant and antimicrobial potentiality of *M. syriaca* in Yellowfin tuna. The extract was added to refined corn oil at different concentrations (0–3900 ppm). Minced tuna was mixed with corn oil containing the extract (tuna ⁄ oil: 3 ⁄1 w⁄ w); the mixture was packed aerobically and stored at 0°C. The final concentration of the *M. syriaca* in tuna was varied in the range of 250 to 975 ppm. The antimicrobial activity

was determined by measurement of total viable count, *pseudomonas ssp.* and lactic acid bacteria.

The use of *M. syriaca* extract depressed the microbial growth with a concentration increasing-effect, and resulted in a maximum count of *pseudomonas sp.* of 5.5 log CFU/ g at 975 ppm, compared to 8.0 logCFU/ g for the non-enriched sample, showing good activity against *pseudomonas sp.* growth. The total viable count and lactic acid bacteria was also inhibited by *M. syriaca* extract, with concentrations of 750 and 975 ppm showing the best antimicrobial activity. The calculation of specific growth rate (k) indicated that all treatments containing *M. syriaca* extract, gave a significant reduction in microbial growth compared to the control samples ($P<0.001$), particularly on growth of pseudomonas. The reduction of growth rates increased with concentration and tended to reach a limit (approximately 58 %, 43 % and 69 %) at concentrations above 500, 750 and 975 ppm for *pseudomonas sp.*, the rate of total viable count and lactic acid bacteria, respectively. The oxidation indexes showed that the extract depressed the increase in both PV and TBARs and the protective effect was higher as concentration of the extract increased.

The above presented results show that the ethyl acetate extract of *M. syriaca* is a potent antioxidant for various foods, while it also posses antimicrobial activity against aerobic microorganisms.

Chapter one

LIPID OXIDATION

1.1 Introduction

Lipids consist of a broad group of compounds that are generally soluble in organic solvents but only sparingly soluble in water. They are major components of adipose tissue and together with proteins and carbohydrates, they consist the principal structural components of all living cells. Glycerol esters of fatty acids, which make 99% of the lipids of plant and animal origin (Nawar, 1996), have been traditionally called fats and oils. Based solely on whether the material is solid or liquid at room temperature, distinction between a fat and oil is of little practical importance, and the terms are often used interchangeably. Lipids can be classified according to their physical properties at room temperature (oils are liquids and fats are solid), their polarity (polar and neutral lipids), their essentiality for human beings (essential and non essential fatty acids) or their structure (simple or complex).

Food lipids are principally triacylglycerides, phospholipids and sterols found naturally in most biological materials consumed as food and added as functional ingredients in many processed foods. As nutrients, lipids, especially triglycerides, are a concentrated caloric source, provide essential fatty acids and are a solvent and absorption vehicle for fat-soluble vitamins and other nutrients. The presence of fat significantly enhances the organoleptic perception of foods, which partly explains the strong preference of fat-rich foods. As a class, lipids contribute to many desirable qualities to foods, including attributes of texture, structure, mouthfeel, flavor and color. However, lipids are also one of the most chemically unstable food components and will readily undergo free-radical chain reactions that not only deteriorate the lipids but also: (a) produce oxidative fragments, some of which are volatile and are perceived as the off-flavors of rancidity, (b) degrade proteins, vitamins and pigments and (c) cross-link lipids and other macromolecules into non-nutritive polymers (Shahidi, 1997; Min et al., 2002).

1.2 Fatty Acid*s*

Fatty acids constitute an essential part of lipids, mainly long chain. The chemical structures of these acids and their physical, chemical and biological properties are basic to understand lipids' properties. In few lipids the acids are replaced by related long-chain compounds such as alcohols, aldehydes or amines. Fatty acids are constituted of saturated acids, monoene acids, methylene and conjugated polyene acids, allenic and acetylenic acids, cyclic acids, branched-chain acids, hydroxyl acids, epoxy and furanoid acids, and *trans* acids (O'keefe, 2002; Gunstone, 1996a). Fats and oils are triglycerides or esters of glycerol and fatty acids. The fatty acids are differentiated by three aspects: (1) chain length, (2) the number and position of double bonds, and (3) the position of the fatty acids regarding the glycerol. Saturated fatty acids are chemically the least reactive and have higher melting point than unsaturated ones with the same chain length. Table 1 shows common saturated and usaturated fatty acids categorized according to chain length. The *cis* configuration of fatty acids is the natural configuration, while the *trans* configuration has different physical and physiological properties, since it develops with processing or heating (O'brien, 2003).

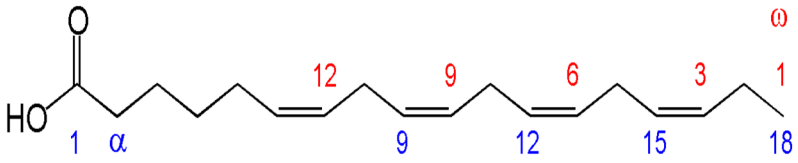
Table 1: Common saturated and unsaturated fatty acids (Enig, 2007)

Saturated Fatty acids	
Short-chain	Propionic (3;0), butyric (4:0), caproic (6:0)
Medium-chain	Caprylic (8:0), capric (10:0), lauric (12:0)
Long-chain	Myristic (14:0), palmitic (16:0), stearic (18:0)
Unsaturated fatty acids	
Short-chain	Crotonic (4:1 n-2)
Medium-chain	Palmitoleic (16:1 n-7), oleic (18:1 n-9),
Long-chain	Linoleic (18:2 n-6), α-linoleic, γ-linoleic, stearidonic (18:4 n-3), linolenic (18:3)

The designation of fatty acids nomenclature is based on either trivial names that are well known before setting chemical structures such as palmitic acid based on palm oil, oleic from olive oil, linoleic and linolenic from linseed oil. Other way of naming fatty acids is

based on systematic nomenclature according to internationally accepted rules of the International Union of Pure and Applied Chemists (IUPAC), and the International Union of Biochemistry (IUB) (O'keefe, 2002).

The shorthand (ω) or (n) is used to denote the location of the double bond, where the nomenclature is based on number of carbons in the fatty acid chain, followed by the number of double bonds, and the position of the double bond closest to the methyl side of the fatty acid molecule , such as stearidonic acid or 18:4 ω-3 or 18:4 n–3 indicates an 18-carbon chain with 4 double bonds, and with the first double bond in the third position from the CH_3 end (Gunstone, 1996a).



stearidonic acid

1.3 Natural Sources of Fats and Oils

The demand for oils and fats for food use is linked to the growth of world population. On the other hand, the supply of these materials from vegetable sources is affected by cultivation conditions and productivity (O'brien, 2003).

Oils and fats come from plant and animal sources as categorized in Table 2. Cotton seed oil is a by-product of the plant. Soybean is grown as oil crop and for its seed meal (a protein-rich animal feed) simultaneously. Rape, sunflower and groundnut are grown as oil crops with residual meal as a by-product. Other sources of plant oils come from oil-bearing trees such as olive, palm, and coconut that continues to bear a useful harvest for many years. Animal fats come from cattle, sheep and pigs or from marine origin (Gunstone, 1996b).

Table 2: Major sources of oils and fats

Plant (vegetables) oils	**Sources**
Vegetable oils (by-products)	Soybean, cottonseed, rice bran, corn

Vegetable oils (primary products)	Soybean, rape, sunflower, groundnut
Tree crops	Olive, palm, palmkernel, coconut
Industrial oils	Linseed, castor, tung
Animal oils and fats	
Milk fat, tallow, lard	animals
Fish oils	Fish

1.3.1 Major Vegetable Oils and Fats

Blackurrant, borage and evening primrose oils gained interest due to the existence of γ-linolenic (18:3 *n*-6), which is believed to be important in treatments of multiple sclerosis, arthritis, eczema, premenstrual syndrome and other diseases.

Cocoa butter (*Theobroma cacao*) is a solid fat, obtained from the cocoa bean along with cocoa powder. It is in high demand due to its characteristic melting behavior, which makes it suitable for use as a fat ingredient of chocolate. It has a sharp melting curve just below body temperature and its rapid melting produces a cooling sensation in the mouth. The fat is rich in palmitic acid (24-30%), stearic (30-36%), and oleic acid (33-39%).

Coconut oil (*Cocos nucifera*) is rich in lauric acid (45-50%) and in other short medium-chain acids. (C_8-C_{14}), and these so-called lauric oils are in high demand in both food and non-food uses.

Corn oil (maize oil, *Zea mays*) is extracted from the germ of corn after it has been separated from the grain. The latter is used for fodder and for the production of corn starch, sugar, and syrup. Corn oil belongs to the group of oils with high levels of linoleic and oleic fatty acids (O'brien, 2003; Gunstone et al., 1994). The refined oil has good oxidative stability and is used in salads, for frying and after partial hydrogenation, in margarines. Growing demand for bio-ethanol as a fuel additive is expected to lead to an increased production of maize and hence of corn oil.

Cottonseed oil (*Gossypium hirsutum*) is extracted from the cotton plant grown for its fibre with oil being produced only as a by-product (11-12% of the gross value of the crop). The major growing areas include China, USA, FSU, India and Pakistan.

Cottonseed oil is characterized by a relatively high content of palmitic acid (20-30%) and is used as a salad oil, frying oil, and in spreads.

Olive oil (*Olea europea*) is a tree crop grown predominantly in the Mediterranean region, characterized by its high level of oleic acid (65-85%) and by its high distinctive flavor. It has high oxidative stability because of the presence of natural antioxidants.

Palm oil and palmkernel oil (*Elaeis guinesis)* produces two oils with quite different fatty acid composition. The soft fleshy mesocarp yields palm oil rich in palmitic (about 40%), oleic (about 40%), and linoleic acid (about 10%). The seed or kernel produces a second oil, palmkernel oil, at 10-20% of the level of palm oil. Palmkernel and coconut oils are the major lauric oils with about 50% of lauric acid and other medium-chain acids.

Soybean oil (*Glycine max)* is highly unsaturated being rich in linoleic acid (containing more than 50%), and also containing some linolenic acid (4-11%). Much of the oil is consumed after partial hydrogenation, particularly to reduce the level of linolenic acid.

Sunflower oil (*Helianthus annuns).* The seed oil is low in saturated acids, contains virtually no *n*-3 acids and rich in linoleic acid (up to 70%). It is an important component of margarines rich in polyunsaturated acids.

Sesame oil (*Sesame indicum)* is grown particularly in China, India, Sudan and Mexico. It contains saturated (around 14%), oleic (33-54%) and linoleic acids (35-59%). Its high oxidative stability is due to the presence of sesamol, a natural antioxidant (Gunstone, 1996b).

1.3.2 Oils and Fats of Animal Sources

Butter fat is an important source of dietary lipids. Milk fat has a very complex fatty acid composition with high levels of short and medium-chain acids.

Lard and animal tallow from pigs, cattle and sheep are produced by rendering of carcass tissue. These animals' fats contain low but significant levels of acids with an odd number of carbon atoms, with branched-chains, and with trans-unsaturation. The major acids are palmitic and oleic acid.

Marine and Fish oils obtained from 'oily fish', e.g herring, pilchard, menhaden and anchovy, form a significant proportion (2-3%) of the world's available edible oils. The

fish livers of cod, halibut and shark contain approximately 50% oil and are important source of vitamin A and D. Marine oils are however, partially hydrogenated in order to obtain fats of adequate oxidative stability for conventional food application in margarine shortenings. The polyunsaturated fatty acids are the most characteristic feature of marine oils. The major saturated acids are palmitic (16:0) and myristic (14:0). The monoethylenic fatty acids are composed mainly of palmitoleic 16:1(n-7), oleic 18:1(n-9, and its isomer *cis*-vaccenic acid 18:1 (n-7) (Gunstone, 1994).

1.4 Mechanisms of Lipid Oxidation

During storage and distribution, foods are exposed to a wide range of environmental conditions, such as temperature, humidity, oxygen and light that can trigger several reaction mechanisms leading to food degradation. As a consequence of these mechanisms, foods may be altered to such an extent that they may be either rejected by the consumer of may become harmful to the health. Among the major modes of food deterioration is lipid oxidation. The presence of unsaturated fatty acids in foods is a prime reason for the development of rancidity due to the reaction that occurs at the double bond with oxygen leading to formation of free radicals and hydroperoxides that decompose further to secondary products, like ketones and aldehydes, and contribute to off-flavors.

The oxidation process is accelerated by metals, light, heat, and by several initiators during processing, harvesting, handling, storage, manufacturing, refining, and distribution steps. (Man, 2000; Gunstone 1996c).

1.4.1 Autoxidation

The degradation of fats by autoxidation has been the subject of research for more than 100 years because of its economical significance in food stability and safety, biological integrity and health, as well as in many applications in the chemical industry (Brimberg, 2003). Autoxidation is a radical chain process where the unsaturated fats (RH) react with oxygen to form hydroperoxides in three basic steps: initiation, propagation and termination (Frankel, 1985).

1.4.1a Initiation

The initiation reactions produce free radicals by some catalytic means, i.e. the allylic radical $R^{\bullet}$ when hydrogen is abstracted from the olefinic acid molecule RH. The resultant $R^{\bullet}$ reacts with an oxygen molecule to form an unstable peroxyl free radical $ROO^{\bullet}$. The peroxyl radical reacts with another fat molecule (RH) thus producing hydroperoxides (ROOH) and new free radicals (Hämäläinena et al., 2001). The activation energy required for this process originates either from high temperature storage conditions, light, or other sources. At the initiation stage the reactions proceed at slow rate; the duration of the initiation stage varies among different fatty substances and is related to the level of unsaturation and the presence or absence of natural antioxidants.

1.4.1b Propagation

At the propagation stage, the oil acquires a rancid flavor. Most of peroxides formed are unstable and easily broken forming more free radicals, which in turn participate in new reactions. This chain reaction continues until either the unsaturated compound has been exhausted or the free radicals have inactivated each other.

1.4.1c Termination stage is the mutual annihilation of free radicals. The automatic termination of oxidation is difficult, since it is not likely for all free radicals formed to inactivate each other. The following reactions describe the autoxidation of fats and oils:

1-Initiation

$$RH + O_2 \longrightarrow R^{\bullet} + {}^{\bullet}OOH$$

2-Propagation

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + RH \longrightarrow R^{\bullet} + ROOH$$

3-Termination

$$R^{\bullet} + R^{\bullet} \longrightarrow RR$$

$$R^{\bullet} + ROO^{\bullet} \longrightarrow ROOR$$

$$ROO^{\bullet} + ROO^{\bullet} \longrightarrow ROOR + O_2$$

Where **RH** = Lipid molecule**, R$^{\bullet}$=** Allylic radical**, ROO$^{\bullet}$=** Peroxyl radical, **ROOH**= Hydroperoxide.

Figure 1 presents the autoxidation of linoleic acid

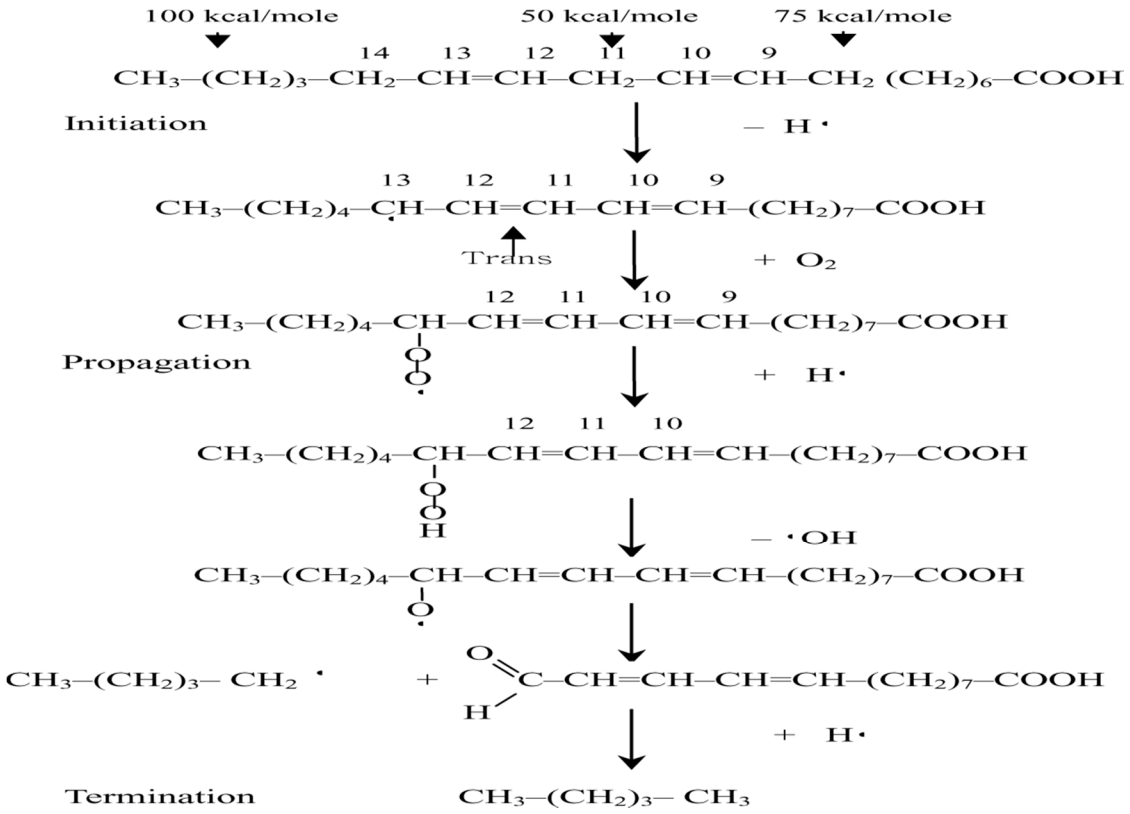


Figure 1: the initiation, propagation and termination of thermal oxidation of linoleic acid (Choe & Min, 2007).

1.4.2 Photoxidation

Another important pathway for the formation of allylic hydroperoxides from unsaturated fats is by exposure to light in the presence of oxygen and sensitizer. It is important to distinguish between the sensitized photoxidation and photolytic autoxidation, which can

be initiated by UV-catalyzed decomposition of peroxides and hydroperoxides. Photosensitized oxidation generally involves light excitement of the electrons in the outer orbitals of the sensitizer molecule to the singlet state (^{1}sensitizer*) followed by intersystem crossing to the triplet (^{3}sensitizer*):

$$^1\text{Sensitizer} + h\nu \longrightarrow {}^1\text{Sensitizer}^* \longrightarrow {}^3\text{Sensitizer}^*$$

Photosensitized oxidation has two pathway types: In type I phathway, the sensitizer presumably reacts, after light absorption, with substrates (A) to form intermediates, which then react with ground state (triplet) oxygen to yield the oxidation products (Min et al., 2002)

$$^3\text{Sensitizer}^* + A \longrightarrow (\text{Sensitizer-A})$$
$$(\text{Sensitizer-A}) + {}^3O_2 \longrightarrow A\,O_2 + {}^1\text{Sensitizer}$$

Triplet oxygen lipid oxidation of foods has been studied for 70 years as part of the effort to improve oxidative stability of the foods. Unsaturated fatty acids are more susceptible to oxidation than saturated fatty acids, a property that is primarily due to the lower activation energy in the initiation of free radical formation for triplet oxygen oxidation. However triplet oxygen oxidation does not fully explain the initiation step of lipid oxidation.

In the type II pathway, a photosensitizer such as chlorophyll, pheophytins, riboflavin, and myoglobin in food can absorb energy from light and transfer it to triplet oxygen to form singlet oxygen, which is more electrophilic than the triplet state oxygen (Frankel, 1985)

$$^3\text{Sensitizer}^* + {}^3O_2 \longrightarrow {}^1O_2{}^* + {}^1\text{Sensitizer}$$

Where ^{1}Sensitizer*: activated singlet state sensitizer

^{3}Sensitizer*: activated triplet sensitizer

$^1O_2{}^*$: activated singlet state oxygen

$^3O_2$: ground state triplet oxygen

Singlet oxygen oxidation can be very rapid in foods due to the very low activation energy required for the chemical reaction. It can react rapidly (-1500 times faster than $^3O_2$) with moieties of high electron density, such as C=C bonds. The resulting hydroperoxides can then cleave to initiate a conventional free radical chain reaction.

The reaction of $^1O_2{}^*$ with unsaturated fats proceeds by entirely different mechanism than free radical autoxidation. The activated singlet oxygen reacts directly with double bonds. So oxygen is inserted at either carbon of a double bond, which is shifted to yield an allylic hydroperoxide. By this mechanism hydroperoxides are formed at each unsaturated carbon of a fat molecule. The rate of type I pathway is mostly dependent on the type and concentration of the sensitizer and substrate. Compounds that are readily oxidizable, such as phenols, and easily reducible tend to favor type I, while the rate of type II pathway is mostly dependent on the solubility and concentration of oxygen in food systems. Oxygen is more soluble in nonpolar lipids, than it is in water. In contrast, water-based foods such as milk may tend toward type I. Types I and II will enhance the oxidation by either formation of reactive substrate species or production of singlet oxygen (Min et al., 2002).

In many foods containing lipid, special measures are taken to reduce or prevent oxidation, such as removal of oxygen, addition of antioxidants, and use of gas barrier packaging materials.

1.4.3 Metal catalysis

Certain transition metals, which have two or more valence states with a suitable oxidation-reduction potential between them play a catalytic role in lipid oxidation. Several mechanisms for metal catalysis of lipid oxidation have been postulated (Love, 1985). They include:

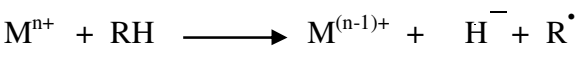
1-Acceleration of hydroperoxide decomposition

$$M^{n+} + ROOH \longrightarrow M^{(n+1)} + OH^- + RO^{\bullet}$$

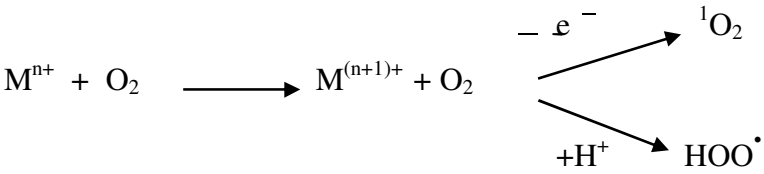
$$M^{n+} + ROOH \longrightarrow M^{(n-1)+} + H^+ + ROO^{\bullet}$$

$$2ROOH \longrightarrow ROO^{\bullet} + RO^{\bullet} + H_2O$$

2- Direct reaction with unoxidized substrate



3-Activation of molecular oxygen to give singlet oxygen and peroxy radicals.



Metals and organometalic compounds are catalysts of the oxidation and are, thus, primary pro-oxidants. According to Brimberg (2003) and De Leonardis (2002), the addition of cupper increased the oxidation rate of methyl linoleate more than iron as a catalyst of autoxidation.

1.5 Oxidation products

Oxidation products at early stages are considered as **primary oxidation** products.

Monohydroperoxides are the major initial reaction products of fatty acids with oxygen (Galdovic et al., 1997). The major hydroperoxide products resulting from autoxidation of methyl linoleate ester detected by Hämäläinen et al. (2001) were 9-hydroperoxy-10-*trans*, 12-*cis*-octadecadienoic acid methyl ester (9-OOH) and 13-hydroperoxy-9-*cis*, 11-*trans*-octadecadienoic acid methyl ester. Although hydroperoxides are tasteless and odorless, they decompose to volatile carbonyl compounds as products of degradation called **secondary oxidation products**, which contribute to the sensation of rancidity. The hydroperoxides are decomposed to short-chain compounds (volatile) including aldehydes, ketones, alcohols, acids, esters, lactones, ethers, and hydrocarbons (Gunstone, 1996c). Among them are unsaturated aldehydes, such as 2,4-decadienal, and 2,4-nondienal, which contribute to the characteristic flavor of oils that are not deteriorated, and can be considered desirable. However saturated aldehydes such as hexanal, heptanal, octanal, nonanal and 2-decanal, have distinctive off-odors in olfactometry analysis of heated oil, such as the fruity and plastic off-odors from heated high oleic acid oils.

The presence of hydroperoxides of conjugated dienes at early stages of corn oil oxidation

was detected by Guillen (2009), in structures such as alkanals, (E)-2-alkenals, (E,E)-2,4-alkadienals, 4-hydroxy-(E)-2-alkenals, 4-hydroperoxy-(E)-2-alkenals and 4,5-epoxy-(E)-2-alkenals, which can be either free or joined to truncated structures of triglycerides. Malonaldehyde is formed from polyunsaturated fatty acids with at least 3 double bonds. (Gordon, 2004). According to Frankel (1983), primary oxidation products of autoxidation or photosnsitization are found to be source of malonaldehyde. Five membered hydroperoxy epidioxides and 1,3-dihydroperoxides were found to be the most important precursors of malonaldehyde**. Further oxidation products** are suspected as carcinogenic or toxic, where they are able to degrade amino acids to produce Strecker aldehyde or the α-keto acid, depending on the decarboxylation of the amino acid Secondary lipid oxidation products (alkadienals and ketodienes, among others) are also able to produce this reaction, but these compounds need further oxidation (Figure 2).

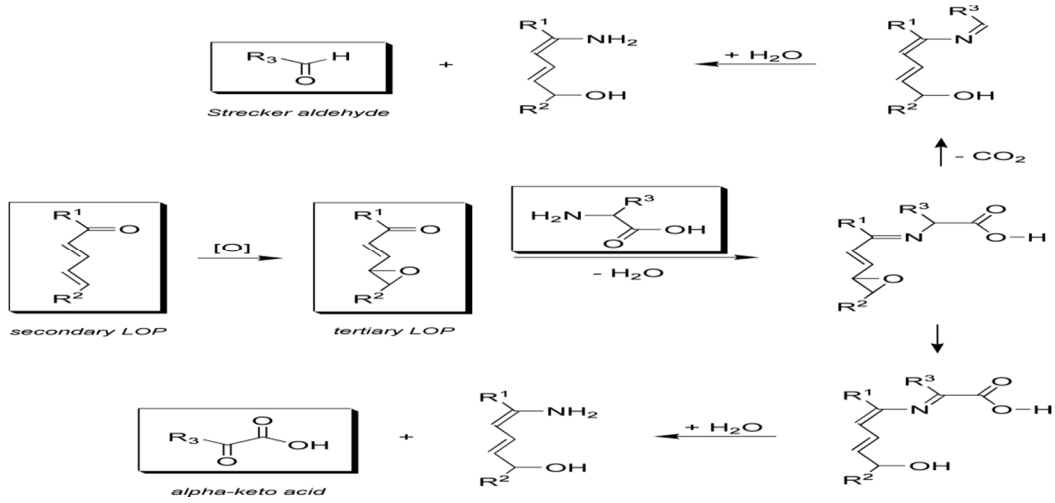


Figure 2: Oxidation of secondary lipid oxidation products to tertiary and the later reaction of tertiary oxidation products with amino acids to produce Strecker aldehydes and α-keto acids.

1.6 Thermal oxidation: Baking and Frying

The lipids in food undergo a variety of chemical changes as a result of exposure to heat treatment through, for example, cooking, baking, boiling, roasting, canning, pasteurization, drying, frying, etc. Such changes are of extreme importance to both the consumer and the processor, because they may have an adverse effect on the flavor, nutrition, and toxicology. The chemistry of lipid oxidation at high temperature is complex

since both thermolytic and oxidative reactions are simultaneously involved. Both saturated and unsaturated fat molecules undergo chemical decomposition when exposed to heat in the presence of oxygen (Nawar, 1985).

1.6.1 Changes occurring during oxidation in Frying

Both shallow and deep-fat frying are considered among the most important applications of oils and fats in food. Deep-fat frying is a common method of food preparation that imparts desired sensory characteristics, i.e. fried food flavor, golden brown color and crisp texture. During frying at 190°C, oils thermally and oxidatively decompose and form volatile compounds that alter functional, sensory and nutritional quality. In deep-fat frying processes, the applied heat is transferred from the oil to the food and water is evaporated while oil is absorbed by the food (Warner, 2002). During frying several chemical and physical changes occur. Thermal reactions are hydrolysis, oxidation and polymerization that are common chemical reactions in frying oil and produce volatile or nonvolatile compounds.

1.6.1a Changes in oil

Hydrolysis occurs when food is placed in oil at frying temperatures. Air and water initiate a series of interrelated reactions. Water and steam hydrolyze triglycrides, which produce mono- and diglycerides and free fatty acids. **Oxidation** occurs when **o**xygen present at the oil surface is introduced by addition of food, activating a series of reactions involving formation of free radicals, hydroperoxides and conjugated dienoic acids. The chemical reactions that occur during oxidation, contribute to the formation of volatile and nonvolatile decomposition products. The oxidation mechanism in frying is similar to autoxidation at 25°C. However, the unstable primary oxidation products decompose rapidly at 190°C into secondary oxidation products (section 1.5). The thermal oxidation rate is faster than the autoxidation, but specific and detailed scientific information and comparisons of oxidation rates between thermal oxidation and autoxidation are not available. The mechanism of thermal oxidation involves the initiation, propagation, and termination of the reaction as shown in Figure 1.

Polymerization

The major decomposition products of frying oil are nonvolatile polar compounds and triacylglycerol dimers and polymers. Dimers or polymers are either acyclic or cyclic depending on the reaction process and the kinds of fatty acids of the oil. The amounts of cyclic compounds are relatively small compared to the nonvolatile polar dimers and polymers (Dobarganes et al. 2000). Dimers and polymers are large molecules with a molecular weight formed by a combination of -C-C-, -C-O-C-, and -C-O-O-C- bonds (Kim et al., 1999). Dehydroxydimer, ketodehydrodimer, monohydrodimer, dehydrodimer of linoleate, and dehydrodimer of oleate are dimers found in soybean oil during frying at 195 ◦C (Christopoulou & Perkins 1989). Dimers and polymers have hydroperoxy, epoxy, hydroxy, and carbonyl groups, and -C-O-C- and -C-O-O-C- linkages. Dimerization and polymerization in deep-fat frying are radical reactions. Allyl radicals are formed preferably at methylene carbons α to the double bonds. Dimers are formed from the reactions of allyl radicals by C-C linkage. The formation of acyclic polymers from oleic acid during heating is shown in Figure 3. Triacylglycerols react with oxygen and produce alkyl hydroperoxides (ROOH) or dialkyl peroxides (ROOR). They are readily decomposed to alkoxy and peroxy radicals by RO-OH and ROO-R scission, respectively. Alkoxy radicals can abstract hydrogen from oil molecule to produce hydroxy compounds, or combine with other alkyl radicals to produce oxydimers. Peroxy radicals can combine with alkyl radicals and produce peroxy dimers. Cyclic polymers are produced within or between triacylglycerols by radical reactions and the Diels-Alder reaction (Figure 4). The formation of cyclic compounds in frying oil depends on the degree of unsaturation and the frying temperature (Meltzer et al., 1981).

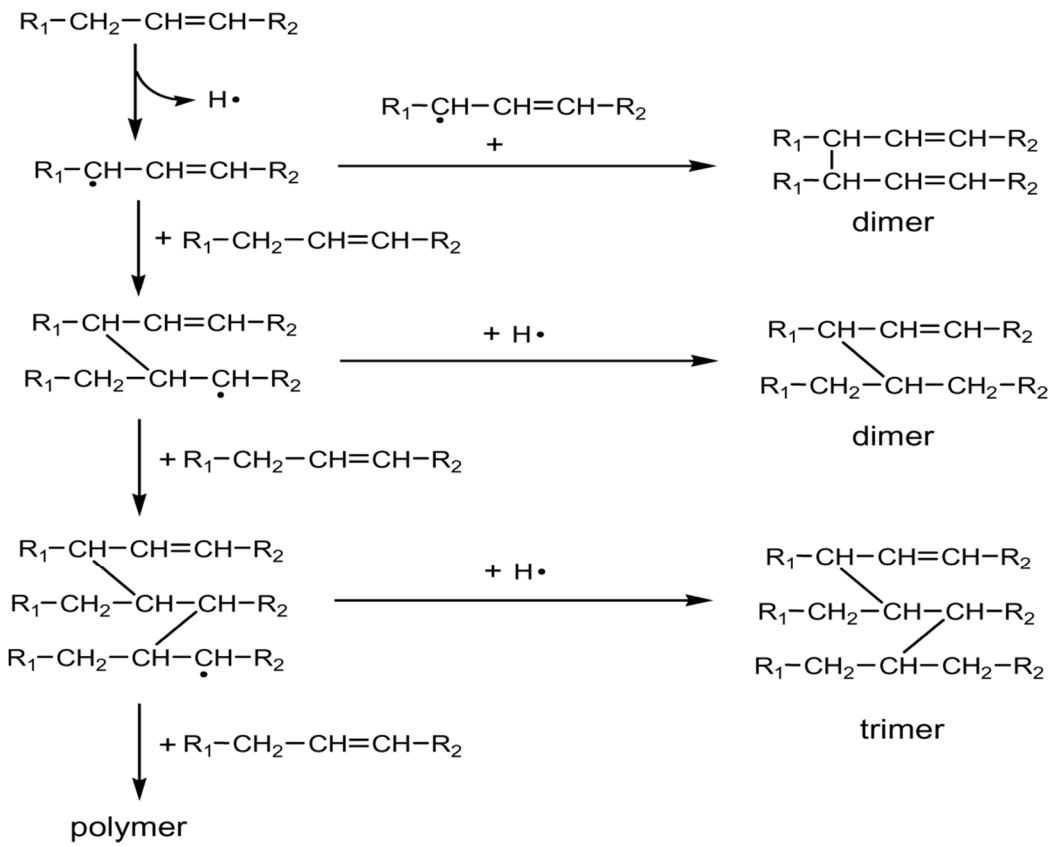


Figure 3: Acyclic polymer formation from oleic acid during deep-fat frying

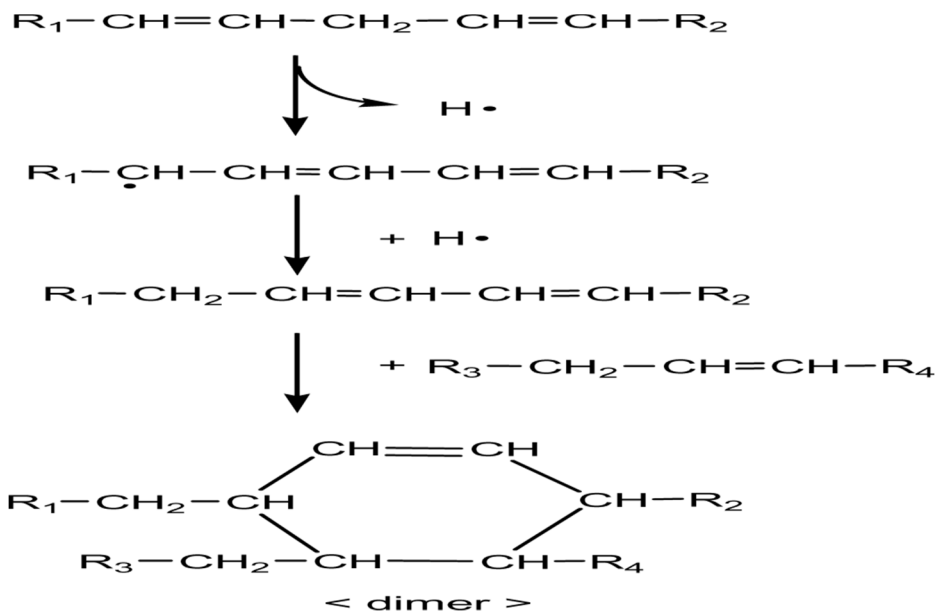


Figure 4: Cyclic compound formation from linoleic acid by Diels-Alder reaction during deep-fat frying

1.6.1b Changes in food

Due to oxidation in oil, many changes in food happen mainly changes in flavor and nutritional alue. Tocopherols, essential amino acids, and fatty acids in foods are degraded during deep-fat frying**.** Overfried foods at higher temperature and longer frying time than the optimum frying have darkened and hardened surfaces and a greasy texture due to the excessive oil absorption. (Choe & Min, 2007).

The volatile compounds resulting from thermoxidation evaporate in the atmosphere with steam and the remaining volatile compounds in oil undergo further chemical reactions or are absorbed in fried foods. The nonvolatile compounds in the oil change the physical and chemical properties of oil and fried foods, affecting flavor stability, quality and texture of fried foods during storage (Choe & Min, 2007).

Polymers formed in deep-fat frying are rich in oxygen and accelerate further degradation of the oil, increase the oil viscosity (Tseng and others 1996), reduce the heat transfer, produce foam during deep-fat frying, and develop undesirable color in the food. Polymers also cause the high oil absorption to foods. Figure 5 summarizes the physical and chemical changes that occur in deep-fat frying.

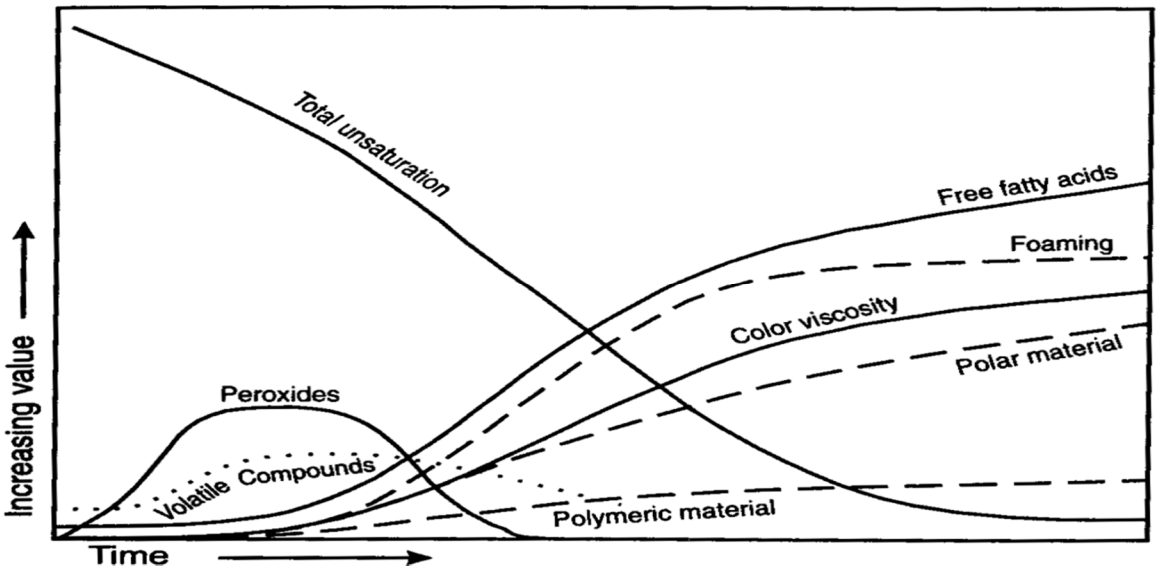


Figure 5: Physical and chemical changes in deep-fat frying (Choe et al., 2007)

The type of fried food affects the resulting composition of the frying oil as fatty acids are released from fat-containing foods, such as chicken, and their concentration in the frying

oil increases with continued use. Food particles accumulating in the oil also increase the deterioration rate of the frying oil, therefore filtering the oil through adsorbents helps removing these particles and oxidation products to enhance oil stability.

1.6.2 Effect of temperature on lipid oxidation

The increase of temperature causes a very strong reduction of the induction period and enhances the oxidation. The effect of temperature is complicated by a reduction in oxygen solubility in liquids at increased temperature, and by changing in partitioning of antioxidants between phases if more than one phase is present. For a sunflower oil-in-water emulsion from which tocopherols had been removed, the time to peroxide value of 50 meq/kg decreased from 8 days at 30°C to 3 days at 50 °C (Gordon, 2004). Applying higher, frying temperatures accelerates thermal oxidation and polymerization of oils. The conjugated dienes and trans-fatty acid content was increased when temperature of treatment increased from 170°C to 190°C (Tyagi, 1996). High frying temperature decreased polymers with peroxide linkage and increased polymers with ether linkage or carbon to carbon linkage (Kim et al., 1999). The rate constants of the degradation reactions in deep-fat frying between (155°C-190°C) was increased slightly with temperature, following an Arrhenius-type equation. The increase of oxidised triglycerides was greater at higher temperature (Houhoula et al., 2003). The intermittent heating and cooling of oils causes higher deterioration than continuous heating due to increase of oxygen solubility when the oil is cooled down (Clark, 1991). The effect of time and temperature on the accumulation of polar compounds in cottonseed oil during deep-fat frying was studied by Houhoula et al. (2003). The polar content increased by increasing temperature and as frying time. (section 1.6.1). Also, in a kinetic study of oil deterioration done by Houhoula et al. (2002), the thermoxidative alterations induced by heating the oil were measured and compared with those observed in frying at the same temperature. The major difference observed between frying and heating was related to the *p*-AV increase, which presented a considerably higher rate during heating.

1.6.3 Other factors affecting lipid oxidation in thermal oxidation

Beside temperature, there are other factors that affect lipid oxidation during thermal oxidation such as: exposure to air, light, trace metals (Naz et al., 2005) and moisture which enhance their chemical reactivity.

The thermal oxidation in deep-fat frying depends on factors such as replenishment of fresh oil, frying conditions, original quality of frying oil, food materials, type of fryer, antioxidants, oxygen concentration, high frying temperatures for prolonged hours, the contents of free fatty acids, polyvalent metals, and unsaturated fatty acids of oil. The factors that affect the extent of oil deterioration in frying are indicated in Table 3. In a study by Melton et al. (1994), the increase of polar compounds was correlated with the numbers of frying or days of frying in successive frying experiments. On the other hand, conjugated compounds were reported to increase initially and then reach a plateau.

Table 3: Factors that affect extent of oil deterioration in frying process (Warner, 2002)

Oil/Additives	**parameter/factor**
Unsaturation of fatty acid	Oil temperature
Type of oil	Frying time
Type of food	Aeration/oxygen absorption
Metals in oil /food	Frying equipment
Initial oil quality	Continuous or intermittent heating or frying
Degradation products in oil	Frying rate
Antioxidants	Heat transfer
Antifoam additives	Turnover rate; addition of makeup oil
	Filtering of oil /fryer cleaning

In order to inhibit the frying oil deterioration, it is important to choose fresh oil with good initial quality, no prior oxidation, low levels of polyunsaturated fatty acids, and low amounts of catalyzing metals. Addition of antioxidants or antifoaming agents may also improve the frying oil quality.

1.6.4 Baking is considered one of the major applications of food processing. The products range from bread, layered doughs to cakes and biscuit (cookies). Many studies are concerned about the physical and nutritional value of baked foods, due to its economic importance, since these products are widely used and are often stored for extended periods before consumption (Reddy, 2005).

Fats used to make doughs are almost entirely plastic fats, i.e., mixtures of solid and liquid components that appear solid at certain temperatures, and deform when pressure is applied. Fats exert their influence with flour and sugar, which are major components of a baked product. Baking fats may include butter or margarine, which are more than 80% fat and also contain aqueous phase, or they may be shortenings with 100% fat, which give pastry the crispness and flakiness. The prime purpose of fat in cake is to assist in aeration, while in short pastry it is of secondary importance (Gunstone, 2002). Baking is controlled by varying the temperatures and regulating time of baking in the individual zones within the oven. Fat-type affects the consistency of dough during processing, acts as a lubricant and contributes to the plasticity of the cookie dough (Maache-Rezzoug et al. 1998), prevents excessive development of the gluten proteins during mixing and imparts desirable taste (Jacob, 2007).

During storage fat is susceptible to oxidation leading to rancidity in baked goods affecting the texture, color, and sensory parameters of baked products (Bajaj, 2006). After making cookies it is important to use a suitable packaging system which reduces the amount of oxygen transmitted to the cookies, as it affects accelerating rate of oxidation of cookies during storage. In a study by Lu & Xu (2010), it was found that high concentration of initial oxygen has a significant impact on the fat oxidation in packaged cookies, where vacuum packaging was the most efficient in delaying oxidation by reducing peroxide values.

Lipid oxidation in thermal treatment of fat-rich model systems food such as cookies affects also the level of the acrylamide formation which is considered a carcinogenic (IARC, 1994), and this effect is more evident in sugar-free system where lipid become the main sources of carbonyls (Capuano et al., 2010).

1.7 Effect of Pressure on Lipid Oxidation

There is currently a great deal of interest in the application of ultra high pressure in food processing. Most review articles that point out the potential of ultra high pressure treatment, as a novel food preservation and processing technique, state that ultra high pressure-treated foods keep their original flavor, color and freshness. However, although detailed, systematic data on the effects of ultra high pressure on microbial and enzymatic inactivation are reported in the literature, the effect on lipid oxidation is less documented. The main studies concern lipid oxidation of meat and revealed that high-pressure treatment may induce lipid oxidation in meat depending on processing time and especially on the pressure level applied (Cava et al., 2009; Dissing et al.,1997; Cheah, 1997; Mariutti et al., 2008). It has been also mentioned by Ma et al. (2007) that high pressure treatment combined with thermal treatment increases the level of lipid oxidation in chicken and beef muscle. Indrawati (2000) found, by studying ultra high pressure induced lipid oxidation in seed oils and olive oil, that the peroxide value of the olive oil was not markedly changed under pressure treatment; on the other hand, sunflower and grape-stone oil, showed an evident increase in peroxide value and *p*-anisidine value, since they have the highest level of unsaturated fatty acids, which affect lipid oxidation and make them less resistant against oxidation.

1.8 Oxidation in lipid-based emulsions

Lipid–based emulsions may exist naturally as in milk, or manufacturally as in mayonnaise, salad dressings, cream, ice-cream, butter and margarines. Powdered coffee whiteners, sauces and many desserts are examples of foods that were emulsions at a certain stage during their production but subsequently were converted into another form. The bulk physiochemical properties of food emulsions, such as appearance, texture, and stability depend ultimately on the components of the food and their interactions with each other. The main ingredients of food emulsions are water, lipids, proteins, carbohydrates, minerals, sugars, and small-molecule surfactants (McClements, 2002). An emulsion is a dispersion of oil droplets in water (oil-in-water, O/W) or dispersion of water droplets in oil (water-in-oil, W/O) as shown in Figure 5.

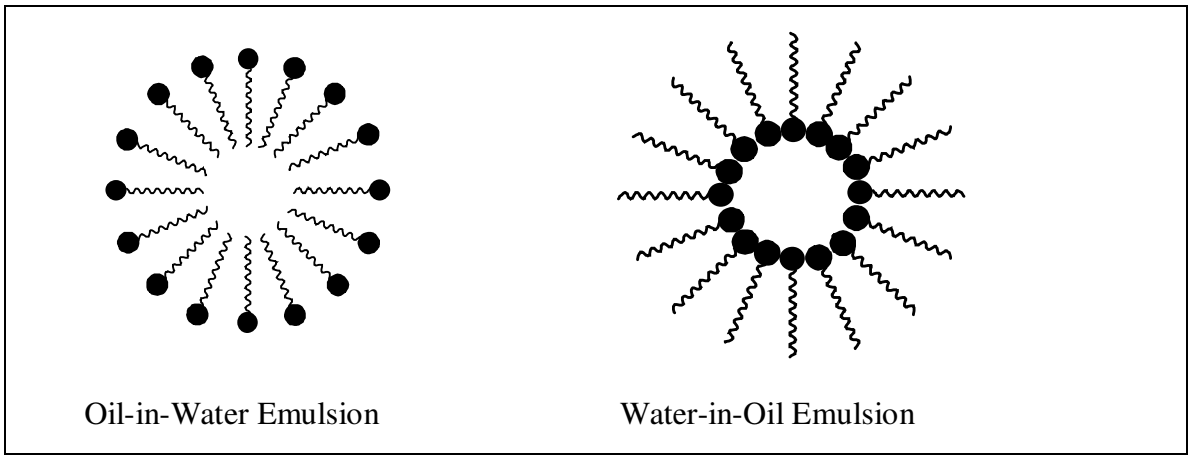


Figure 5: Interfacial interactions in two main emulsion systems of oil and water

An emulsion is formed by dispersion of one liquid layer in another immiscible layer (primary homogenization), that can be followed by another step to reduce the size of the droplet (secondary homogenization). To form a stable emulsion, the droplets must be prevented from merging after they have been formed. This is achieved by adding sufficiently high concentration of surface-active substance, known as an emulsifier. During the homogenization process, the emulsifier rapidly adsorbs to the droplet surfaces forming a protective membrane that prevents the droplets from getting close together. Emulsifiers have the ability to decrease the interfacial tension between oil and water phases and thus reduce the energy required to deform and disrupt a droplet. The most important types of lipid-based emulsifiers used in the food industry are small-molecule surfactants (e.g. Tweens, Spans, and salts of fatty acids) and phospholipids (e.g. lecithin). Proteins are also used as emulsifiers as the use of low molecular weight emulsifiers (e.g. Tween) is not extensively applicable industrially. Certain proteins can form positively charged emulsion droplets that are less susceptible to metal-promoted oxidation (Hu et al., 2003). All lipid-based emulsifiers are amphiphilic molecules that have a hydrophilic "head" with a high affinity for water and lipophilic "tail" with high affinity for oil (Figure 5), (McClements, 2002).

The oil can undergo oxidation and affect emulsion stability leading to deterioration and loss in nutritional value. Lipid oxidation is a major cause of quality deterioration in food emulsions (Let et al., 2003). The oxidative stability of emulsions is affected by processing and storage parameters, such as temperature, emulsifier type, metal chelation

(Haar, 2008), pH (Diamakou et al., 2007), and oil freshness (Let et al., 2003), while a change in homogenization pressure leading to variation of emulsion average droplet size had no effect on emulsions' stability according to Diamakou et al. (2007). The oxidative stability of emulsion with Tween was the least among other emulsifiers such as lecithin and caseinate. It was found that oxidation generally progressed faster at lower pH, and the addition of EDTA, as a metal chelator, generally reduced oxidation. The different effects of the emulsifier types could be related to their ability to chelate iron and scavenge free radicals, interfere with interactions between the lipid hydroperoxides and iron as well as to form a physical barrier around the oil droplets (Haahr, 2008).

1.9 Methods to Estimate Lipid Oxidation in Foods

Different methods have been developed to characterize the extent of lipid oxidation in foods, and to determine whether or not a particular lipid is susceptible to oxidation (Man, 2000). Many studies imply one or more of the methods to compare oil stability under several conditions. After the sample is oxidized under standard conditions, the extent of oxidation is measured by chemical, instrumental, or sensory methods (Antolovich et al., 2002**).** Chromatographic techniques are also used for the analysis and characterization of specific oxidation products. They are powerful methods for monitoring lipid oxidation because they provide a detailed profile of the fatty acids and other molecules present in lipids. Sensory evaluation is also used and based on flavor and odor evaluation. It provides most useful information related to consumer acceptance. The sensory analysis is highly sensitive but it depends on training and test panel (Frankel, 1993).

1.9.1 Accelerated Oxidation Tests are used as a technique for rapid determination of oil and oil-based foods' oxidation estimating shelf life and susceptibility of the food to oxidation. Normally, oxidation can take a long time to occur, *e.g.,* a few days to few months, which is impractical for routine analysis. A number of accelerated oxidation tests have been developed to speed up this process. These methods artificially increase the rate of lipid oxidation by exposing the lipid to heat, oxygen, metal catalysts, light or enzymes. These accelerated tests are specific to the analysis of oxidation in foods with

results usually expressed as an induction time, which is the time required for the sample to start oxidation (Man, 2000; Antolovich, 2002).

1.9.1.1 Active oxygen method (AOM)

A liquid sample is held at 80-100°C while air is constantly bubbled through it. Stability is expressed as hours of heating until rancidity occurs, which may be determined by detection of a rancid odor or by measuring the peroxide value (Frankel 1993; AOCS Cd12-57).

1.9.1.2 Oven test (schaal test)

This method is based on measuring the oxidation of a known weight of oil placed in an oven at a specified temperature (60-70 °C) for a period of days until rancidity starts. Oxidation is determined by measuring parameters such as conductivity, peroxide value or diene conjugation and sensory analysis (AOCS recommended practice Cg 5-97). The usual substrates used in this method include lard, edible oils or a model substrate such as methyl linoleate (Antolovich, 2002). The samples are examined at regular intervals and the peroxide value or conjugated diene increase is plotted against time. The end of induction period is determined, when sudden increase in the slope of the plot appears.

1.9.1.3 Oil stability index (OSI) (Rancimat)

The oil stability index (OSI) method, also commonly known as the Rancimat method, allows oxidative stability to be determined automatically under standardized conditions (AOCS, Cd 12b-92). The end point corresponding to a sudden rise of volatile acids generated from the oil samples heated at high temperature under constant aeration is detected. These compounds are trapped in water and monitored by electro-conductivity. The oxidative stability of lard and tallow was examined by four accelerated stability tests, and it was found that Rancimat may be the least reliable method compared to other methods of accelerated stability. Thus, it was recommended that more than one accelerated stability test should be used to determine antioxidant effectiveness (Liang, 1998).

1.9.1.4 OXITEST is an innovative method applied instrumentally offering a quick and precise estimation for the oxidative stability of foods and oils in the presence of an oxidative environment at high temperature (up to 95°C) and pressure (0 - 8 bar). Oxidation process is speeded up and the growing rancidity is measured automatically. The instrument has two separate oxidation chambers for performing two analyses on the same food (duplicates) or for analysing different samples in the same operating conditions simultaneously. Dedicated software lets the operator follow and record the oxidative process inside the two reaction chambers and calculate the induction period. The information are obtainable from instrument response regarding the induction period of the autoxidation process, and the rate of acceleration of the autoxidation. The amount of oxygen consumed by the food product during oxidation can be obtained as well. Unlike traditional systems, OXITEST allows making the analysis directly on the complete food without having previously separated the fats it contains, saving a considerable amount of time. OXITEST instrument showed to have high accuracy and the results of oxidation test were highly correlated to the results of the same tests done manually. In a comparative study done by Comadini et al., 2009, the OXITEST was employed to determine the induction periods of vegetable oils; the results were highly correlated with those achieved with OSI technology, ($r = 0.9785$ ($p < 0.05$) for oilseeds and palm oil and $r = 0.9501$ ($p < 0.05$) for extra-virgin olive oils).

1.9.2 Determination of primary oxidation products

1.9.2.1 Determination of hydroperoxides

A. Peroxide Value is among the most commonly used methods for measuring the increase in concentration of hydroperoxides (ROOH), that are formed in the initial stages of oxidation, thus giving an indication of the progress of lipid oxidation in early stages. One of the most commonly used methods to determine peroxide value utilizes the ability of peroxides to liberate iodine from potassium iodide. The lipid is dissolved in a suitable organic solvent and an excess of potassium iodide KI is added:

$$ROOH + KI(excess) \rightarrow ROH + KOH + I_2$$

Once the reaction has gone to completion, the amount of hydroperoxide (ROOH) that has reacted can be determined by measuring the amount of iodine formed. This is done by titration with sodium thiosulfate ($Na_2S_2O_3$) and a starch indicator:

$$I_2 + \text{starch} + 2Na_2S_2O_3 \text{ (blue)} \rightarrow 2NaI + \text{starch} + Na_2S_4O_6 \text{ (colorless)}$$

The amount of sodium thiosulfate required to titrate the reaction is related to the concentration of peroxides in the original sample expressed in meq active oxygen/ kg oil. The method has been standardized by IUPAC (standard method 2.501) and AOCS (Cd 8-53).

B. Other methods

As far as primary oxidation compounds are concerned, it has been shown that ^1H Nuclear Magnetic Resonance (^1H NMR) can also be used to evaluate the presence of hydrogen atoms of hydroperoxy groups in edible oils (Guillén, 2007).) High performance liquid chromatography (HPLC) is also used to measure the concentrations of hydroperoxides and used in studies of mechanism or product arising from oxidation of lipids (Rossell, 1994; Song et al., 1992).

1.9.2.2 Conjugated dienes and trienes

Almost immediately after peroxides are formed, the non-conjugated double bonds (C=C-C-C=C) that are present in natural unsaturated lipids are converted to conjugated double bonds (C=C-C=C), resulting from stabilization of the radical state by double bond rearrangement. The conjugated diene structures absorb at a wavelength of 232–234 nm, and this absorption provides the conjugated diene value (CD), whereas conjugated trienes absorb at 268nm. Thus oxidation can be followed by dissolving the lipid in a suitable organic solvent and measuring the change in its absorbance with time using a UV-Visible spectrophotometer. In the later stages of lipid oxidation the conjugated dienes are broken down into secondary products (which do not adsorb at UV-visible light strongly) that leads to a decrease in the absorbance. This method is therefore useful for monitoring the early stages of lipid oxidation, and measures the increase of concentration of primary reaction products. However, it is efficiently used to estimate oxidation in deep fat frying (Houhoula et al, 2004). The method has been standardized by IUPAC (standard method

2.505) and AOCS (Ti 1a-64). Both CD and PV are considered as classical indexes of primary oxidation products (White, 1995; Farhoosh, 2009).

1.9.3 Determination of secondary oxidation products

As mentioned above, primary oxidation compounds in turn degrade to give so-called secondary oxidation compounds that are detected by different methods.

1.9.3.1 *p*-Anisidine Value measurement is based on the reaction of aldehydes, especially 2-alkenals, with *p*-anisidine, where the reaction products are measured spectrophotometrically at 350 nm. The carbonyl compounds including pentanal, deca-2,4-dienal and octa-3,5-dien-2-one are suggested to be the major contributors to off-flavors associated with the rancidity of many food products (Antolovich, 2002; AOCS Official Method Cd,18–90).

1.9.3.2 Carbonyl value (CV) is an estimation of total carbonyl compounds in edible fats and oils known as shown in a developed method for measuring (CV) by Endo et al. 2001. Carbonyl compounds are more stable than hydroperoxides and their measurement is a good index of oxidative changes in lipids (Farhoosh, 2008).

1.9.3.3 Thiobarbituric acid (TBA) test measures the concentration of relatively polar secondary reaction products, mainly malonaldehyde (MDA). The MDA reacts with thiobarbituric acid (TBA) to form a pink pigment (Figure 6) measured spectrophotometrically at 532–535 nm.

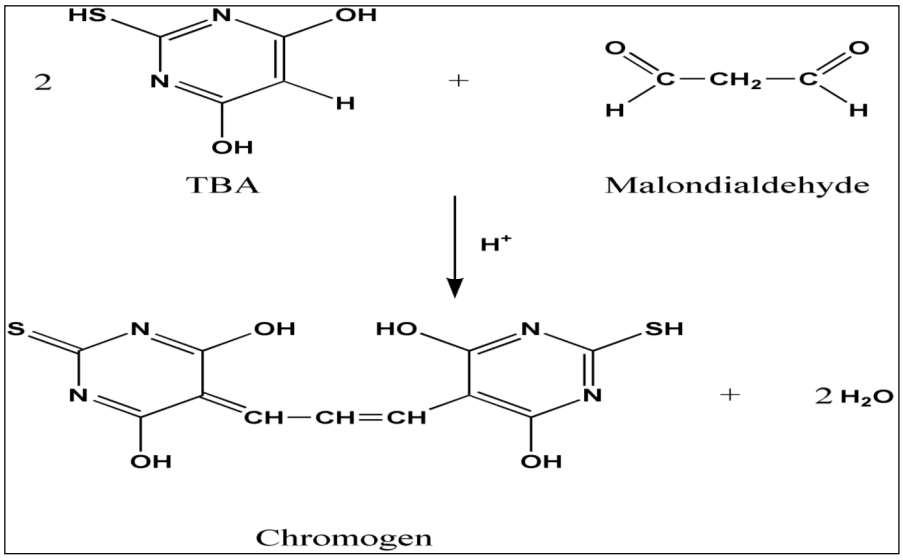


Figure 3 Chromophore formed by condensation of MDA with TBA

The method has been standardised by the official method AOCS Cd 19-90. Determination of TBARs has been applied to estimate oxidation stability of o/w emulsion (Diamakou et al., 2007; Mattia et al., 2009).

1.9.3.4 Iodine Value (IV) gives a measure of the average degree of unsaturation of a lipid, expressed as the grams of iodine absorbed per 100g of lipid, measured through iodometric titration. The higher the IV, the greater is the degree of unsaturation. The iodine value is used to follow processes such as hydrogenation and oxidation that involve changes in the degree of unsaturation (Pocklington, 1990; AOCS Cd(1-25).

1.9.3.5 Polar content

The deterioration of fried oils and fats can be monitored by measuring the polar compounds which include mono- and di-glycerides, and other products formed during heating of foods. Non-polar compounds are primarily unaltered triglycerides. The polar compounds in a sample can be separated from non polar compounds using chromatographic techniques. Polar compounds are measured by dissolving the fat sample in light petroleum ether-diethyl ether (87:13), then applying the solution to silica gel column. Non-polar compounds are eluted, the solvent evaporated, the residue weighed

and the polar compounds can be estimated by the difference. The quality of the determination can be verified by eluting polar content and separating polar and non-polar compounds using Thin Layer Chromatograpghy (TLC). The method has been standardized by the IUPAC (standard method 2.507), and AOCS (Cd 20-91). Limitation of polar content of an accepted oil should not exceed 27% (Pike, 2003).

Quantification of polymerized triacylglycerols, oxidized triacylglycerols, and diacylglycerols in polar compounds can be achieved by using high-performance size-exclusion chromatography (HPSEC), a rapid procedure that gives detailed information on the main groups of compounds in fats and oils associated with hydrolysis, oxidation, and thermal polymerization, especially in frying fats.

The level of polar compounds is a good indicator of the quality of used frying fats and oils, giving information of the total amount of newly formed compounds having higher polarity than that of triacylglycerols and being the basis of the present recommendations and regulations (Dobarganes et al., 2000).

1.9.3.6 Analysis oil oxidation by Near Infrared Spectroscopy (NIR)

The (NIR) transmittance spectroscopy was used for rapid determination of the oxidation level of vegetable oils (Yildiz et al., 2001). A number of new methods have been developed over the past few years for measuring lipid oxidation, among them the analysis by NIR, that is used in determination of free fatty acids (FFA), and level of unsaturation. The determination of oxidation and crystallinity and the identification of hydroxyl groups have also been achieved by mid-IR spectroscopy. Fourier transform-infrared (FT-IR) analysis has also been used to determine *cis* and *trans* contents and FFA contents in fats and oils and to quantify the PV. Moreover, (Guillén & Cabo, 1999) used frequency data from the FT-IR spectra to evaluate the degree of oxidation of edible oils. The (NIR) spectroscopy has also been used for the the determination of iodine value of fat and the fatty acid composition of fats and oils. NIR spectroscopy offers several advantages over conventional methods, including high speed, multiplicity of analyses from a single spectrum, nondestruction of the sample, and applicability to small amounts of sample. NIR analysis is also economical because reagents are not needed, labor requirements are low owing to minimal sample preparation, and no chemical wastes are produced. Only a

few studies, however, have used NIR techniques for measuring lipid oxidation, and those focused primarily on the spectral properties of purified hydroperoxides from methyl oleate and linoleate. Results obtained in a study by Yildiz et al. (2001) indicate that NIR spectroscopy is a useful technique for measuring oxidation in soybean oil.

References

Antolovich M., Prenzler P., Patsalides E., McDonald S. and Robards K.: Methods for testing antioxidant activity, *Critical Review. Analyst*, **127**, 183–198**,** 2002.

AOCS recommended practice Cg 5-97, Oven storage test for accelerated aging of oils. *Official methods and recommended practices of the AOCS,* 5th edn, 2nd printing; 2006.

AOCS Official method Cd 8-53, Peroxide Value, Acetic acid-chloroform method in *Official methods and recommended practices of the AOCS,* 4th edn, D. Firestone, AOCS press, IL, USA, 1994.

AOCS Official Method Cd, 18–90, *p*-Anisidin value, In: *Official methods and recommended practices of the american oil chemists' society*, 5th edn. AOCS Press, IL, USA, 1998.

AOCS official method Ti, 1a–64, Conjugated dienes In: *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 5th edn. AOCS Press, IL, USA, 1998.

AOCS official method Cd 19-90, Thiobarbituric Acid Value Direct Method (modified), in *Official Methods and Recommended Practices of the American Oil chemist's society*, 5th edn., AOCS press, Champaign, IL, USA, 1997.

AOCS Cd 20-91. Official methods and recommended practices of the American Oil Chemists' Society, Champaign, Il, USA: American Oil Chemists' Society ed. Firestone D., 1991.

AOCS Cd 1-25 Iodine value of fatty acids and oils *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 4th edn., Champaign, 1 Il, USA, 1993.
AOCS Cd 12–57 *Official Methods and Recommended Practices of the American Oil Chemists' Society,* edited by David Firestone, Vol. 1. American Oil Chemists' Society, Champaign, Il, USA, 1980.

Bajaj S., Urooj A.: effect of incorporation of mint on texture, color and sensory parameters of biscuits. *Intern. J. Food Prop.*, **9**, 691-700, 2006.

Brimberg U., Kamal-Eldinb A.: On the kinetics of the autoxidation of fats: influence of pro-oxidants, antioxidants and synergists , *Eur. J. Lipid Sci. Technol.*105, 83–91, 2003.

Cava R., Ladero L., González S., Carrasco S.,M. Ramírez: Effect of pressure and holding time on colour, protein and lipid oxidation of sliced dry-cured Iberian ham and loin during refrigerated storage. *Innov. Food Sci. and Emerg. Technol.*, **10** , 76–81, 2009.

Capuano E., Oliviero T., Açar O., Gökmen V. & Fogliano V.: Lipid oxidation promotes acrylamide formation in fat-rich model systems, *Food Res. Intern.*, **43**, 4, 1021-1026, 2010.

Choe E., Min D.: Chemistry of deep fat frying oils, *J. food sci.*, Review, **72**, 5, 77-78, 2007.

Clark W., Serbia G.: Safety aspects of frying fats and oils. *Food Technol.*, **45,** 84-89, 1991.

Comandini P, Verarso V., Maiocchi P., Caboni M.: Accelerated oxidation: Comparative study of a new reactor with oxidation stability instrument. *Eur. J. of Lipid Sci. Technol.*, **111**, 9, 933-940, 2009.

Cheah P. & Ledward D.: Catalytic Mechanism of Lipid Oxidation following High Pressure Treatment in Pork Fat and Meat, *J. Food Sci.,* **62**, 6, 1135-1138**,** 1997.
Dissing J., Bruun-Jensen L., Skibsted L.: Effect of high-pressure treatment on lipid oxidation in turkey thigh muscle during chill storage, Z Lebensm Unters Forsch A 205, 11–13, 1997.

De Leonardis A., Macciola V.: Catalytic effect of the Cu(II)- and Fe(III) cyclohexanebutyrates on olive oil oxidation measured by Rancimat *Eur. J. Lipid Sci. Technol.,* **104**,156–160, 2002.

Diamakou C., Kiokias S.,Tsaprouni I. and Oreopoulou V.: Effect of processing and storage parameters on the oxidative Deterioration of Oil-in-Water Emulsions. *Food Biophysics*, **2,** 38-45, 2007.

Dobarganes C, Marquez-Ruiz G, Velasco J. :Interactions between fat and food during deep-frying. *Eur. J. Lipid Sci. Techno.l*, **102,**521–528, 2000.

Endo, Y., Li, C., Tagiri-Endo, M., & Fugimoto, K. A modified method for the estimation of total carbonyl compounds in heated and frying oils using 2-propanol as a solvent. *Journal of the American Oil Chemists' Society*, **10**, 1021–1024, 2001.

Frankel, E. & Neff W.:Formation of malonaldehyde from lipids oxidation *, Biochimica et Biophysica Acta,***754**, 264-270, 1983.

Farhoosh R.,Pazhouhanmehr S. :Relative contribution of compositional parameters to the primary and secondary oxidation of canola oil. *Food Chem.* **114**, 1002–1006, 2009.

Gertz, C., Klostermann, S., & Kochhar, S.: Testing andcomparing oxidative stability of vegetable oil and fats at frying temperature. *Eur. J. Lipid Sci. Technol*.,**102,**(8–9), 543–551., 2000.

Enig M. (2007): Role of medium-chain triglycerides in weight management in Obesity: epidemiology, pathophysiology, and prevention, Bagchi D., Preuss H., CRC, FL. U.S.A.

Frankel E.: In search of better methods to evaluate natural antioxidants and oxidative stability. *Trends Food Sci. Technol.*, **4**:220-225, 1993.

Frankel E. (1985): Chemistry of autoxidation, in *Flavor Chemistry of Fats and oil*, Min D., Smouse T., American Chemical Society, p7-9.

Galdovic N., Zupancic- kralj L., Plavec, J.: Determination of primary oxidation products of linoleic acid and triglycerols. *J. of chromatogr. A*, **767**, 63-68, 1997

Gordon M. (2004): Factors affecting lipid oxidation in *Understanding & measuring the shelf-life of food,* Steele R., CRC FL, USA.

Guillen M., Goicoechea E. Oxidation of corn oil at room temperature: Primary and secondary oxidation products and determination of their concentration in the oil liquid matrix from ^{1}H nuclear magnetic resonance data, *Food Chem.*, **116**, 183–192, 2009

Guillén, M & Goicoechea, E.: Detection of primary and secondary oxidation products by Fourier transforms infrared spectroscopy (FTIR) and ^{1}H nuclear magnetic resonance (^{1}H NMR) in sunflower oil during the storage. *J. Agric. Food Chem.,* **55**(26), 10729–10736, 2007.

Gunstone F., Harwood J., Padley F. (1994): Occurance and characteristics of oils and fat in *The Lipid Handbook*, 2nd edn., Chapman & Hall, electronic publishing division, p 47-167.

Gunstone F. (2002): Food Application of Lipids in *Food Lipids*, Akoh C., Min D., Marcel Dekker Inc., NY, U.S.A., p729-748.

Gunstone F.(1996a): Fatty Acids-Nomenclature, structure, isolation and structure determination, biosynthesis and chemical synthesis in the *Fatty Acid and Lipid chemistry,* Blakie Academic and Professional, London, UK., p l-15.

Gunstone F. (1996b): The major sources of oils, fats and other lipids in the *Fatty Acidand Lipid chemistry,* Blakie Academic and Professional, London, UK., 1996: p 61-86

Gunstone F. (1996c): Reactions associated with double bonds in the *Fatty Acid and Lipid chemistry,* Blakie Academic and Professional, London, UK. 1996: pl62-164.

Hämäläinena T., Sundberg S., M. Marjukka, Kaltiaa S., Hasea T., Hopiab A.: Hydroperoxide formation during autoxidation of conjugated linoleic acid methyl ester *Eur. J. Lipid Sci. Technol.,* **103**, 588–593, 2001.

Haahr M, Jacobsen C.: Emulsifier type, metal chelation and pH affect oxidative stability of *n*-3-enriched emulsions, *Eur. J. Lipid Sci. Technol.*, **110***,* 949–961, 2008

Houhoula D., Oreopoulou V.& Tzia C.: A Kinetic Study of Oil Deterioration During Frying and a Comparison with Heating, *JAOCS*, **79**, 2, 2002.

Houhoula D., Oreopoulou V.: The effect of process time and temperature on the accumulation of polar compounds in cottonseed oil during deep-fat frying*, J. Sci. Food Agric.,* 83, 314-319, 2003.

Houhoula D., Oreopoulou V. and C. Tzia: Antioxidant efficiency of oregano in frying and storage of fried products. *Eur. J. Lipid Sci. Technol.*, **106,** 746–751, 2004.

Hu, M., McClements J., Decker E: Lipid oxidation in corn oil-in-water emulsions stabilized by casein, whey proteins and soy protein isolate. *J. Agric. Food Chem.*, **51**, 6, 1696-1700, 2003.

Indrawati A., Loey V., Smout C., Hendrick M. (2000): High Hydrostatic Pressure Technology in *Food preservation*, Zeuthen P., Bagh-sorensen L., Woodhead publishing in food science, Cambridge, England, p428-421.

IUPAC standard method 2.507 Standard Methods for the Analysis of Oils, Fats and Derivatives, 7th edn., Eds. *C. Paquot*, *A. Hautfenne*, Blackwell Scientific Publications, Oxford, UK, 1987.

IUPAC standard method 2.501. Determination of the peroxide value (P.V.) in IUPAC *Standard Methods for the Analysis of Oils Fats and Derivatives*, 7th edn. Eds. Paquot C., Hautfenne A., Blackwell Scientific Publications, Oxford, UK, 1987.

IUPAC standard method 2.505.Evidence of purity and deterioration from ultraviolet spectrophotometry in *IUPAC Standard Methods for the Analysis of Oils Fats and Derivatives*, 7th edn. Eds. Paquot C., Hautfenne A., Blackwell Scientific Publications.

IARC (International Agency for Research on Cancer): Some industrial chemicals. IARC *Monogr Eval Carcinog Risks Hum* **60**: 389–433, 1994.

Jacob J., Leelavathi K.: Effect of fat-type on cookie dough and cookie quality Journal of Food Engineering **79**, 299–305, 2007.

Kim I-H, Kim C-J, Kim D-J: Physiochemical properties of methyl linoleate oxidized at various temperatures, *Korean J. Food sci. Technol.,* **31**,600-605, 1999.

Let M., Jacobsena C., Frankel E., Meyer A.: Oxidative flavour deterioration of fish oil enriched milk**,** *Eur. J. Lipid Sci. Technol.* **105,** 518–52, 2003.

Love J. (1985): Factors affecting lipid oxidation-metal catalysts and chelators in *Flavor Chemistry of Fats and oil*, Min D., Smouse T., American Chemical Society, p 61-78.

Li-Xin L., Xu F.: Effects of initial oxygen concentration and oxygen-barrier property of film on fat oxidation rate of packed cookies. *Eur Food Res Techno,l* **231**,347–351, 2010.

Ma H., Ledward D., Zamri A, Frazier R., Zhou G.: Effects of high pressure/thermal treatment on lipid oxidation in beef and chicken muscle Food Chemistry **104**, 1575–1579, 2007.

Man D., Jones A.: *Shelf-life Evaluation of foods*, 2nd edn., ASPEN publishers inc. Gaithersburg, Maryland, p 3-6, 2000.

Maache-Rezzoug, Z., Bouvier, J., Allaf, K., & Patras, C.: Effect of principal ingredients on rheological behaviour of biscuit dough and on quality of biscuits. *Journal of Food Engineering,* 35, 23–42. 1998.

Mariutti L., Orlien V., Bragagnolo N., Skibsted L.: Effect of sage and garlic on lipid oxidation in high-pressure processed chicken meat, *Eur Food Res Technol*, **227**,337–344, 2008.

Melton, S., Jafar S., Sykes D., and. Trigiano M.: Review of Stability Measurements for Frying Oils and Frying Food Flavor, *Ibid.* **71**:1301–1308, 1994.

Mattia C., Sacchetti G., Mastrocola D., Pittia P.: Effect of phenolic antioxidants on the dispersion state and chemical stability of olive oil O/W emulsions, *Food Res. International*, **42**, 1163–1170, 2009.

Marquez-Ruiz G. & Dobarganes M. (1997): Analysis of Lipid Oxidation products by Combination of Chromatographic Techniques in *New techniques and applications in Lipid Analysis*, Mcdonald R. & Mossoba M., AOCS press, p 216-231.

Min D., Boff J. (2002): Lipid Oxidation of Edible Oils in *Food Lipids*, Akoh C., Min D., Marcel Dekker, Inc., N Y, U.S.A., p335-362.

McClements D. (2002): Lipid-based Emulsions and emulsifiers, in *Food Lipids*, Akoh C., Min D., Inc., N.Y., U.S.A., p63-85.

Nawar W. (1985): Chemistry of thermal oxidation of Lipids, in *Flavor Chemistry of Fats and oil*, Min D., Smouse T., American Chemical Society, p39-60.

Nawar W. (1996): Lipids in Food Chemistry, ed. Fennema O., Marcel. Dekker Inc., NY, USA.

Naz S., Siddiqi R., Sheikh H. and Sayeed S.: Deterioration of olive, corn and soybean oils due to air, light, heat and deep-frying, *Food Res. Inter.*, 38, 2, 127-134, 2005.

O'brien R. (2003): Raw materials, in *fats and oils, formulating and processing for Application,* CRC press, p1-50.

O'keefe S.(2002): Nomenclature and Classification of Lipids in *Food Lipids*, Akoh C., Min D., Marcel Dekker, Inc., NY, U.S.A., p1-40.

Pike O. (2003): Fat characterization in *Food Analysis*, 3rd edn., Neilson S., Plenum Publishers, N.Y.,U.S.A.

Pocklington W: Determination of the Iodine value of oils and fats, results of a collaborative study, *Pure and Appl. Chem.*, **62,** 12, 2339-2343, 1990.

Rossell B. (1994): Measurement of rancidity in *Rancidity in Foods*, 3rd edn., J. Allen & R. Hamilton, AN ASPEN Publication, N.Y., U.S.A., p34-62.

Reddy V., Urooj A., Kumar A.: Evaluation of antioxidant activity of some plant extracts and their application in biscuits. *Food Chem.,* **90**, 317–321, 2005.

Song J., Chang C., Park D.: Specific determination of lipid hydroperoxide in vegetable oils by a HPLC-EC Assay. *J. Korean Biochem.*, **25**, 4, 337-341, 1992.

Tyagi V., Vasishtha A.: Changes in the characteristics and composition of oils during deep-fat frying. *J. Am. Oil Chem. Soc.,* **73**, 499-506, 1996.

Shahidi F. (1997): Natural antioxidants: an overview in *Natural antioxidants: chemistry, Health effects and applications.* AOCS press, USA p1-2.

Warner C. (2002): Chemistry of Frying Oils in *Food Lipids*, Akoh C., Min D. Marcel Dekker, Inc. NY, U.S.A., p205-220.

White, P. (1995): Conjugated diene, anisidine value and carbonyl value analysis. In *Methods to assess quality and stability of oils and fat-containing foods*, Warner K. &. Eskin N., Champaign: *AOCS Press*. 158–178.

Chapter Two

Food Antioxidants

2.1 Introduction

Over the past decades, research dealing with antioxidants has been intensified covering a wide variety of multidisciplinary fields that affect food, nutrition and health/well-being. Antioxidants can be prepared synthetically or it can be extracted from natural sources to be used as natural antioxidants. Both synthetic and natural antioxidants are widely used in many food products. They have become an indispensible group of food additives, due to their capability to increase shelf life of the food without damaging sensory or nutritional analysis (Madhavi et al., 1996).

The use of antioxidants dates back to 1940s. Gum guaiac was the first antioxidant approved for the stabilization of animal fats, specially lard. Antioxidants function mainly by disrupting the free radical chain reaction or by decomposing the lipid peroxides that are formed into stable end products. In general, antioxidants are very effective at very low levels, 0.01% or less. At higher levels most of them behave as pro-oxidant because of their involvement in the initiation reactions. Numerous chemical and instrumental methods have been developed for the determination of various antioxidants and their activity in terms of oxidative stability of model or food systems. However, in recent years sensory quality is involved in judging the quality of food under storage. Assessment of antioxidants in different methods and the combination of all approaches explains the large variety of ways in which results of antioxidant testing are reported. The measurement of antioxidant activities, especially of antioxidants that are mixtures, multifunctional or are acting in complex multiphase systems, cannot be evaluated satisfactorily by a simple antioxidant test without due regard to the many variables influencing the results (Antolovich et al., 2002).

Approval of an antioxidant for using in food requires extensive toxicological studies, including mutagenic and carcinogenic, so as to be accepted up to a certain limit or "generally recognized as safe" (GRAS), because some antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have proven to produce tumors in animals at high doses. The effective use of antioxidants requires a basic understanding of the chemistry of lipid oxidation, the mechanism of action of antioxidants and other properties such as synergism and degradation (Madhavi et al., 1996). Antioxidants comprise both substances used to preventing fats in foods from becoming rancid as well as dietary antioxidants. Recently, the impact of antioxidants on

health systems of human body has become an issue of interest to researchers and the public worldwide. If the amount of free radicals and oxidative stress exceeds the capacity of body's antioxidant defense system, oxidative damage may result. The oxidation of lipids, carbohydrates, proteins and nucleic acids in the body has been linked with cardiovascular and neurological diseases, chronic inflammations and cancer (Halliwell, 2007). Antioxidants can delay or prevent oxidative events and do not initiate further oxidative processes during their action (Galati et al., 2002). During the recent years, the epidemiological evidence on the health-beneficial effects of diets rich in polyphenols (Mink, 2007), has triggered a wide scientific interest to polyphenol research.

2.2 Mechanism of Antioxidants

Antioxidants can delay or inhibit lipid oxidation at low concentration. They inhibit the oxidation of foods by scavenging free radicals, chelating pro-oxidative metals, quenching singlet oxygen and photosensitizers, and inactivating lipoxygenase. Antioxidants show interactions, such as synergism (tocopherols and ascorbic acids), antagonism (tocopherol and caffeic acid), and simple addition (Choe et al., 2009). Antioxidants can be classified into two main types: primary or chain-breaking and secondary or preventive.

2.2.1 Primary antioxidants

2.2.1.a Free radical scavenging

Primary antioxidants (AH) are capable (when present in trace amount) to react with alkyl or peroxyl radicals ($L^{\cdot}$, $LOO^{\cdot}$, $LO^{\cdot}$) before they further react with unsaturated lipid molecules They scavenge free radicals of foods by donating hydrogen and producing relatively stable antioxidant radicals (equations 1-3):

$$L^{\cdot} + AH \longrightarrow LH + A^{\cdot} \quad (1)$$

$$LOO^{\cdot} + AH \longrightarrow LOOH + A^{\cdot} \quad (2)$$

$$LO^{\cdot} + AH \longrightarrow LOH + A^{\cdot} \quad (3)$$

Examples of this type of antioxidants are phenolic compounds tocopherols, BHT, BHA, TBHQ, propyl gallate (PG), lignans, flavonoids, phenolic acids, ubiquinone carotenoids,

ascorbic acids, and amino acids (Choe et al., 2009). The direct antioxidant capacity of phenolic compounds is essentially due to the ease with which a hydrogen atom from an aromatic hydroxyl group can be donated to a free radical (Choe et al., 2006).The effectiveness of antioxidants to scavenge free radicals of foods depends on the bond dissociation energy between oxygen and a phenolic hydrogen, pH related to the acid dissociation constant, and reduction potential and delocalization of the antioxidant radicals (Choe 2006 et al.; Cao et al. 2007). Hydrogen transfer from antioxidants to the peroxy or alkyl radicals of foods is more thermodynamically favorable when the bond dissociation energy for O–H in the antioxidants is low (Cao et al., 2007). The bond dissociation energy for O–H of phenolic antioxidants is affected by surrounding solvents; it is higher in polar solvents such as acetonitrile and *tert*-butyl alcohol than nonpolar benzene (Zhang et al., 2005). Thus, polar solvents decrease the radical scavenging activity of the antioxidants due to the intermolecular hydrogen bonding between oxygen or nitrogen in a polar solvent and OH group in phenolic antioxidants (Amorati et al., 2007). The antioxidant free radical ($A^{\cdot}$) may further react with peroxy radicals to form stable peroxy-antioxidant compounds:

$$A^{\cdot} + LOO^{\cdot} \longrightarrow LOOA \quad (4)$$

$$A^{\cdot} + LO^{\cdot} \longrightarrow LOA \quad (5)$$

2.2.1b Photosensitizer inactivation

Photoactivated food sensitizers (such as chlorophylls and riboflavin) transfer the energy to triplet atmospheric oxygen to form singlet oxygen, or transfer an electron to the triplet oxygen to form a superoxide anion radical. These reactive oxygen species react with food components to produce free radicals. Carotenoids having fewer than 9 conjugated double bonds prefer the inactivation of photosensitizers instead of singlet oxygen quenching; singlet oxygen quenching is preferable by carotenoids with 9 or more conjugated double bonds (Viljanen et al., 2002). The energy of the photosensitizer is transferred to the singlet state of carotenoids to become a triplet state of carotenoids, which is changed to

the singlet state by transferring the energy to the surrounding or emitting phosphorescence (Stahl et al., 1992).

2.2.2 Secondary Antioxidants

Secondary antioxidants are compounds that retard chain initiation by various mechanisms other than the pathway followed by primary antioxidants (Jadhav, 1995). The secondary antioxidants reduce the rate of autoxidation of lipids by such processes as binding metal ions, scavenging oxygen, decomposing hydroperoxides to nonradical products, absorbing UV radiation, and deactivating singlet oxygen. They usually require the presence of another minor component for effective action. Typical examples are sequestering agents, metal ions, reducing agents (ascorbic acid), tocopherols or other phenolics (Madhavi, 1996).

2.2.2.a Metal chelating

Metals reduce the activation energy of the oxidation, especially in the initiation step, to accelerate oil oxidation (Jadhav et al**.,** 1995). Metals catalyze food radical formation by abstracting hydrogen. They also produce hydroxy radicals by catalyzing decomposition of hydrogen peroxide or hydroperoxides. Crude oil contains transition metals such as iron or copper, often existing in chelated form rather than in a free form (Decker, 2002). Oil refining decreases metal content. Edible oils manufactured without refining, such as extra virgin olive oil and roasted sesame oil contain relatively high amounts of transition metals (Choe et al., 2005). Metal chelators decrease oxidation by preventing metal redox cycling, forming insoluble metal complexes, or providing steric hindrance between metals and food components or their oxidation intermediates (Graf et al., 1990). EDTA and citric acid are the most common metal chelators in foods. Most chelators are water-soluble, but citric acid can be dissolved in oils with some limitation to chelate metals in the oil system. Phospholipids also act as metal chelators (Koidis & Boskou, 2006). Flavonoids can also bind the metal ions (Rice-Evans et al., 1996) and the activity is closely related with the structural features. Lignans, polyphenols, ascorbic acid, and amino acids such as carnosine and histidine can also chelate metals (Decker et al., 2001).

2.2.2b Singlet oxygen quenching

Singlet oxygen has high energy and reacts with lipids at a higher rate than triplet oxygen. Tocopherols, carotenoids, curcumin, phenolics, urate, and ascorbate can quench singlet oxygen (Choe et al., 2005). Singlet oxygen quenching includes both physical and chemical quenching. Physical quenching leads to deactivation of singlet oxygen to the ground state of triplet oxygen by energy transfer or charge transfer (Min, 1989). There is neither oxygen consumption nor product formation. Singlet oxygen quenching by energy transfer occurs when the energy level of a quencher (Q) is very near or below that of singlet oxygen:

$$^1O_2 + Q \rightarrow {}^3O_2 + {}^3Q \qquad (6)$$

$$^3Q \rightarrow {}^1Q \text{ (no radiation)} \qquad (7)$$

2.3 Interactions of antioxidants in foods

Interactions among antioxidants can be synergistic, antagonistic, or merely additive.

2.3.1 The additive interaction means that a net interactive antioxidant effect is the same as the sum of individual effects. Polyphenolic compounds such as epigallocatechin gallate, quercetin, epicatechin gallate, epicatechin, and cyanidin showed additive effects on free radical scavenging activity with ascorbic acid or α-tocopherol (Murakami et al., 2003).

2.3.2 Synergism is a phenomenon in which a net interactive antioxidant effect is higher than the sum of the individual effects. A typical example of antioxidant synergism is between α-tocopherol and ascorbic acid in autoxidation and photooxidation of lipids (Van Aardt et al., 2005). Several mechanisms are involved in synergism among antioxidants: a combination of two or more different free radical scavengers, in which one antioxidant is regenerated by others; a sacrificial oxidation of an antioxidant to protect another antioxidant (Decker, 2002). When there are two or more antioxidants whose antioxidant mechanisms are different, the antioxidation can also show a synergism (Decker, 2002). A combination of metal chelators and free radical scavengers is a good example. Metal

chelators mainly act during the initiation step of lipid oxidation and free radical scavengers do so at the propagation step (Choe et al., 2006).

2.3.3 Antagonism is a phenomenon in which a net interactive antioxidant effect is lower than the sum of the individual antioxidant effects. Antagonism has been observed between α-tocopherol and rosmarinic acid or caffeic acid, between catechin and caffeic acid, and between caffeic acid and quercetin. Antagonism among antioxidants in the oxidation of food components can arise by regeneration of the less effective antioxidant by the more effective antioxidant (Peyrat-Maillard et al., 2003).

2.4 Types of Antioxidants

2.4.1 Synthetic Antioxidants are widely used in food industry as preservation agents and also needed by human body as supplement antioxidants to prevent free radical damage. For many years, food-grade synthetic antioxidants are used safely to ensure food quality and nutritional value, by slowing the oxidation of fats and oils in fresh and processed foods, extending the shelf life and inhibiting rancidity. Synthetic antioxidants are used to stabilize the refined oils and fats in foods, including frying oils and animal fats. They are used also to ensure the quality of a vast array of foods, including chips, crackers, cookies, cake mixes, cereal-based snack foods, sauces, dehydrated soups and processed nuts, seasonings and even dietary supplements. Figure 1 shows some of the structure of commonly used synthetic antioxidants. Synthetic antioxidants are derived from petroleum-based products. The authorization for using synthetic antioxidants depends on the product end-use and regulatory rules of each country on labeling of antioxidants in food products. In the United States, the following synthetic antioxidants have been evaluated by the FDA and are the most widely used: TBHQ (tertiary-butylhydroquinone), BHA (butylated hydroxy anisole), BHT (butylated hydroxyl toluene) and PG (propyl gallate) (Rajalakshmi, 1996). According to regulatory agencies in Europe the maximum level of AP (ascorbyl palmitate), BHA, OG (octyl gallate) and DG (dodecyl gallate) in food should not exceed 200 mg/kg, whether antioxidants were used individually or combined. For BHT it shouldn't exceed 100 mg/kg, therefore determination and analysis of antioxidants in food is important (Xiu-Qin et al.,2009).

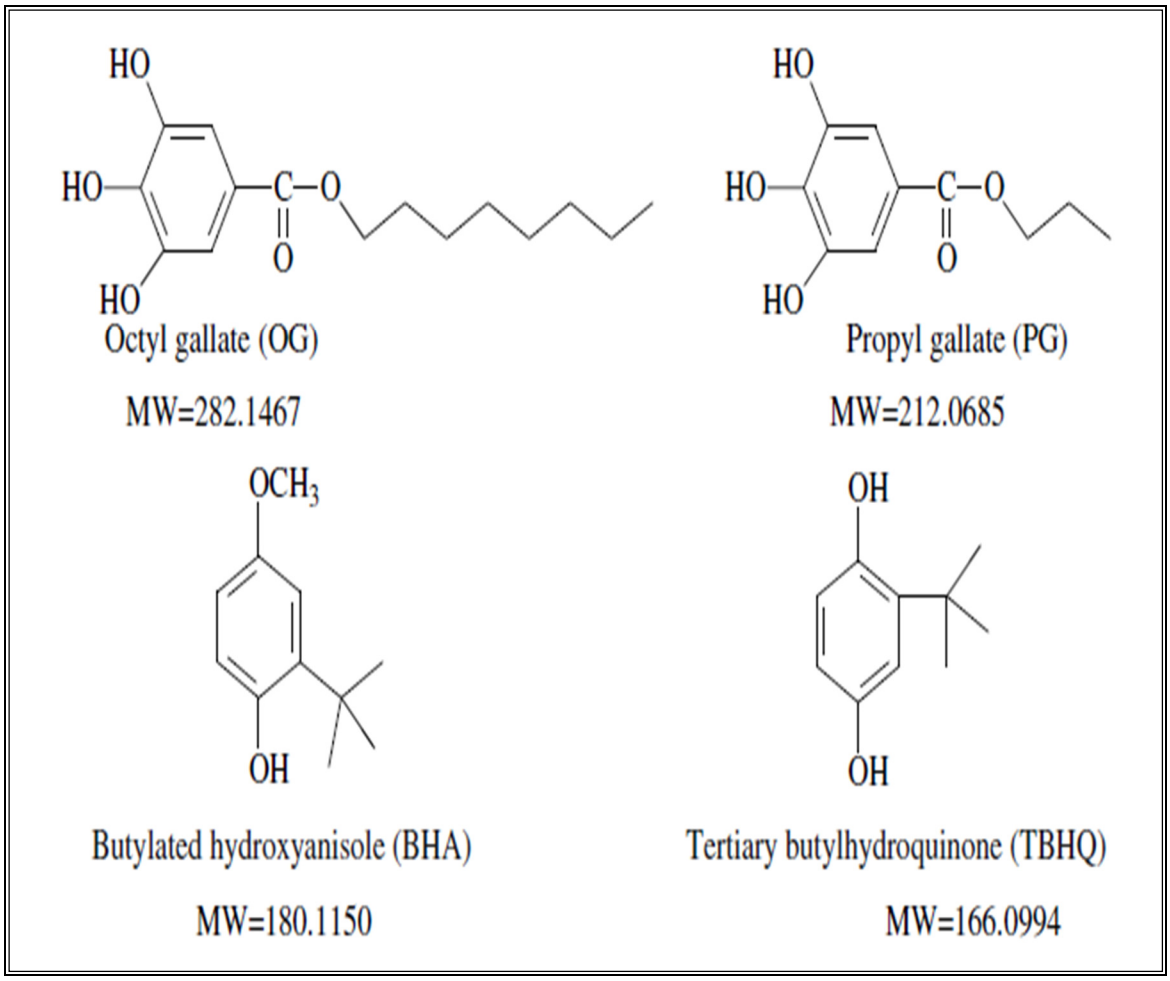


Figure 1: molecular structure of most commonly used synthetic antioxidants

Synthetic antioxidants are cheaper and easier to process than natural antioxidants. When used in approved quantities, they are safe and effective. The choice of antioxidant depends on oil type, "carry through" properties and compatibility in food system to be used in. TBHQ offers excellent "carry through" properties, making it ideal for potato chips, snacks fried in vegetable oils and in baking applications. In animal fats such as lard, BHA, which has excellent solubility in fats and oils, brings added stability to biscuits and cakes.

However, many synthetic antioxidants have the drawback that they are volatile and decompose easily at high temperature. Incompatibility between food additives and foods can lead to discoloration. High water or salt content can cause color formation with some antioxidants. PG can discolor when it comes into contact with iron. Currently used

synthetic antioxidants have been suspected to cause or promote negative health effect (Barlow, 1990). A variety of experimental studies have been reported on BHA and BHT. The International Agency for Research on Cancer (IARC) has evaluated BHA and found sufficient evidence for carcinogenicity in experimental animals (Williams et al., 1999). On the other hand, it was found that BHA and BHT pose no cancer hazard at current levels of food additive use (Williams et al., 1999; Botterweck et al., 2000). For all these reasons stronger restrictions have been placed on their application, and there is a growing interest of food industry toward substituting them with naturally occurring antioxidants.

2.4.2 Natural Antioxidants

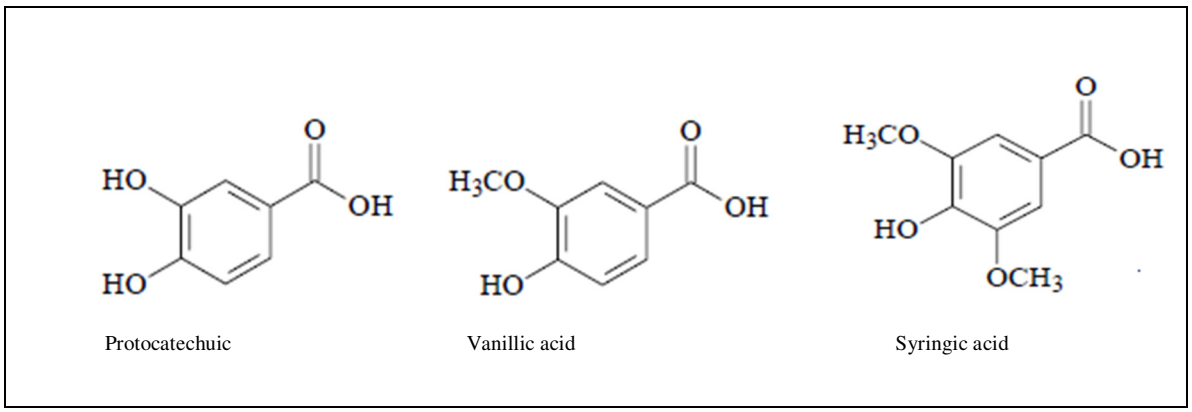
According to Pokorny (2007), the main advantages of employing natural antioxidants in foods compared to synthetic ones are: (1) being readily accepted by most consumers, (2) their wide availability from variable natural resources, (3) being accepted by health officials, (4) the possibility of being replaced by food components, (5) being labeled as flavorings, (6) being proven to affect sensory properties of the food positively, and (7) their functionality as preservatives.

Extensive research has been done on the isolation, purification, and identification of the various antioxidants. Phenolic compounds, tocopherols and ascorbic acid are the most important natural antioxidants. Carotenoids, protein-related compounds, Maillard reaction products, phospholipids, and sterols also show natural antioxidant activities in foods (Choe, 2009).

Phenolic compounds are plant secondary metabolites produced either from phenylalanine via the acetate pathway or from its precursor shikimic acid via the shikimate pathway (Harborne, 1989). All phenolic compounds are characterised by the presence of a phenolic ring. They can be divided into different subclasses as a function of the number of phenol rings and the structural elements binding these rings to each other (Manach et al, 2004). Based on this classification, distinctions are made between the phenolic acids, flavonoids, stilbenes and lignans. Among the polyphenol subclasses, phenolic acids and flavonoids are the most common and have often been described to be the most important as regarding the intake and potential effects in humans (Bravo, 1998).

Plant phenolics are thought to act at several levels in the oxidative sequence as hydrogen donators or singlet oxygen quenching agents decreasing local oxygen concentrations. Antioxidant potentiality of phenolics could be also due to their action as transition metal chelating agent or due to their capability to scavenge radicals.

Phenolic acids are one of the important subclasses of polyphenols. They can have a simple hydroxybenzoic acid structure (such as protocatechuic, ellagic, gallic, gentisic, salicylic, and vanillic acid), or hydroxycinnamic acid structure (such as coumaric, ferulic, caffeic, chlorogenic, rosmarinic and sinapic acid) (Manach et al., 2004) (Figure 2).



Protocatechuic Vanillic acid Syringic acid

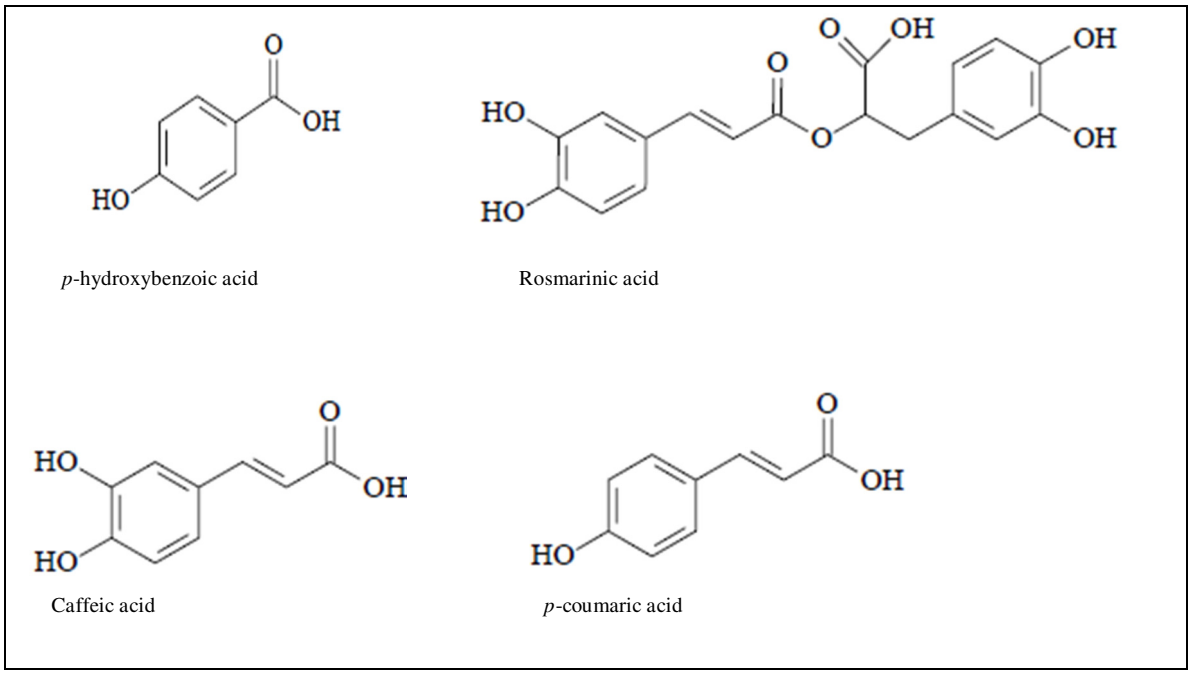


Figure 2: Common Phenolic compounds

Flavonoids are major plant polyphenols and are derivatives of diphenylpropanes and a heterocyclic 6-membered ring with oxygen. They occur in all parts of plants as complex mixtures of different members. Their structure, consisting of two hydroxy substituted aromatic rings joined by a three carbon link (a C_6-C_3-C_6 configuration) (Figure 3) renders them hydrogen and electron donors. Thus they are effective scavengers of free radicals. The antioxidant activity of flavonoids is related to their structure especially the hydroxy substitution of the aromatic A- and B-ring and the substitution pattern of C-ring (Figure 4). According to the structure of C-ring, six major subgroups are distinguished that are commonly found in plant: flavonols (Figure 5) with 2,3-double bond, 3-OH and 4-ketogroup, flavones (Figure 6) with 2,3-double bond and 4-ketogroup, dihydroflavonols (Figure 7)or flavanonols with 3-OH and 4-ketogroup, flavanones (Figure 8) with 4-ketogroup, flavonols or catechines with 3-OH (Figure 9) and anthocyanines. (Figure 10).

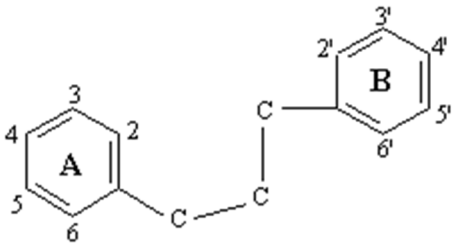


Figure 3: A-B ring of flavonoids (C6-C3-C6 structure)

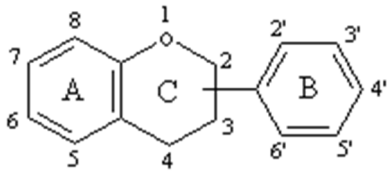


Figure 4: Basic skeleton of flavonoids (A-B-C ring)

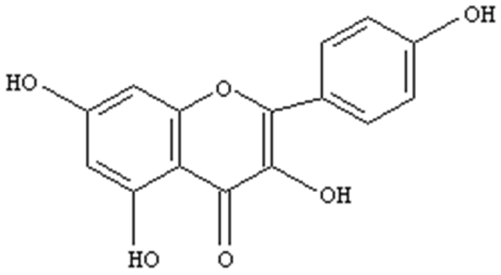


Figure 5: Flavonol structure (Kaemferol, Quercetin, Myricetin).

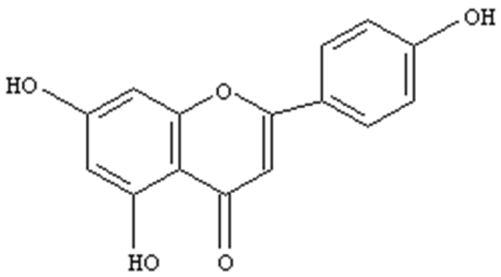


Figure 6: Flavone structure (Apigenin, Luteolin).

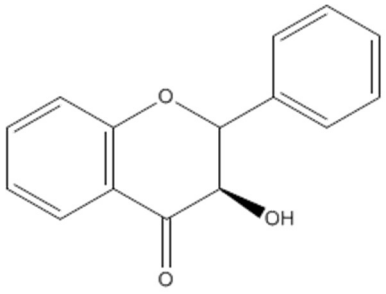


Figure 7: Dihydroflavonol

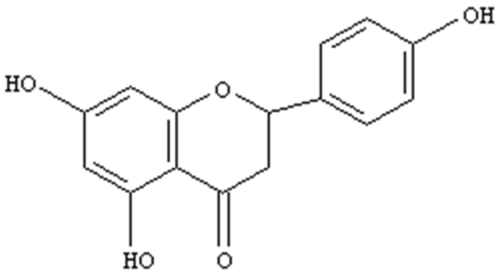


Figure 8: Flavanone structure (hesperidin, naringenin)

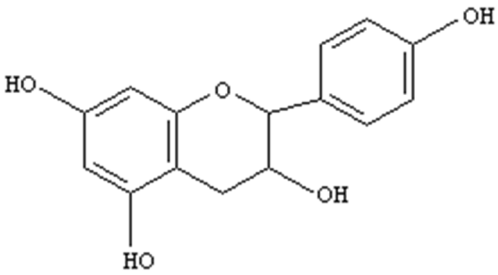


Figure 9: flavanols (catechin, epicatechin)

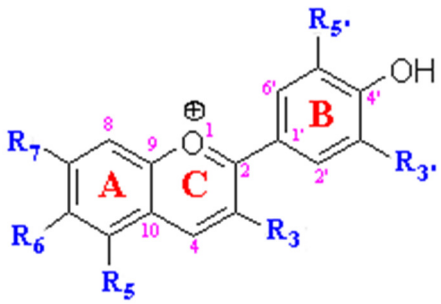


Figure 10: basic structure of anthocyanidine

The donation ability of flavonoid hydroxyls is variable according to possible substitution patterns. The o-di-OH arrangement, usually encountered in B-ring, is active for radical scavenging (Wu, 1996; Zhang, 2000), while the contribution of the 5,7-di-OH-A-ring, which is commonly found in natural flavonoids, is considered to be minor due to the low reactivity of the meta configuration of the hydroxyls (Ancerwicz et al., 1998; Sang et al., 2003). A certain body of research is concerned with the effect of the structural differences of the C-ring. A fully substituted C-ring seems favorable regarding the activity, while compounds that lack one or more structural elements presented contradictory results. Tsimogiannis and Oreopoulou (2007) studied the effect of C-ring and 5,7-di-OH-A-ring against oxidation of certain flavonoids. The results revealed that fully substituted C-ring (such as quercitin) was the most significant element for maximal antioxidantive effect. Flavonoids missing the 2,3-double bond or the 3-OH (taxifolin, luteolin) were less active, and the lack of both of the above structural elements (eriodictyol) resulted in the least effect. The glycosylation of flavonoids results in lower antioxidant activity than the corresponding aglycons. The solubility of flavonoids in fats and oils is very low and their role in the oxidation of oil is limited; however, they can contribute to decreasing the oxidation of oil in food emulsions (Zhou et al. 2005).

Tocopherols are monophenolic compounds and derivatives of chromanol. They are very soluble in oil and thus are the most important antioxidants in edible fats and oils. Tocopherols are more frequently found in vegetable oils than animal fats, especially soybean, canola, sunflower, corn, and palm oils (Choe, 2009), (Figure 11).

Lignans are phenylpropanoids derived from phenylalanine. They include sesamol, sesamin, sesamolin, sesaminol, sesamolinol, pinoresinol, and secoisolariciresinol. The major lignans in unroasted sesame oil are sesamin, sesamolin and sesamol. Secoisolariciresinol and secoisolariciresinol diglucoside are found in flaxseed (Eliasson et al., 2003).

Ascorbic acid, sodium ascorbate, and calcium ascorbate are water soluble and have a limitation as antioxidants for fats and oils. Ascorbyl palmitate is used in fat-containing foods to decrease their oxidation (Choe, 2009), (Figure11).

Carotenoids are polyenoic terpenoids having conjugated trans double bonds. They include carotenes (β-carotene and lycopene), which are polyene hydrocarbons, and xanthophylls (lutein, zeaxanthin, capsanthin, canthaxanthin, astaxanthin, and violaxanthin) having oxygen in the form of hydroxy, oxo, or epoxy groups. Carotenoids are fat soluble and play an important role in the oxidation of fats and oils.
Carotene is the major carotenoid in oils, and β-carotene is the most studied. Palm oil is one of the richest sources of carotenoids (Choe, 2009).

Protein-related compounds Such as hypoxanthine, xanthine, glycine, methionine, histidine, tryptophan, proline, lysine, ferritin, transferritin, and carnosine show their antioxidant activities in the oxidation of lipid-containing foods (Reische et. al., 2002).

Enzymes such as glucose oxidase, superoxide dismutase, catalase, and glutathione peroxidase are known to decrease the oxidation of foods (Yuan & Kitts 1997). Application of enzymes and proteins as antioxidants is limited to unprocessed oil because oil processing denatures the enzymes and proteins.

Maillard reaction products from amines and reducing sugars or carbonyl compounds from lipid oxidation slow down lipid oxidation (Saito 1997). There are a number of Maillard reaction products, but the responsible compounds for the antioxidant activity have not been clearly determined to date.

Phospholipids such as phosphatidylethanolamine and phosphatidylcholine exist in crude oil, but most of them are removed by oil processing such as degumming. Although phospholipids are generally known as antioxidants, they can increase lipid oxidation depending on the environment such as presence of iron (Choe, 2009)

Sterols exist mainly in edible oils (corn oil, rapseed oil, soybean and olive oil). Antioxidant activity of β-sitosterol was lower than those of ferulic acid and tocopherol in the autoxidation of soybean oil (Devi et al. 2007).

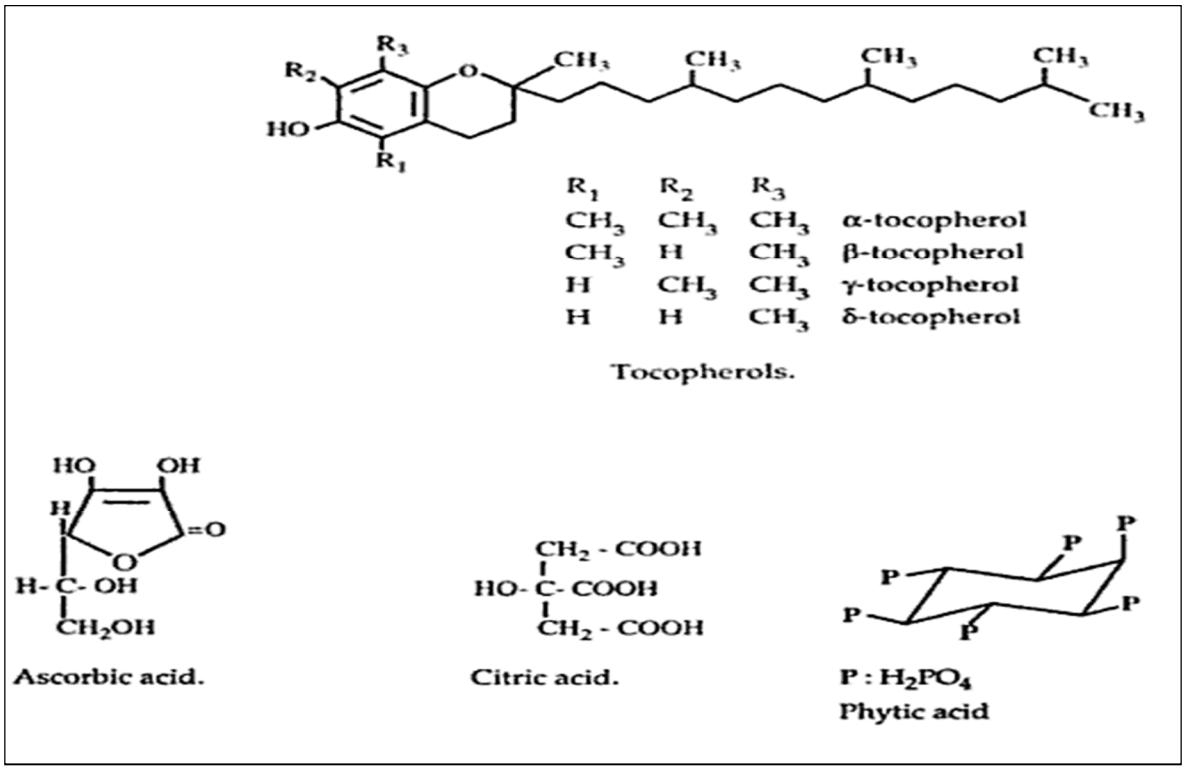


Figure 11: Structures of some categories of natural antioxidants

2.5 Application of Antioxidants in Food

Antioxidants for food application should have reasonable cost, be nontoxic, stable, and effective at low concentration, have carry-through effect, and should not change the flavor, color, and texture of the food matrix (Schuler 1990). The effects of antioxidants on the oxidation of foods are dependent on their concentration (Frankel, 1996), polarity, and the medium of the food (Cuvelier, 2000; Samotyja, 2007), and also the presence of other antioxidants (Decker 2002). The increase of concentration to a certain level is expected to increase the antioxidant efficiency.

2.6 Evaluation of Antioxidant Activity

2.6.1 In vitro antioxidant activity in food systems or lipid substrates

The interest in antioxidants and their action has resulted in the development of an array of methods to assess in-vitro antioxidant capacity in foods. The antioxidant activity is measured by monitoring the oxidative stability of the food lipids enriched with antioxidant compared to non-enriched samples. After the sample is oxidized under standard conditions, the extent of oxidation is measured by chemical, instrumental, or sensory methods (the common methods are detailed in the first chapter (1.6). Most of these studies aim at measuring the extension of the induction period, which is also expressed as an antioxidant index or a protection factor. The induction period is the time required for the sample to start oxidizing rapidly, corresponds roughly to the initiation step, and coincides with the oneset of off-flavor development of lipids.

A practical measure of antioxidant activity hit atleast two objectives: whether the test substance has a detectable antioxidant or prooxidant effect under the test conditions; and a comparison of the quantitative effect or likely effect, of specified concentrations of different test materials on the substrate. For quantitive measures of antioxidant activity most authors report activities as comparative results, *e.g.*, peroxide values, TBARS assays or absorbance increase at 235 nm after a fixed time period, *e.g.*, induction times. However, there appear to be no standard units for reporting such activity (efficiency, effectiveness, assay, capacity, action, *etc*.) independent of the test procedure.

Antioxidant activity is a function of many parameters: time or rate; temperature; substrate; concentration of antioxidant; concentration of other substances, *e.g.*, oxygen, peroxides or other antioxidants/pro-oxidants, *etc*. and partitioning behavior.

Various chemical and physico-chemical procedures are used to monitor oxidation processes. The desirable features of a test of antioxidant activity are the use of a substrate and conditions in the test that mimic the real situation and the ability to quantify the result by reference to a suitable standard (Antolovich, 2002, Rajalakshmi, 1996).

2.6.2 Ability to scavenge free radicals

In addition to the methods involving the incorporation of the antioxidant in a food or lipid substrate, strategies have been developed for measuring the antioxidant activity as the ability to scavenge free radicals generated in aqueous and lipophilic phases. The ability to scavenge specific radicals may be targeted as, for example, hydroxyl radical, superoxide

radical or nitric oxide radical. One approach involves the generation of a free radical species and direct measurement of its inhibition due to the addition of antioxidant(s).
The radical that is generated varies and systems have been described using horseradish peroxidase–H_2O_2, *o*-phenylenediamine–H_2O_2, copper(II)–cumene hydroperoxide, trichloromethyl peroxyl radical, DPPH, and azo compounds such as the chromogenic redox indicator ABTS.

2.6.2.1 DPPH Antiradical efficiency

The DPPH• radical scavenging assay reflects the capacity of a sample to reduce this nitrogen centered radical mainly by single electron transfer mechanism; the hydrogen atom transfer mechanism has supposedly a marginal role (Huang *et al*, 2005). DPPH absorbs at 515 nm when it is free, but when added to an antioxidant, the absorption is reduced due to radical scavenging which reduces the concentration of DPPH. The antioxidant activity can be determined by monitoring the decrease in this absorbance. Results are reported as the EC_{50}, that is, the amount of antioxidant necessary to decrease by 50% the initial DPPH concentration. The DPPH test was used by to determine antioxidant activity of *origanum dictamus* (Kouri et al., 2007) and to study the antioxidant activity of 5',7',3,4-hydroxy-substituted flavonoids (Tsimogiannis & Oreopoulou, 2004).

Some of the most common tests and basic units used in evaluating antioxidant activity are summarized in Table 1.

Table 1: The common tests and basic units used for measuring antioxidant activity

Test	Measurement	Units
Peroxide value (PV)	Peroxides and hydroperoxides	meq/kg of active oxygen.

Conjugated dienes (CD)	1,4-Dienes produced by early stages in lipid autoxidation	Absorbance/unit mass mg/ kg linoleic acid equivalents.
Thiobarbituric acid reactive substabces (TBARs)	Thiobarbituric acid derivatives of malondialdehyde absorbing at 532–535 nm	mg/ kg
Hexanal formation, pentane formation, hexane formation, etc.	Specific oxidation end- product formed	mg/ kg of product formed
$ABTS^{\cdot+}$ assay Total radical trapping (TRAP) DPPH	Absorbance of radical at suitable wavelength	Inhibition time for appearance of radical or decay rate
Electron spin resonance(ESR) spin trap test	Intensity/rates of change in concentration of antioxidant or spin-trap derivative radicals	mg/ L of radical species

References

Ancerewicz J, Migliavacca E, Carrupt P., Testa B, Bree F, Zini R, Tilliment J., Labidalle S., Guyot D., Chauvet-Monges A., Crevat A., Le Ridant A.: Structure-Property relationships of trimetazidine derivatives and model compounds as potential antioxidants. *Free Rad. Biol. Med.*, **25**, 1,113–120.

Amorati R., Cavalli A., Fumo M.,, Masetti M., Menichetti S., Pagliuca C., Pedulli G., Viglianisi C.: Kinetic and thermochemical study of the antioxidant activity of sulfur-containing analogues of vitamin E. Chem Eur J ,13,8223–30, 2007.

Antolovich M., Prenzler P., Patsalides E., McDonald S. and Robards K.: Methods for testing antioxidant activity, *Critical Review. Analyst*, **127**, 183–198**,** 2002.

Barlow S. (1990): Toxicological aspects of antioxidants used as food additives, In *Food Antioxidants.* Hudson BJF (ed.) Elsvier. London, 253-307.

Botterweck A., Verhagen H., Goldbohn R., Kleinjans J., Van den Brandt P.: Intake of butylated hydroxyanisole and butylated hydroxytoluene and stomach cancer risk: results from analyses in the Netherlands Cohort Study. 2000, **38**, 7, 599-605.

Bravo L.: Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.,* **56**,317-333, 1998.

Cao W., Chen W., Sun S., Guo P., Song J., Tian C.: Investigating the antioxidant mechanism of violacein by density functional theory method. *J. Mol. Struct.: Theochem* 2007, **817**, 1–4.

Choe E, Min D.: Mechanisms and factors for edible oil oxidation. Comp Rev Food Sci 5, 169–86. 2006.

Choe E, Min D.: Chemistry and reactions of reactive oxygen species in foods. *J. Food Sci.* **70**,142–159, 2005

Choe E. & Min D.: Mechanism of Antioxidants in Oxidation of Foods, *Compr. Rev. Food Sci. Food Safety,* 2009**, 8**, 345-358.

Cuvelier M, Bondet V, Berset C. 2000. Behavior of phenolic antioxidants in a partitioned medium: structure-activity relationship. *J. Am. Oil Chem. Soc.* 77:819–23.

Decker E, Ivanov V, Zhu BZ, Frei B.: Inhibition of low-density lipoprotein oxidation by carnosine and histidine. *J. Agric. Food Chem.*, **49**, 511–516, 2001.

Decker E. (2002): Antioxidant mechanisms. In *Food Lipids* eds. Akoh C, Min D, 2nd ed., Marcel Dekker Inc., NY, USA, pp 517–42.

Devi R., Jayalekshmy A., Arumughan C.: Antioxidant efficacy of phytochemical extracts from defatted rice bran in the bulk oil system. *Food Chem.* **104**, 658–64, 2007.

Eliasson C, Kamal-Eldin A, Andersson R, Aman P. High-performance liquid chromatographic analysis of secoisolariciresinol diglucoside and hydroxycinnamic acid glucosides in flaxseed by alkaline extraction. *J. Chromatogry,* **1012**, 151–159, 2003.

Graf E., Eaton J.: Antioxidant functions of phytic acid. *Free Radical Biol Med* **8**, 61–9, 1990.

Harborne, J. (1989): General procedures and measurement of total phenolics In Methods in *plant biochemistry*:Volume 1 Plant Phenolics, Academic Press,London, UK,pp: 1-28.

Jadhav S., Nimbalkar S., Kulkarni A., Madhavi D.(1995): Lipid Oxidation in Biological and Food Systems in *Food Antioxidants*, *Technological, Toxicological, and health perspectives*, Madhavi D., Deshpande S., Salunkhe D., Marcel Dekker, NY, USA.

Koidis A, Boskou D.: The contents of proteins and phospsholipids in cloudy (veiled) virgin olive oils. *Eur. J. Lipid Sci. Technol.* **108**, 323–8, 2006.

Kouri G., Tsimogiannis D., Bardouki H., Oreopoulou V.: Extraction and analysis of antioxidant components from Origanum dictamnus, *Innov. Food Sci. Emerg. Technol.,* **8**, 155–162, 2007.

Frankel E.: Antioxidants in lipid Foods and their impact on Food quality, *Food Chem.*, **57**, 1, 51-55, 1996.

Galati, G., Sabzevari, O., Wilson, J. & O`Brien, P:. Prooxidant activity and cellular effects of the phenoxyl radicals of dietary flavonoids and other polyphenolics. *Toxicology* **177**, 91-104,2002.

Huang, D., Ou, D. & Prior, D.: The chemistry behind antioxidant assays. *J. Agric. Food Chem.,* **53**, 1841-1856, 2005.

Halliwell, B. Dietary polyphenols: Good, bad, or indifferent for your health? *Cardiovasc Res* **73**:341-347, 2007.

Madhavi D., Deshpande S., Salunkhe D. (1996): Introduction In *Food Antioxidants*, *Technological, Toxicological, and health perspectives*, Madhavi D., Deshpande S., Salunkhe D., Marcel Dekker, NY,USA.

Manach, C., Scalbert, A., Morand, C., Remesy, C. & Jimenez, L. (2004) Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.,* **79**, 727-747.

Min D, Lee S., Lee E. (1989). Singlet oxygen oxidation of vegetable oil. In *Flavor chemistry of lipid foods*: Min D., Smouse T., Champaign, IL, USA: AOCS Press. pp 57–97.

Mink, P., Scrafford, C., Barraj, L.: Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women. *Am. J. Clin. Nutr.,* **85**, 895-909, 2007.

Murakami M, Yamaguchi T, Takamura H, Matoba T.: Effects of ascorbic acid and α-tocopherol on antioxidant activity of polyphenolic compounds. *J. Food Sci.* **68**, 1622–1625, 2003.

Peyrat-Maillard MN, Cuvelier ME, Berset C.: Antioxidant activity of phenolic compounds in 2,2_-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced

oxidation: synergistic and antagonistic effect. *J Am. Oil Chem. Soc.*, **80**, 1007–1012, 2003.

Pokorny J.: Are natural antioxidants better and safer than synthetic antioxidants? *Eur. J. Lipid Sci. Technol.* 2007, **109**, 629–642.

Reddy V., Urooj A., Kumar A.: Analytical, Nutritional and Clinical Methods Evaluation of antioxidant activity of some plant extracts and their application in biscuits, *Food Chem.,* **90**, 317–321, 2005.

Xiu-Qin L., Chao J., Yan-Yan S., Min-Li Y., Xiao-Gang C: Analysis of synthetic antioxidants and preservatives in edible vegetable oil by HPLC/TOF-MS, *Food Chem.* **113**, 692–700, 2009.

Rice-Evans C., Miller N., Paganga G.: Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.,* **20**, 933–56, 1996

Reische D., Lillard D., Eitenmiller R. (2002): Antioxidants. In *Food Lipids*, eds Akoh C, Min D, Marcel Dekker Inc., NY, USA, pp 489–516.

Rajalakshmi D. & Narasimhan S.(1996): Sources and Methods of Evaluation in *Food Antioxidants*, *Technological, Toxicological, and health perspectives*, Madhavi D., Deshpande S., Salunkhe D., Marcel Dekker NY, USA.

Samotyja U, Malecka M.: Effects of blackcurrant seeds and rosemary extracts on oxidative stability of bulk and emulsified lipid substrates, *Food Chem*, **104**, 317–23, 2007.

Schuler P. (1990): Natural antioxidants exploited commercially. In *Food Antioxidants*, Hudson BJF (ed) Elsvier, London, UK, 99-170.

Saito H., Ishihara K.: Antioxidant activity and active sites of phospholipids as antioxidants. *J. Am. Oil Chem. Soc.*, **74**, 1531–1536, 1997.

Stahl W, Sies H.: Physical quenching of singlet oxygen and cis-trans isomerization of carotenoids. *Ann NY Acad Sci,* **691**, 10–19, 1992.

Tsimogiannis D., Oreopoulou V.: Free radical scavenging and antioxidant activity of 5,7,3',4'-hydroxy-substituted flavonoids, Innovative *Food Science and Emerging Technologies* **5**, 523– 528, 2004.

Yuan Y., Kitts D. (1997): Endogenous antioxidants: role of antioxidant enzymes in biological systems. In: *Natural antioxidants: chemistry, health effects and applications* editor, Shahidi F., Champaign, IL.: AOCS Press. pp 258–270.

Van Aardt M., Duncan S., Marcy J., Long T., O'Keefe S., Nielsen-Sims S.: Effect of antioxidant (α-tocopherol and ascorbic acid) fortification on light-induced flavor of milk. *J. Dairy Sci.,* **88**, 872–880, 2005.

Viljanen K, Sunberg S,Ohshima T, Heinonen M.: Carotenoids as antioxidants to prevent photooxidation. *Eur J Lipid Sci Technol.,* **104**, 353–9, 2002.

Williams G., Iatrolous M. & Whysne J.: Safety Assessment of Butylated hydroxyanisole and Butylated Hydroxytoluene as Antioxidant Food Additives. *Food Chem. Toxicol.* **37** 1027-1038, 1999.

Zhang H-Y, Wang L-F.: Solvent effects are important in elucidating radical-scavenging mechanisms of antioxidants. A case study on genistein. *J. Biomol. Struct. Dyn.* , **22**, 483–6, 2005.

Zhou B, Wu L., Yang L., Liu Z.: Evidence for alpha-tocopherol regeneration reaction of green tea polyphenols in SDS micelles. *Free Radical Bio Med* **38**, 78–84. 2005.

Wu C., Zhen-Quin Z., Tian-Xi H., Da-Yuan Z..: Structure-activity relationship of natural flavonoids in hydroxyl radical-scavenging effects, *Zhongguo Yaoli Xuebao*, **23**,7, 667-672, 2002.

Chapter 3

Majorana Syriaca: an antioxidant potent from *Lamiceae*

3.1 ***Lamiaceae*** *(syn. Labiatae*) herbs consist of more than 200 genus and 3500 species (Watson & Dallwitz, 2007), such as sage (*Salvia officinalis L.*), thyme (*Thymus vulgaris L.*), rosemary (*Rosmarinus officinalis L.*), two oregano species (*Origanum vulgare L.*, *Origanum onites L.*), etc. Many of them are part of a typical Mediterranean diet, and consumed world-widely. These herbs have been used also in traditional medicine, but are mainly known for their culinary properties. Oregano, rosemary, sage and thyme have a GRAS status given by the U.S. Food and Drug Administration (U.S. Food and Drug Administration, 2006), meaning that they are generally recognized as safe for human consumption without limitations on intake.

Lamiaceae herbs are a rich source of potentially health-beneficial antioxidant polyphenols (Dragland et al., 2003). This fact, combined with promising data on the antioxidant properties of phenolic compounds, has triggered both scientific and commercial interest towards this family of herbs.

3.2 Phenolic Compounds in *Lamiaceae* Herbs

The antioxidant activity of spices and herbs, including those belonging to the *Lamiaceae* family, is thought to be primarily due to the presence of phenolic compounds and especially phenolic diterpenes, flavonoids and phenolic acids (Kivilompolo et al., 2007;

Kahkonen et. al., 1999). Table 1 shows some phenolic constituents found in this family of herbs such as *R. officinalis, S. officinalis, O. vulgare*. The phenolic content of these herbs depends mainly on the growing location and harvesting time, as was seen for rosmarinic acid (Yesil-Celiktas et al., 2007). The essential oil of these herbs contains antioxidant monoterpenes such as carvacrol and thymol (Alma, 2003), which are mainly responsible for the typical strong aroma of thsese herbs.

In polar extracts of *Lamiaceae* herbs, phenolic acids are the major class of phenolic compounds. Phenolic acids (caffeic, gallic, *p*-coumaric, and rosmarinic) were reported to be the major type of phenolic compounds in methanolic extracts of *O. vulgare*, thyme, rosemary and sage in the study of Shan et al. (2005). Similarly, the major part of aqueous sage extract phenolics consists of phenolic acids, mainly caffeic acid derivatives (Lu & Foo, 2002). As reported by Zheng (2001) rosmarinic acid is the most abundant phenolic compounds found in the acetone extract of sage, oregano and thyme. Whereas in rosemary the main phenolic compounds are diterpene derivatives, carnosic acid and rosemanol, followed by rosmarinic acid. Also some amounts of volatile compounds, for example carvacrol and thymol, as well as flavonoids. Rosmarinic acid has been identified as the predominant phenolic compounds in aqueous extracts of sage and thyme (Zheng & Wang, 2001) and in 60% ethanol extracts of oregano, sage, rosemary and thyme (Kivilompolo & Hyötyläinen, 2007). The main antioxidant constituents in sage were also found to be carnosol, carnosic acid, and rosemarinic acid, followed by other related phenolics (Cuvelier et al., 1994, 1996). The main antioxidant constituents of oregano are flavonoids: luteolin, luteolin-glycoside, apigenin, apigenin glycoside, eriodictyol, taxifolin, and dihydrokaempferol (Justesen 2000; Justesen & Knuthsen, 2001) and rosmarinic acid (Exarchou et al., 2002; Justesen & Knuthsen, 2001). In a study done by (Tsimogiannnis et al., 2006) isolating and characterizing the components of Origanum hercleoticum, eriodictyol, taxifolin, apigenin and carvacrol were detected in a diethyly ether extract, while rosmarinic acid, apigenin and apigenin glycoside were found in ethanol extract.

Table 1: Identified flavonoids in herbs from Lamiaceae

Herb	**Identified Phenolics**	**References**
R. officinalis	luteolin, hesperidin	Okamura et al., 1994
	carnosic acid, carnosol	Bicchi et al., 2000
	p-hydroxybenzoic, syringic, vanillic, protocatechuic and caffeic acid	Zgórka, 2001
	rosmarinic acid	Cuvelier et al., 1996
	gallic, *p*-coumaric, ferulic, chlorogenic and dihydroxycaffeic acid	Kivilompolo et al., 2007 Dorman, 2003
S. officinalis	carnosol, carnosic acid, rosmanol, epi-rosmanol, rosmadial, methyl carnosate	Cuvelier et al., 1994
	protocatechuic acid	Zgórka, 2001
	vanillic, ferulic and rosmarinic acid	Cuvelier et al., 1996
	salvianolic acids (rosmarinic acid dimers)	Lu, 1999
	p-coumaric acid	Lu, 2000
	gallic, vanillic, chlorogenic and dihydroxycaffeic acid	Kivilompolo et al., 2007
	apigenin, luteolin, hesperetin	Cuvelier et al., 1996
	kaempferol	Shan et al., 2005
O. vulgare	*p*-hydroxybenzoic, vanillic, caffeic, *o*-coumaric and	Gerothanassis et al., 1998

	ferulic acid	
	gallic, vanillic, caffeic, *p*-coumaric, ferulic, rosmarinic, chlorogenic and dihydroxycaffeic acid	Kivilompolo et al., 2007 Dorman, 2003
	apigenin, luteolin quercetin kaempferol eriodictyol, naringenin	Justesen, 2001 Exarchou et al., 2002 Shan et al, 2005 Tsimogiannis et. al., 2007 Exarchou et al., 2003

3.3 *Majorana syriaca*

3.3.1 Botanical Review

Majorana syriaca belongs to the *Lamiaceae* family, genus *Origanum.* According to Flora Europaea, the genus *Origanum* is divided into three sections, i.e. *Origanum*, *Majorana*, and *Amaracus* (Bernath, 1996). Ietswaart's classification distinguished ten sections (including the aforementioned ones) consisting of 42 species or 49 taxa, distributed mainly within the Mediterranean regions (Letswaart, 1980; Kokkini, 1996). *Majorana syriaca*, syn. *Origanum syriacum, Origanum maru*, according to Beker et al. (1989) and, Alma, (2003), belongs to the section *Majorana*, which is further subdivided into three geographically distinct varieties, i.e. syriacum, bevanii, and sinaicum (Kokkini, 1996).

3.3.2 Traditional use: Culinary

Majorana syriaca is traditionally used in the Middle East as a food-flavouring ingredient. The leaves of the herb are mixed with other herbs and spices, usually *C. capitatus* and *S. thymbra* to prepare a typical seasoning, known in the Middle East as Za'atar (Ravid, 1983), which is usually added to olive oil and spread on bread. The plant is commonly named in Arabic as "sa'atar" or "za'atar" and there are several wild and cultivated populations in Israel and Palestine (Ishac, 1985, Akguel, 1987). It is also used in teas and cooked or baked foods. It has a strong oregano flavor and also thought to be "Hyssop" mentioned in the bible to have powers of spiritual purification (Alma, 2003). The aroma of the essential oil is used in soaps, perfumes and cosmetics.

3.3.3 Medicinal use

The herb is not only blessed for its aroma and flavor, but also for its fame in folkloric medicine. Extracts of *M. syriaca* from Lebanon have been tested for neurological activity, as the herb is traditionally used against neurological disorders, like Alzheimer's disease (Salah, 2005). It is also used as medicinal herb for improving the inspiratory system, against intestinal and stomach ache, against high blood pressure, and in tooth pastes (Said, et al., 2002, Hudaib, 2008).

3.3.4 Composition of essential oil

According to Putievsky et al. (1996) two chemotypes of *Majorana syriaca* are distinguished: one rich in carvacrol and the other rich in thymol. Ishac (1985) mentioned Palestinian variety is distinguished from other similar types mainly by the constituents of its essential oil, that is considered to consist of approximately 73% carvacrol and 4.5% thymol. In addition to carvacrol and thymol, the other most common compounds of the essential oil of *M. syriaca* are the two monoterpene hydrocarbons, γ-terpinene and *p*-cymene, which are the biogenetic precursors of the two phenolic terpenes, carvacrol and thymol (Putievsky, 1996). The aforementioned compounds were observed in the analysis of all populations of *M. syriaca* with different geographical origin, including different varieties, as presented by Akguel (1987), Baser et al. (1993), Bernath (1996), Halim et al. (1991), Fleisher (1991) and Tume (1993).

3.3.5 Antimicrobial activity

The essential oil of the plant is used as antimicrobial agent and added as preservative in food products such as sausages and meats. It showed a remarkable antifungal activity, compared to essential oils of other aromatic wild plants growing in Israel, and was effective in controlling *Exserohilum turcicum, Fusarium oxysporum, Macrophomina phaseolina,* and *Botrytis cinerea* (Shimoni, 1993). The essential oil of leaves of *Origanum syriacum* grown in Turkey were screened for their antioxidant activity as well as antimicrobial properties and chemical composition by Alma (2003). The antimicrobial activity (both antibacterial and antifungal) increased as concentration increased. It inhibited remarkably the growth of *S. aureus* and *E. coli*.

Many times the antioxidant activity of natural extracts is accompanied by antimicrobial effect, as the essential oil of some plant extracts showed to have not only antioxidant activity, but also antimictobial activity (Sokmen, 2004, Singh et. al. 2007). Most of the antimicrobial activity in essential oils of spices and culinary herbs is thought to be derived from phenolic compounds, while other constituents are believed to contribute little to antimicrobial effects (Nychas, 1995, Singh et al., 2007)

3.3.6 Antioxidant activity of *M. syriaca* and herbs of *Lamiaceae* family

Natural antioxidants from tea, wine, fruits, vegetables and spices (e.g. rosemary and sage) are already exploited commercially either as antioxidant additives or as nutritional supplements (Schuler, 1990). For many years, spices and herbs have been used as additives to enhance the sensory features of food (Pokorny, 1991, Wang *et. al,* 1996). Culinary herbs and spices have been utilized since antiquity to improve the taste and to extend the shelf-life of food (Bishov, 1977). It is now recognized, however, that thyme (*Thymus vulgaris* L.), oregano (*Origanum vulgare* L.) and marjoram (*Origanum majorana* L.) belonging to the *Labiatae* family, have the capability to prevent autoxidation of food lipids (Cuppett, 1998). Thymol and its isomer carvacrol are the main active compounds in the essential oil from thyme (Farag 1989). The ethyl acetate and diethyl ether extracts of *Origanum dictamnus* extracts were effective against autoxidation of cottonseed oil (Kouri, 2007). 2007). Natural additives from *Lamiaceae* herbs are also involved in ready-to-serve foods. Sage and a mixture of spices (sage, red pepper, black pepper, garlic and marjoram) retarded the process of oxidation when added to meat balls that were fried in a medium layer of soybean oil and then stored in a refrigerator (Karpinska, 2001). In studies by Tsimidou *et al.* (1995) using an unsaturated lipid system, oregano at 1% by weight was found to be equivalent to 200-ppm butylated hydroxyanisole (BHA) in controlling the oxidation of mackerel oil. The essential oils from thyme and oregano have been reported to exhibit antioxidant activity in cottonseed oil and lard, respectively (Lagouri, 1993). The antioxidant effect of extracts from oregano in frying and storage of fried products was also studied by Houhoula, 2004. The radical-scavenging capacities of oregano and thyme extracts have been observed in different

model systems (Vichi 2001, Miura, 2002). Natrural antioxidants are also tested in cookies. The addition of extracts of the three plant (*Emblica officianalis*), leaves of (*Moringa oleifera*) and raisins (*Vitis vinifera)* gave an excellent antioxidant effect on the biscuits compared with the effect of BHA. The higher efficiency of the plant extracts could be due to the stability of these natural antioxidant during baking (Reddy, 2004).

The antioxidant activity of *Majorana syriaca* was rarely investigated in details, whereas the majority of studies were concerned about studying the plant among Mediterranean medicinal herbs or as a traditional source of culinary food. So the studies focused on medicinal, botanical and environmental issues rather than extracting antioxidants and using them in practical food applications (Ali-shtayeh et al., 2008, Said et al., 2002, Hudaid et al., 2008). In a master thesis study done by Hinnawi (2010), the ethanolic extract of *Majorana syriaca* showed a high inhibition value of the DPPH that exceeded BHA and total flavonoids compound was calculated to be 146 g mg-1 quercetin equivalent. The antioxidant activity of *Majorana syriaca* was also measured among 51 medicinal plants in Jordan, and it was found to have a recognizable antioxidant activity against $ABTS^{\bullet+}$, a fact that is supported also by measurement of phenolic content that was found to be 22.1 ± 3.2 mg gallic acid equivalent/ dry weight as presented by Tawaha et al. (2007). The essential oil of *Origanum syriacum* showed also to have antioxidant activity that is mainly attributed to its high phenolic compounds (Tepe et. al, 2004). From all above mentioned data it was necessary to proceed into a further investigation for the plant antioxidant potentiality regarding use in food technology.

3.4 Extraction of Natural Antioxidants

Solvent extraction is among the most commonly used procedures to prepare extracts from plants due to their ease of use, efficiency and wide applicability. However the yield of the extraction depends on type of solvents (polarity, ability of penetration in the plant material), extraction time, temperature, sample-to-solvent ratio, as well as chemical composition and physical characteristics of the sample such as particle size of the ground material, where it was found that lowering particle size also enhances the yield of extraction of phenolic compounds (Nepote et al., 2005).

Solvents, such as methanol, ethanol, acetone, ethyl acetate and their combinations have been used for the extraction of phenolics. The polarity of the solvent plays role in extracting antioxidants of similar polarity, so selecting the right solvent affects the amount of polyphenols extracted (Dai & Mumper, 2010). In particular, methanol has been generally found to be more efficient in extraction of lower molecular weight polyphenols while the higher molecular weight flavonoids are better extracted with aqueous acetone. Ethanol is another good solvent for polyphenol extraction and is safe for human consumption (Shai et al., 2005). On the other hand, non polar solvents, such as petroleum ether, were used to extract the less polar compounds such as the constituents of the essential oil. For example, Tsimogiannis et al., (2006) found that carvacrol was the main constituent in pertoleum ether extract of *Origanum heracleoticum.*

The aerial parts of *Sideritis syriaca ssp. syriaca* from *Lamiaceae* herbs were extracted, after defatting, with diethyl ether, ethyl acetate and n-butanol, and the results revealed that ethyl acetate extract contained the highest antiradical efficiency against DPPH. (Armata et al., 2008). The natural antioxidants from *Origanum dictamus* were extracted by successive extractions usin g petroleum ether, diethyl ether, ethyl acetate and ethanol solvents and also by one step single extraction using ethanol (Kouri et al., 2007).

Different bioactive flavonoid compounds including catechin, epicatechin, rutin, myricetin, luteolin, apigenin and naringenin were obtained from spearmint (*Mentha spicata* L.) by two comparable methods of extraction; conventional soxhlet extraction (CSE) and supercritical extraction (SC_{CO2}). Solvents used in (CSE) were pure ethanol, methanol, petroleum ether and 70% ethanol and results revealed that soxhlet extraction had a higher crude extract yield comparing to the SC_{CO2} while SC_{CO2}. at optimum conditions (60°C, 200 bar, 60 min) extracted more flavonoids (Bimakr et al., 2011).

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References

Alma M., Mavi A., Yildirim A., Digrak M., Toshifumi H.: Screening chemical composition and in Vitro antioxidant and antimicrobial activities of the essential oils from *origanum syriacum L.* growing in Turkey, *Biol. Pharm. Bull*., **26,** 12, 1725-1729, 2003.

Akguel A., Bayrak A.: Constituents of essential oils from *Origanum* species growing wild in Turkey. *Planta Med.* , **53**, 114-121,1987.

Baser K., Ozek T., Tumen G., Sezik E.: Composition of the essential oils of Turkish *Origanum* species with commercial importance. *J Essential Oil Res.*, **5,**6, 619-623, 1993.

Beker R., Dafini A., Eisikowitch D., Ravid U.: Volatiles of two chemotypes of *Majorana syriaca* L. (Labiatae) as olfactory cues for the honeybee. *Oecologia*, **79**, 446-451, 1989.

Bernath J.: Some scientific and practical aspects of production and utilization of oregano in central Europe. In: Proceedings of the IPGRI International Workshop on Oregano, 8-12 May, CIHEAM, Valenzano (Italy). pp. 75 – 92, 1996.

Bicchi, C., Binello, A. & Rubiolo, B.: Determination of phenolic diterpene antioxidants in rosemary (Rosmarinus officinalis L.) with different methods of extraction and analysis. *Phytochem. Anal.*, **11**, 236-242, 2002.

Bishov S., Masuoka Y., Kapsali J.: Antioxidant effect of spices, herbs and protein hydrolyzates in freeze-dried model systems: Synergistic action with synthetic phenolic antioxidants. *J Food Proc Preserv.*, **1**, 153–166, 1977.

Cuppett S.: Antioxidant activity of the *Labiatae*. *Adv. Food Nutr. Res.* **42**, 245–271, 1998.

Cuvelier, M.E., Richard, H. & Berset, C. :Antioxidative activity and phenolic composition of pilot-plant and commercial extracts of sage and rosemary. *J. Am. Oil Chem. Soc.* **73**,645-652, 1996.

Cuvelier, M.E., Berset, C. & Richard, H. : Antioxidant constituents in sage (*Salvia officinalis*). *J. Agric. Food Chem.* **42**,665-669, 1994.

Dragland, S., Sanoo, H., Wake, K., Holte, K. & Blomhoff, R.: Several culinary and medicinal herbs are important sources of dietary antioxidants. *J Nutr* **133**, 1286-1290, 2003.

Dorman H., Peltoketo, A., Hiltunen, R., Tikkaneh M.: Characterisation of the antioxidant properties of de-odorized aqueous extracts from selected lamiaceae herbs, *Food chem.*. **83**, 255-262, 2003.

Exarchou, V., Nenadis, N., Tsimidou, M., Gerothanassis, I., Troganis, A. & Boskou, D.: Antioxidant Activities and Phenolic Composition of Extracts from Greek Oregano, Greek Sage, and Summer Savory. *J. Agric. Food Chem.*, **50,** 5294-5299, 2002.

Fleisher A., Fleisher Z.: Aromatic plants of the Holy Land and Sinai., Chemical composition of *Origanum syriacum* L. essential oil. *J Essential Oil Res.*, **3** (2), 121-123, 1991

Farag R., Badel Z., Baroty G.*:* Influence of thyme and clove essential oils on cottonseed oil oxidation. *J Am Oil Chem Soc.*, **66**, 800–804, 1989.

Gerothanassis, I., Exarchou, V., Lagouri, V., Troganis, A., Tsimidou, M. & Boskou, D.: :Methodology for identification of phenolic acids in complex phenolic mixtures by high-resolution two-dimensional nuclear magnetic resonance. Application to methanolic extracts of two oregano species, *J. Agric. Food Chem.*, **46**, 4185-4192,1998.

Halim A., Mashaly M., Zaghloul A., Abd-El-Fattah H., Pooter H., De-Pooter H.: Chemical constituents of the essential oil of *Origanum syriacum* and *Stachys aegyptiaca. Int J Pharmacognosy*, **29,**3, 183-187, 1991.

Hudaib M., Mohammad M., Bustanji Y., Tayyema R., Yousef M., Abuirjeie M., Aburjaia T.: Ethnopharmacological survey of medicinal plants in Jordan, Mujib Nature Reserve and surrounding area, *Journal of Ethnopharmacology*, **120,** 63–71, 2008.

Houhoula D., Oreopoulou V. and C. Tzia: Antioxidant efficiency of oregano in frying and storage of fried products. *Eur. J. Lipid Sci. Technol.*, **106,** 746–751, 2004.

Ishac G., Kharoub G.: The essential oils in Sa'atar. *Magazine of Bethlehem Univ.*, **4**, 162-175, **1985** (in Arabic)

Justesen, U. & Knuthsen, P. (2001) Composition of flavonoids in fresh herbs and calculation of flavonoid intake by use of herbs in traditional Danish dishes. *Food Chem.*, **73**, 245-250.

Kahkonen M., Hopia A., Vuorela H., Rauha J., Pihlaja K., Kujala T., Heinonen M., *J. Agric. Food Chem.*, **47**, 10 3954-3962,1999.

Karpinska M., Borowski J., Danowska-Oziewicz M.: The use of natural antioxidants in ready-to-serve food, Food Chemistry **72**, 5-9,2001.

Kivilompolo**,** M. & Hyötyläinen, T.: Comprehensive two-dimensional liquid chromatography in analysis of Lamiaceae herbs: characterisation and quantification of phenolic acids. *J Chromatogr A,* **1145**, 155-164, 2007.

Kokkini S., Karousou A., Dardioti N., Lanaras T.: Autumn essential oils of Greek oregano (*Origanuum vulgare subsp. Hirtum*), *Phytochemistry*, **44**, 883-886, 1996.

Kouri G., Tsimogiannis D., Bardouki H., Oreopoulou V.: Extraction and analysis of

antioxidant components from Origanum dictamnus, *Innov. Food Sci. Emerg. Technol.,* **8**, 155–162, 2007.

Letswaart J. (1980): A taxonomic revision of the genus *Origanum* Labiatae). The Hague: Leiden Univesity Press, Netherlands.

Lagouri, V., Blekas, G., Tsimidou, M., Kokkini, S. & Boskou, D.: Composition and antioxidant activity of essential oils from oregano plants grown wild in Greece. *Z. Lebens. Unters. Forsch.,* **197,** 20-23,1993.

Lu, Y. & Foo, L.:Rosmarinic acid derivatives from Salvia officinalis, *Phytochemistry,* **51**, 91-94, 1999.

Lu,Y. & Foo, L.: Polyphenolics of Salvia – A review. *Phytochemistry,* **59**,117-140, 2002.

Lu, Y. & Foo, L.Y. (2000): Flavonoid and phenolic glycosides from Salvia officinalis. *Phytochemistry* **55**, 263-267.

Lu,Y. & Foo, L.Y. Polyphenolics of Salvia – A review. *Phytochemistry,* **59**, 117-140, 2002.

Miura K., Kikuzaki H.,Nakatani N.: Antioxidant activity of chemical composition from sage (*Salvia officinalis* L.) and thyme (*Thymus vulgaris* L.) measured by the oil stability index method. *J. Agric. Food Chem.*, **50**, 1845–1851, 2002.

Nychas, G. (1995): Natural antimicrobials from plants. In: *New methods of food preservation,* eds. Gould, G., Blackie Academic Professional, London, UK, pp. 58–89.

Nepote, V., Grosso, N.R.; Guzman, C.A. Optimization of extraction of phenolic antioxidants from peanut skins. *J. Sci. Food Agric.* **2005**, *85*, 33-38.

Okamura, N., Haraguchi, H., Hashimoto, K. & Yagi, A.: Flavonoids in Rosmarinus officinalis leaves. Phytochemistry 37, 1463-1466, 1994.

Pokorny, J.: Natural antioxidants for food use. Trends in Food Sci.Technol., **9**, 223-227, 1991.

Putievsky E., Dudai N., Ravid U. (1996): Cultivation, selection and conservation of oregano species in Israel. In: *Proceedings of the IPGRI International Workshop on Oregano,* 8-12 May, CIHEAM, Valenzano (Italy), pp. 102-109.

Ravid U., Putievsky E.: Constituents of essential oils from *Majorana syriaca*, *Coridothymus capitatus* and *Satureja thymbra*. *Planta Med.*, **49**, 248-249, 1983.

Reddy V., Urooj A., Kumar A.: Analytical, Nutritional and Clinical Methods Evaluation of antioxidant activity of some plant extracts and their application in biscuits, *Food Chem.,* **90**, 317–321, 2005.

Shan, B., Cai, Y.Z., Sun, M. & Corke, H.: Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. J. Agric. Food Chem., 53, 7749-7759, 2005.

Shimoni M., Putievsky E., Ravid U., Reuveni R.: Antifungal activity of volatile fractions of essential oils from four aromatic wild plants in Israel. J. Chem Ecology, 19, 1129-1133, 1993.

Singh, G., Kiran S., Marimuthu,P., Isidorov V. & Vinogorov V.: Antioxidant and antimicrobial activities of essential oil and various oleoresins of *Elettaria cardamomum* (seeds and pods), *J. Sci. Food Agric.,* **10,** *51*22–5142, 2007.

Salah S., Jager A.: Screening of traditionally used Lebanese herbs for neurological activities. *J. Ethnopharmacology,* **97**, 145-149, 2005.

Said, O., Khalil, K., Fulder, S., Azaizeh, H.,:Ethnopharmacological survey of medicinal herbs in Israel, the Golan Heights and the West Bank region. *J.of Ethnopharmacology* **83,** 251–265. 2002.

Schuler P. (1990): Natural antioxidants exploited commercially, In *Food Antioxidants*, (ed.). Hudson B. Elsevier, London, UK,pp. 99-170.

Sokmen A., Gulluce M., Akpulat H.,Daferera D., Tepe B., Polissiou M., Sokmen M., Sahin F.: The in vitro antimicrobial and antioxidant activities of the essential oils and methanol extracts of endemic Thymus spathulifolius. *Food Control* **15,** 627–634, 2004.

Salah S., Jager A.: Screening of traditionally used Lebanese herbs for neurological activities. *J Ethnopharmacology,* **97**, 145-149, 2005.

Said, O., Khalil, K., Fulder, S., Azaizeh, H.,:Ethnopharmacological survey of medicinal herbs in Israel, the Golan Heights and the West Bank region. *Journal of Ethnopharmacology* **83,** 251–265. 2002.

Schuler P. (1990): Natural antioxidants exploited commercially, In *Food Antioxidants*, (ed.). Hudson B. Elsevier, London, UK,pp. 99-170.

Sokmen A., Gulluce M., Akpulat H.,Daferera D., Tepe B., Polissiou M., Sokmen M., Sahin F.: The in vitro antimicrobial and antioxidant activities of the essential oils and methanol extracts of endemic Thymus spathulifolius. *Food Control* **15,** 627–634, 2004.

Tepe B.: Antimicrobial & antioxidative activities of the essential oils methanol extracts of Salvia crypthantha and Salvia multicualis, *Food Chem.*, **84**, 519-525, 2004.

Tsimidou M. , Papavergou E. & Boskou D.: Evaluation of oregano antioxidant activity in mackerel oil, *Food Research International*, **28**, 4, 431-433, 1995.

Tume G., Baser K.: The essential oil of *Origanum syriacum* L. var. *bevanii* (Holmes) *J Essential Oil Res.*, **5**(3), 315-316, 1993

Tsimogiannis, D., Oreopoulou V.: Defining the Role of Flavonoid Structure on Cottonseed Oil Stabilization: Study of A- and C-Ring Substitution. *J Amer Oil Chem Soc* **84**, 129–136, 2007.

U.S. Food and Drug Administration (2006): Food additive status list. Electronic reference, retrieved 6.1.2008 from: http://www.cfsan.fda.gov/~dms/opa-appa.html.

Vichi S., Zitterl-Eglseer K., Jugl M., Franz C.: Determination of the presence of antioxidants deriving from sage and oregano extracts added to animal fat by means of assessment of the radical scavenging capacity by photochemiluminescence analysis. *Nahrung*. **45**, 101–104, 2001.

Watson, L. & Dallwitz, M.J. (2007): The families of flowering plants: descriptions, illustrations, identification, and information retrieval. Electronic reference, retrieved 6.11.2007 from: http://deltaintkey.com/angio/www/labiatae.html.

Yesil-Celiktas, O., Girgin, G., Orhan, H., Wichers, H., Bedir, E. & Vardar-Sukan, F.: Screening of free radical scavenging capacity and antioxidant activities of Rosmarinus officinalis extracts with focus on location and harvesting times. Eur. Food Res. Technol. **224**,443-451, 2007.

Zheng, W. & Wang, S.: Antioxidant activity and phenolic compounds in selected herbs. *J Agric. Food Chem.* **49**, 5165-5170, 2001.

Zgórka, G. & Glowniak, K. (2001): Variation in free phenolic acids in medicinal plants belonging to the Lamiaceae family. J Pharm Biomed Anal 26:79-87 *J Agric Food Chem* **49,** 5165-5170, 2001.

Chapter Four

Materials and Methods

4.1 Scope

Natural antioxidants are gaining an increasing attraction in food technology as a substitute for synthetic ones. The main scope of this study is to extract and characterize the components of *Majorana Syriaca* to examine its antioxidant activity being not investigated in details.

First target was to separate the antioxidant components of the plant by solvents of variable polarities, to evaluate the radical scavenging ability of the plant extracts through DPPH scavenging, and to explore the effect of adding the plant extracts on oil stability in accelerated oxidation tests.

The second parts of the work were focusing on using a one-step extraction by ethyl acetate for further oxidations tests. The aim of the following experiments was to investigate the applicability of the plant in lipid-containing food systems and its ability to inhibit oxidation in thermal oxidation (cookies and deep-frying), to examine the plant

extract in important oil-based food applications such as emulsions under different accelerated oxidations

Pressure is used as an alternative for thermal processing; for that reason, it was also interesting to find out the effect of adding the plant extract on stabilizing emulsions samples subjected to high hydrostatic pressure (HHP). Fish products contain also essential fatty acids such as, ω3 and ω6 that are also susceptible to lipid oxidation. Tuna fish was targeted to study in order to find out the effect of adding the plant extract on inhibiting oil oxidation. The effect of adding *Majorana syriaca* on microbial growth was also targeted in tuna fish as a complimentary to its wide range of activity in keeping quality.

4.2 Experimental Design

4.2.1 Analysis and antioxidant activity of extracts from successive extraction

This study is employed on *M. Syriaca* grown up in Palestine. The dried herb was brought from local market of Bethlehem and used in a series of experiments studying intensively its antioxidative effect. In the first part, the goal was to explore its antioxidant activity in terms of solvents of extraction. The dried leaves of *M. syriaca* were ground to pass 5 mm sieve, then an amount of 34 g ground herb was subjected to successive extraction in a Soxhlet apparatus sequentially with solvents of increasing polarity. Amount of each of the solvents used were 400mL, specifically first was added petroleum ether (P), diethyl ether (D), ethyl acetate (EAc), and ethanol (E) in order to separate the components of the plant, determine the antioxidant activity of each fraction and relate it with the composition of the fraction. Furthermore, the main components of the P extract were analyzed according to GS-MS analysis. The main components of the other successive extracts of *M. syriaca* were detected according to analysis of data obtained first by HPLC-DAD analysis that was run in our laboratory, and HPLC–MS analysis done in Vioryl SA labs.

The antiradical efficiency of the extracts was measured using the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging test. Moreover, to test the antioxidant activity of the extracts(P,D,E and EAc), they were added to corn oil at concentrations of 200, 500 and 1000 ppm, and solvent was removed by purging nitrogen (N_2), then the oil was subjected to accelerated oxidation at 70 °C for a period of two weeks at which the oxidation was monitored periodically. By measurement of peroxide value (PV), as primary oxidation indicator, reliable, easy and generally used in oxidation tests. The conjugated dienes (CD) was also presented as primary oxidation indicator used for oils with unsaturated fatty acids like corn oil.

4.2.2 Antioxidant activity of One-step extraction by ethyl acetate (1-EAc)

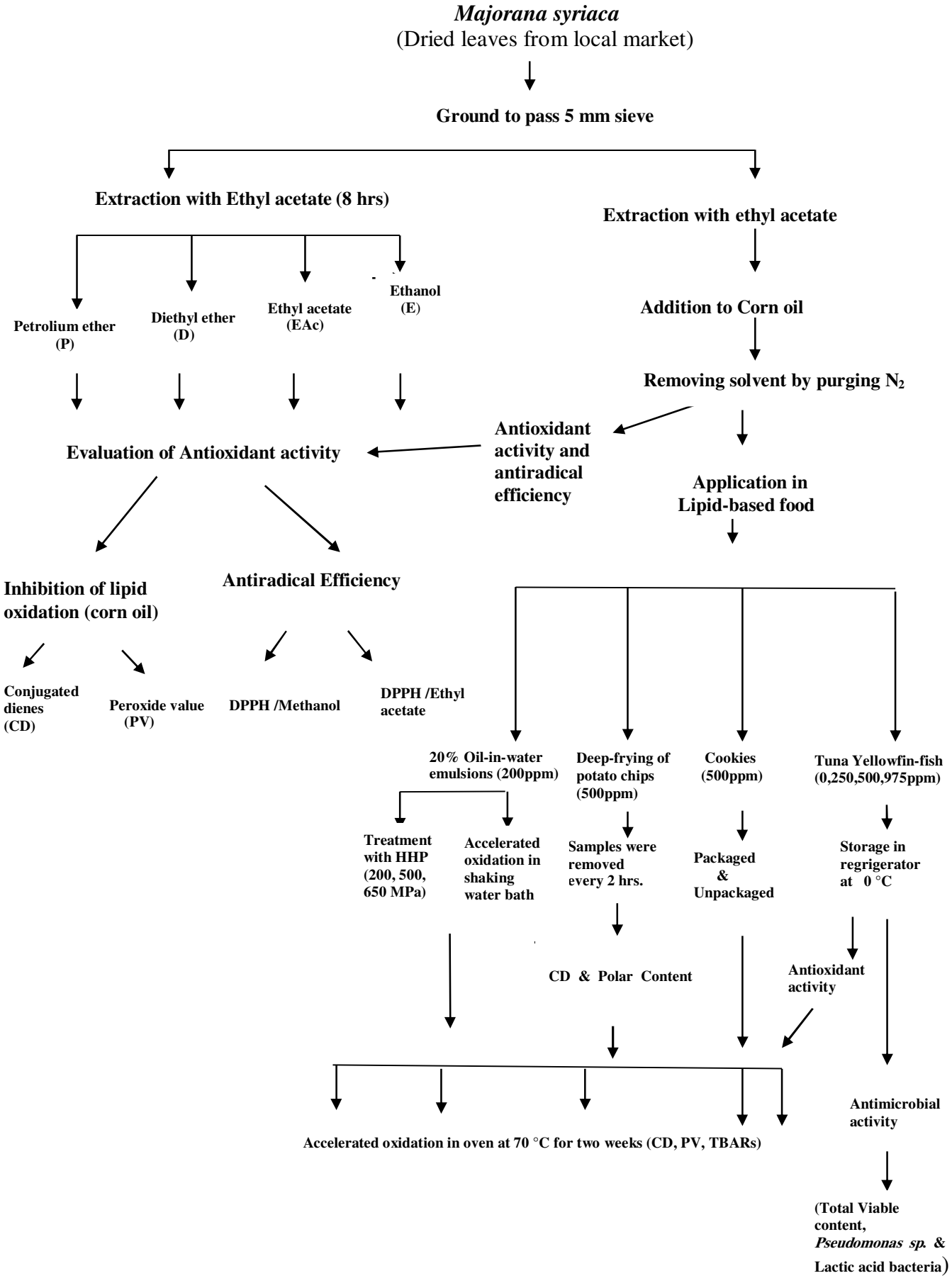
In the second part, *M. syriaca* was subjected to a simplified, one-step extraction by ethyl acetate (1-EAc) to recover the main antioxidant components that are soluble in oil. The main target was to simplify the extraction procedure and to collect the maximum of antioxidants using one step extraction. The antiradical and antioxidant activity of 1-EAc

was estimated as in part one and compared with the activity of the extracts obtained by the successive extractions. The one-step ethyl acetate (1-EAc) was chosed because it is expected to be suitable as a food-grade solvent, with appreciable antiradical and antioxidant efficiency as discussed in section 5.3.

The 1-EAc extract was added to corn oil and the solvent was removed by purging with N_2. The oil enriched with the extract was applied in different food model resembling oil-based food systems at variable conditions, that used were choosed based on their importance in recent food technologies and to and extend previous experiences done in our laboratory (deep-frying).

After examining the antioxidant activity of *M. syriaca* in bulk oil, it was applied in oil used in o/w emulsion to test the antioxidant activity within oil in emulsion system. The bulk corn oil was enriched with 200 ppm of the 1-EAc extract, and was used in making 20% oil-in-water emulsions, that were involved in two main experiments: in the first experiment the emulsions were subjected to oxidation in a shaking water bath at 40°C and 60°C for two weeks. In the second experiment, the emulsions were subjected to high hydrostatic pressure (HHP) treatment, followed storage in oven at 70°C for accelerated oxidation tests. The oil enriched with extract from *M. syriaca* was also employed in the preparation of high-temperature processed products, i.e. deep-fried potato chips, and baked cookies. More specifically, the extract was added at a concentration of 500 ppm to the fresh bulk corn oil used in frying for 16 h. The stability of frying oil was monitored by measuring polar content and CD every 2h. Then, the potato chips, were packaged and stored in oven at 70°C for accelerated oxidation tests. In the same manner, cookies were prepared by using corn oil enriched with 500 ppm of the extract. Samples of packaged and unpackaged cookies, were subjected to accelerated oxidation tests. The final approach was to test the antioxidant and antimicrobial potentiality of *M. syriaca* in a meat-type food, since they also contain lipids and susceptible to oxidation, and microbial spoilage. Since *M. syriaca* showed in literature antimicrobial activity, that has not been yet explored in tuna fish, and as a complementary to studies that were run in our lab about fish products, so this part of the study the focused on antioxidant and antimicrobial effect of the plant extract in Yellowfin tuna stored in refrigerator at 0°C. The extract

concentration varied in the range of 250 to 975 ppm, and the antioxidant activity was monitored periodically during storage, while the antimicrobial activity was assessed by determination of Total Viable content, Pseudomonas sp. and lactic acid bacteria. The oxidation tests in experiments of storage or accelerated oxidation included CD and PV, beside TBARs used specifically in emulsions and tuna tests. An outline of the experimental design is presented in Diagram 1.


Majorana syriaca
(Dried leaves from local market)
Ground to pass 5 mm sieve
Extraction with Ethyl acetate (8 hrs)
Extraction with ethyl acetate
Petrolium ether (P)
Diethyl ether (D)
Ethyl acetate (EAc)
Ethanol (E)
Addition to Corn oil
Removing solvent by purging N_2
Antioxidant activity and antiradical efficiency
Evaluation of Antioxidant activity
Application in Lipid-based food
Inhibition of lipid oxidation (corn oil)
Antiradical Efficiency
Conjugated dienes (CD)
Peroxide value (PV)
DPPH /Methanol
DPPH /Ethyl acetate
20% Oil-in-water emulsions (200ppm)
Deep-frying of potato chips (500ppm)
Cookies (500ppm)
Tuna Yellowfin-fish (0,250,500,975ppm)
Treatment with HHP (200, 500, 650 MPa)
Accelerated oxidation in shaking water bath
Samples were removed every 2 hrs.
Packaged & Unpackaged
Storage in regrigerator at 0 °C
CD & Polar Content
Antioxidant activity
Accelerated oxidation in oven at 70 °C for two weeks (CD, PV, TBARs)
Antimicrobial activity
(Total Viable content, *Pseudomonas sp.* & Lactic acid bacteria)

4.3 Materials

4.3.1 Materials

Plant source: Dried leaves of *M. syriaca* were brought from the local market of Bethlehem and ground in a mill (Retch ZM 1, Haan, Germany), to pass 5 mm sieve before extraction.

For cookies, white flour and sods were kindly donated from Mili Pan. Dakou (Avlona, Greece) and sugar was purchased from the local market.

Potatoes for deep-frying were brought from the local market.

Corn oil, a fresh and refined oil was kindly donated from (Minerva SA, Inofita, Greece), and used for the autoxidation experiments.

Raw tuna (Yellowfin tuna – *Thunnus albacares*) were brought from (CEFRICO, Vigo, Spain) slices (weight: 100 ± 10 g) were air-shipped to the laboratory, directly after slicing at the fish processing location (CEFRICO, Vigo, Spain).Samples were ice packed (0° C) in vacuum and transported within 1 day of purchase.

Sterilized Ringer solution (Merck Ringer Tablets in distilled water; Merck, Darmstadt, Germany) was used in microbial growth test.

4.3.2 Solvents and Reagents

Solvents of extraction: Petroleum ether, diethyl ether, ethyl acetate and ethanol 95% were used for the extraction of plant components. All the reagents were of analytical grade except ethanol (Sigma-aldrich-USA).

DPPH (purity 95%) was a product of Aldrich (St. Louis, MO, USA).

Tween-20 (2%) an emulsifier used for emulsions was purchased by Acros Organics (Geel, Belgium).

Sterilized ringer solution (Merck Ringer tables in distilled water; Merck, Darmstadt, Germany) was used in microbial growth test.

4.4 Instruments

A ventilated oven (Heraeus Instruments GmbH, Hanau, Germany), was used for the accelerated oxidation tests.

A **Vacuum oven** (Vacutherm, Heraeus, Germany) was used in drying samples of extracts under vacuum for calculation of solid content.

An evaporator (Buchi, Flawil, Switzerland) was used for evaporation of solvents.

A laboratory fryer (Bluesky, Zhe jiang, China) keeping temperature within a range of ±1° C was used.

A blender (Philips Cucina HR 2860, Germany) was used in mincing tuna fish. Another blender (Waring commercial, Torrington, CT, USA) was used for the extraction of lipids from tuna, cookies and potato chips, and also in the preparation of the emulsions.

A two-stage, high-pressure **homogenizer** APV Lab 1000 (Albertslund, Copenhagen Denmark) was used for making emulsions.

A low temperature incubator (Sanyo MIR 153; Ora-Gun, Gunma, Japan) were used for storage of tuna fish during experiment. Temperature in the incubators was recorded automatically (with electronic, programmable miniature data loggers (COX TRACER®, Belmont, NC, USA)).

The high pressure unit (Food Pressure Unit FPU 1.01, Resato International BV, Roden, Holland) was used in HHP treatement (Figure1a & b).

Figure 1: High hydrostatic pressure unit (HHP)

The high pressure unit comprised a pressure intensifier and a multivessel system consisting of six vessels of 45 mL capacity each, with a maximum operating pressure and temperature of 1000 MPa and 90°C, respectively (Figure 1). The pressure transmitting

fluid used was polyglycol ISO viscosity class VG 15 (Resato International BV, Roden, Holland). Process temperature in the vessels was achieved by liquid circulation in the outer jacket controlled by a heating cooling system.

A sealer (BOSS NT42N, Bad Homburg, Germany) was used for packaging emulsion samples.

Instruments used in the analysis and measurements

A digital spectrophotometer (Unicam Helios; Spectronic Unicam EMEA, Cambridge, UK) was used for experiments (Figure 2).

Figure 2: Digital spectrophotometer used for experiments.

For measurement of polar content, a glass column of 2.5 cm diameter and 38 cm height. (Winzer-Germany) was used.

Emulsion droplet size was measured with a static light scattering technique using Malvern Mastersizer Instrument (Micro, Malvern, Worcs, England) and NFD methodology.

GC-MS analysis was performed using an HP 6890 GC system (+1) coupled to an HP 5973 mass selective detector (Palo Alto, CA, USA).

The HPLC apparatus consisted of an HP 1100 gradient pump and a diode array detector (DAD) (Hewlett-Packard, Waldbronn, Germany). A Hypersil C18 column ODS 5 mm, 25064.6 mm (MZ Analysentechnik, Mainz, Germany) was used under thermostated conditions at 30 °C. ChemStation for LC 3D software (Agilent Technologies 1999–2000) was used for analysis of compounds.

For HPLC-MS analysis, Mass spectra were obtained *via* liquid chromatographic introduction into a 1100 Series LC/MSD Trap SL mass spectrometer (Agilent Technologies, USA), equipped with a HP 1100 Series gradient HPLC system (Agilent Technologies). The chromatographic separation of the compounds was achieved using a Zorbax Eclipse XDB-C18 column (4.66250 mm, 5 mm).

4.5 Extraction of Antioxidants

The ground material was subjected to **successive extraction in a Soxhlet** apparatus (Figure 3) using solvents ranging in polarity, starting by petroleum ether diethyl ether, ethyl acetate, and ethanol, until exhaustion of the color (8-16 h).

Figure 3: Soxhlet extraction apparatus

The distillation apparatus was fixed as shown and the dried leaves of the plant were gound to pass 5 mm sieve and moisture content in ground material was determined (1.45%). Then the ground material was filled in a cartoon tube (34 g), the solvent was

added to the round bottom flask to the limit (400 mL). The duration of each extraction was 8h, and the temperature was controlled for mild boiling point of each solvent. (D, 35°C, P, 66°C, EAc, 76°C, E, 78 °C).The extract was collected and the solid content was calculated by measuring the solid residue after evaporating a sample of 1 mL of the extract in vacuum oven (measured in triplicate). The yield of each extract was calculated as:

Yield = [(Solid content *volume collected)/weight of input material]*100%

The amounts of the total extracted compounds were determined in triplicate and expressed on dry basis.

Additionally, a one-step extraction using ethyl acetate only was run; the ground material was subjected to extraction with ethyl acetate in a Soxhlet apparatus until the exhaustion of the color.

4.6 Sample preparations

To test the extract from *M. syriaca* in different food applications, samples of lipid-based foods were preapared in the laboratory as an approach. First was used the bulk corn oil, followed o/w emulsion, potato chips, cookies and tuna.

4.6.1 Oil samples

In part one and two, the activity of the extracts (P, D, EAc, E) obtained from successive extraction, and from the one-step extraction with ethyl acetate only, were tested against the autoxidation of corn oil. The oil was enriched with each of the extracts at concentrations 200, 500, and 1000 ppm (expressed as dry matter in oil). A specific volume of each extract was added in the corresponding quantity of the oil, followed by continuous stirring and purging with nitrogen until total removal of the solvent. Each sample (50.00 g) as well as pure corn oil were transferred into identical 100-mL open beakers and subjected to accelerated oxidation at in a ventilated oven. The values of PV and CD was measured every two days, during the oxidative process through a period of two weeks where it was needed to achieve high level of oxidation.

4.6.2 The oil-in-water emulsions were prepared by using oil enriched with ethyl acetate extract obtained from *M. syriaca* by one-step extraction. An oil containing 200 ppm extract (as prepared in 4.5.1) was used in emulsion preparation. Oil-in-water (20% w/w) emulsions were prepared by mixing the oil containing the extract for 10 min in a blender (Waring Commercial, Torrington, CT, USA) with distilled water containing 2% Tween-20 as emulsifier. The pre-emulsion passed through a high-pressure valve, two-stage APV Lab 1000 homogenizer (Albertslund, Copenhagen Denmark) at 200 bars.

The o/w emulsions were subjected to accelerated oxidation in shaking water bath at 40 °C and 60 °C. On the other hand, in another experiment the emulsions were subjected to HHP as described in 4.5.3.

4.6.3 The high hydrostatic pressure treatment (HHP) was conducted as follows: Samples (bulk oil or oil-in-water emulsion) of 5 g were packaged in polypropylene bags and sealed to expel headspace air (BOSS NT42N, Bad Homburg, Germany).

HHP experiments were conducted in triplicate at two different pressures, 200 and 650 MPa for time 6 min. The temperature was kept constant at 25 (± 1.5)°C. Non-HHP treated emulsions were used as control samples. The high pressure unit (mentioned in section 4.3.2 p 97) was set and vessels were isolated.

4.6.4 Deep-frying of potato chips

Potatoes were peeled, soaked in water, and sliced to thickness of 2 ± .01 mm. A laboratory fryer, keeping temperature within a range of ±1 °C was used. Frying experiments were conducted with pure oil and oil containing 500 ppm of *M. Syriaca* extract, which was obtained from the one-step extraction with ethyl acetate according to part two (as explained 4.5.1). Frying experiments were conducted with 2 kg of refined corn oil at 185 °C directly after slicing the potato chips as follows: 100 g of sliced potatoes, after the removal of excess water with a filter paper, were put into a frying basket (stainless steel) and fried for 3 min. The basket was lowered into and raised from

the fryer manually. Successive fryings were run every 40 min with a new batch of 100 g of potatoes each time, until 16 h of frying had elapsed. Oil samples (10 g) were removed from the fryer every 2 h. Batches of potato chips were removed from the fryer every 2 h, separated into portions of 5 g each, packaged by polypropylene bags, and thermosealed. The bags then were kept in an oven at 70 °C for accelerated oxidation tests until opened for analysis. The oxidation rate was monitored by measuring CD and PV of the oil extracted from potato chips every two days.

4.6.5 Preparation of cookies

Cookies were prepared using pure corn oil or corn oil enriched with 500 ppm of *M. Syriaca* extract, obtained from the one-step extraction with ethyl acetate. The components of the cookies formula (150 g oil, 800 g flour, 10 g sods, 150 g sugar and 250 g water) were mixed at low speed for 2 min and at high speed for 5 min, and the obtained dough was shaped as cookies of 3 mm thickness and 4 mm diameter. The cookies were baked at 160 °C for 30 min. After baking they were left to cool at room temperature. The baked cookies were divided into 2 parts: one part was separated in portions of 25 g and packaged with laminated paper (PVC) and another part was not packaged. Both types were subjected to accelerated oxidation at 70 °C for about two weeks. The oxidation rate was monitored by measuring conjugated dienes content and peroxide value of the oil extracted from the cookies.

4.6.6 Preparation of minced tuna fish

Yellowin tuna slices 100 ± 10 g were air-shipped to the laboratory, directly after slicing at the fish processing location. Samples were ice packed (0° C) in vacuum and transported within 1 day of purchase.

Figure 4 Samples of raw fish material.

Preparation of tuna fish was as follows: Fish slices were removed from the packaging in a laminar flow hood, cut and minced using a clean and sanitized blender (Philips Cucina HR 2860, Germany). Afterwards, the batch was divided into portions of 150 g, and each portion was mixed with corn oil (tuna/oil: 3/1 w/w) containing different concentrations of *M. syriaca* extract (0, 250, 500, 750 and 975 ppm) until the samples were homogenized. Minced fish without oil was also studied. Samples of 20 g were aerobically packed in polyethylene bags and stored at controlled isothermal conditions of 0°C ±0.2°C in low temperature incubators (Sanyo MIR 153; Ora-Gun, Gunma, Japan). Temperature in the incubators was constantly recorded with electronic, programmable miniature data loggers (COX TRACER®, Belmont, NC, USA). Duplicate samples were taken in appropriate time intervals (24 or 48 h) during a 15-day period to allow for the study of lipid oxidation and microbial growth. All determinations were made in duplicate and in two different single specimens.

4.6.7 Oil extraction for oxidation tests

In potato chips experiment an amount of 5 g of potato chips was blended with 30 mL of (1:1) petroleum ether/diethyl ether in a blender (Waring Commercial, Torrington, CT,

USA) for 2 min. The mixture was filtered and the solid was reblended again with 20 mL of (1:1) petroleum ether/diethyl ether for 1 min.

In cookies, an amount of 15 g of cookies was ground and blended with 50 mL of (1:1) petroleum ether/diethyl ether in a blender (Waring Commercial, Torrington, CT, USA) for 2 min. The mixture was filtered and the solid was re-blended again with 40 mL of (1:1) petroleum ether/diethyl ether for 1 min.
The filtrates received from cookies or potato chips were combined and the solvent was removed through evaporation in a vacuum oven at 50 °C. The oil was collected and used in oxidation measurements.

In tuna experiment, oil was extracted according to the method of Lee et al. (1996). Twelve grams of sample were added to 50 mL of chloroform ⁄ methanol (2:1) and blended (Waring commercial) for 90 s. The mixture was filtered and the solid was re-blended again with 30 mL of chloroform⁄ methanol (2:1). The mixture obtained was filtered and the filtrates were combined. To separate the combined filtrate into two phases (water-methanol and chloroform), 20 mL of 0.5% NaCl was added. The mixture was shaken gently and allowed to stand until a clear separation was visible. The chloroform layer was collected and left to evaporate in a vacuum oven at 45 °C (Heraeus, Germany) for 2 h. The obtained oil was weighed and processed for oxidation measurements.

4.7 Antioxidant activity assays

4.7.1 DPPH test

The antiradical efficiency of the extracts obtained in part one and two, was determined using the DPPH test. The absorption of DPPH radical at 515 nm was monitored, according to the method proposed by Brand-Williams et al., 1995 as follows: A constant volume (3.9 mL) of a daily prepared DPPH solution of 6.06 x10^{-5} M in methanol or ethyl acetate was placed in a cuvette, and 0.1 mL of an extract solution in the solvent used in the analysis was added. The extract solutions were prepared by mixing the appropriate volume of the liquid extract with the solvent in order to obtain concentrations in the range of 300–10,000 g extract/kg DPPH in the cuvette. The cuvette was capped, shaken, placed

in the spectrophotometer and the absorbance was recorded at 515 nm. In a second cuvette, a blank DPPH solution was screened to determine the decomposition rate of the radical to get accurate measurements.

The DPPH has a deep purple colour and absorbs strongly at 515 nm, whereas the yellowish reduction product does not. A calibration curve correlating the DPPH absorption with concentration was constructed by measuring the absorption of solutions containing DPPH at known concentrations. The antioxidant activity was evaluated by the EC_{50} parameter, which represents the mass (dry material) of extract necessary to reduce 50 % of the DPPH. Obviously, the lower the EC_{50}, the higher is the antiradical activity.

4.7.2 Peroxide value

The oxidative process of bulk corn oil samples, or of oil extracted from chips, cookies or tune was monitored by the determination of the peroxide value according to IUPAC official method 2.501 (1987). An amount of 1.0 g of oil sample was dissolved in 25 mL of a solution of acetic acid: chloroform (2:1), then 1 mL of saturated potassium iodide was added and the sample was kept in dark for 5 minutes. The sample was diluted by 50 mL distilled water and titrated with sodium thiosulfate (0.01 N). The PV was calculated as follows:

$$PV = (V*T)/m *1000 \qquad \text{equation (1)}$$

Where:

V represents volume of titration (mL)

T is the Normality of sodium thiosulfate (0.01N)

m is the mass of oil in test (g)

and PV is expressed in meq oxygen (O_2) kg^{-1} oil.

In the experiment with tuna, where oil was in combination with tuna, the method was slightly changed. Oil was extracted from tuna samples according to the method of Lee, Trevino and Chaiyawat (1996). An amount of 12 g of sample was added to 50 mL of chloroform/methanol 2:1, and blended for 90 s at high speed. The mixture was filtered

and the solid was re-blended again with 30 mL of chloroform/methanol 2:1. The mixture obtained was filtered and the filtrates were combined. To separate the combined filtrate into two phases (water-methanol and chloroform), 20 mL of 0.5 % NaCl were added. The mixture was shaken gently and allowed to stand until a clear separation was visible. The chloroform layer was collected and left to evaporate in a vacuum oven at 45 °C (Heraeus, City) for 2 h. The obtained oil was weighed and PV was measured according to IUPAC official method 2.501.

4.7.3 Conjugated dienes

Conjugated dienes were determined according to the IUPAC standard method 2.505 (IUPAC, 1987). For oil samples, an amount of 0.1 g was diluted in 100 mL isooactane and mixed thoroughly. The absorbance was measured at 232 nm using a UV–VIS scanning spectrophotometer (Unicam Helios). The amount of CD in the oxidized oil was calculated by monitoring absorbance at 232 nm and using the relative molecular mass (280 g/mol) and the molar absorptivity of linoleic acid (e=26,000) (Kiokias et. al., 2006). The concentration was calculated as follows:

CD= 1.0769/C*A (g CD/100g oil) equation (2)

Where

C is the final concentration of the oil in cuvette in (g/L)

And

A is the absorption

For o/w emulsions, a modification of the method has been used. More specifically, the emulsion sample (20 μL) was added to a mixture of 10 mL isooctane/2-propanol (2:1 v/v) and vortexed (1 min), and the absorption measured. The amount of CD was calculated according to equation 2.

For Potato chips, the measurement of PV and CD was applied on the oil extracted from samples as follows: An amount of 5 g of potato chips was blended with 30 mL of (1:1) petroleum ether / diethyl ether in a blender (Waring Commercial, Torrington, CT, USA) for 2 min. The mixture was filtered and the solid was reblended again with 20 mL of (1:1) petroleum ether / diethyl ether for 1 min.

The oil was extracted from cookies as follows: An amount of 15 g of cookies was ground and blended with 50 mL of (1:1) petroleum ether / diethyl ether in a blender (Waring Commercial, Torrington, CT, USA) for 2 min. The mixture was filtered and the solid was re-blended again with 40 mL of (1:1) petroleum ether / diethyl ether for 1 min.

4.7.4 Thiobarbituric acid reactive substances (TBARs) were determined according to an adapted method of McDonald & Hultin, 1987. An amount of 3g of sample was added to 15.0 mL of water and vortexed thoroughly. A portion of 1.0 mL was taken, added to 2.0 mL of TBA solution (prepared by mixing 15 g trichloroacetic acid, 0.375 g thiobarbituric acid, 1.76 mL 12 N HCl and 82.9 mL H_2O) in test tubes, and placed in a boiling water bath for 15 min.

The tubes were cooled to room temperature for 10 min and centrifuged (2000 x g) for 15 min. The absorbance of the supernatant was measured at 532 nm. The concentration of TBARs was calculated from a standard curve prepared by 1,1,3,3-tetraethoxypropane.

4.7.5 The content of polar compounds was evaluated by the column chromatographic method IUPAC 2.505, 1987 using a glass column of 2.5 cm diameter and 38 cm height. (Winzer-Germany). The column was filled with 30 mL of petroleum ether and diethyl ether (87:13). A solution of 25 g silica was dissolved in 80 mL of the petroleum ether and diethyl ether mixture and added to the column. Then the column was filled with 150 mL of the petroleum ether and diethyl ether solution and left to settle down leaving 10 cm on top of filling material. An amount of 2.5 ± 0.1 g of oil was collected from the frying batch, weighed in volumetric flask, dissolved in the petroleum ether and diethyl ether solution and mixed well. The solution was then added to the column and the column was opened to let the solution to pass through silica gel for 1 h with stable and slow rate. The nonpolar content was collected in a round bottom flask and weighed after evaporating the

solvents by a rotatory evaporator (Buchi, Flawil, Switzerland). The polar content was calculated as follows:

polar content= $(m-m_i)/m \times 100\%$ equation (3)

Where m is the mass of oil sample (2.5 ± 0.1 g), and m_i is nonpolar amount (g).

4.7.6 Estimation of antioxidant activity

To describe the efficiency of antioxidants added to the bulk oil system during accelerated oxidation period, a term called oxidation factor is used. The oxidation factor OF indicates the change of peroxide values, conjugated dienes and TBARs (OF_{PV}, OF_{CD}, OF_{TBARs} observed in the sample with the additive during the oxidation experiments compared to the relevant change of control samples and is calculated by the following equations:

$$OF_{PV} = (PV_{final} - PV_{initial})_{sample\ with\ additive}/(PV_{final} - PVi_{nitial})_{control\ sample} \quad \text{equation 4}$$

$$OF_{CD} = (CD_{final} - CD_{initial})_{sample\ with\ additive}/(CD_{final} - CD_{initial})_{control\ sample} \quad \text{equation 5}$$

$$OF_{TBARs} = (TBARs_{final} - TBARs_{initial})_{sample\ with\ additive}/(TBARs_{final} - TBARs_{initial})_{control\ sample} \quad \text{equation 6}$$

4.8 Dissolved oxygen measurement

The Winkler test (Winkler, 1888; Sapogova et. al. 2002) was used for the determination of the amount of oxygen dissolved in oil and oil-in-water emulsion samples, immediately after HHP treatment. An amount of 25 mL of the test sample was put in a volumetric flask (25 mL) and 0.1 mL of $MnSO_4$ (48%) was added immediately and mixed thoroughly and carefully without letting in air. Then 0.2 mL of alkaline KI (15% in KOH 70%) was added and mixed again without letting in air. A pinkish-brown precipitate appears which means that dissolved oxygen in the mixture has oxidized the Mn(II) ions to Mn(III). Following, 0.3 mL of sulfuric acid (50%) was added to the pinkish solution, mixed and allowed to stand for 2 min. The mixture was titrated by $Na_2S_2O_3$ (0.31%) using starch (0.1%) as an indicator. The amount of dissolved oxygen was calculated from the stoichiometric equation:

1 mole of O_2 ⟶ 4 moles of $Mn(OH)_3$ ⟶ 2 moles of I_2 equation (7)

4.9 Droplet size measurement

Emulsion droplet size was measured with a static light scattering technique (Gelin, Poyen, Courthaudon, Le Meste, & Lorient, 1994) by using a Malvern Mastersizer Instrument (Micro, Malvern, Worcs, England) and NFD methodology. The mean droplet diameter-$d_{3.2}$ (μm) of the emulsions was calculated as following:

$$d_{3.2}=\Sigma n_i d_i^3 / \Sigma n_i d_i^2 \qquad \text{equation (8)}$$

where n_i is the number of droplets with diameter d_i.

4.10 Microbial growth tests

Microbiological analysis and enumeration were run as follows: A representative sample (10 g) was transferred to a sterile stomacher bag with 90 ml sterilized Ringer solution (Merck Ringer Tablets in distilled water; Merck, Darmstadt, Germany) and was homogenized for 60 s with a Stomacher (BagMixer® interscience, France). Samples (0.1 ml) of 10-fold serial dilutions of fish homogenates were spread on the surface of the appropriate media in Petri dishes for enumeration of different spoilage bacteria (Koutsoumanis et al., 2002). The total aerobic viable count was enumerated on plate count agar (PCA; Merck) after incubation at 25°C for 72 h. *Pseudomonas* sp. were enumerated on cetrimide agar (CFC; Merck) after incubation at 25°C for 48 h. For *Lactobacillus* enumeration, the pour-plate method on de Man-Rogosa-Sharpe Agar (MRS; Merck) was used, followed by incubation at 25°C for 96 h. Two replicates of at least three appropriate dilutions were enumerated. The microbial growth was modelled using the Baranyi growth model (Baranyi & Roberts, 1995) applied by DMfit software for the curve fitting. Kinetic parameters, such as the rate (k) of the microbial growth, were estimated and % reduction rate was determined as:

% reduction = 100 (k_o-k_f)/ k_o equation (9)

where k_o and k_f are the rates of microbial growth in control and treated samples, respectively.

4.11 Analysis of the extracts

4.11.1 GC-MS analysis

The petroleum ether extract was analyzed by GC-MS, since it is expected to extract the less polar compounds, which are volatile; therefore, GC-MS analysis was applicable for their identification. The constituents of the extract were separated using an HP-1MS column (30 m length, 0.25 mm i.d., 1.00 mm film thickness; Palo Alto, CA, USA), as described by Tsimogiannis et al. (2006): The oven temperature was kept stable for 2.5 min at 40 °C and then increased to 300°C at a rate of 20°C/min. Helium was used as the carrier gas at an average velocity of 28 cm/s (0.6 mL/min flow). The tentative identification of the compounds was made by comparison of their mass spectra with the data of NIST and Wiley mass spectral libraries. The analysis was conducted at the laboratory of Chemical process Engineering, in National Technical University of Athens.

4.11.2 HPLC-DAD analysis

The HPLC-DAD method proposed by Mercken (2000) was used in order to separate the components of the fractions obtained with diethyl ether, ethyl acetate, and ethanol. The HPLC apparatus consisted of an HP 1100 gradient pump and a diode array detector (DAD). A Hypersil C18 column ODS 5 mm, 250x 4.6 mm was used under thermostated conditions at 30 °C. The analysis was performed as described by Kouri et al (2006). The solvent system consisted of water (A), methanol (B) and acetonitrile (C), each containing 0.2% trifluoroacetic acid. The initial composition of the mobile phase was 90% A, 6% B and 4% C. With linear gradients, the composition changed to 85% A, 9% B and 6% C within 5 min, 71% A, 17.4% B and 11.6% C within 30 min, and 0% A, 85% B and 15% C within 60 min. The flow rate was maintained at 1 mL/min. The elaboration of the

chromatographic data was carried out on a ChemStation for LC 3D software (Agilent Technologies 1999–2000).

4.11.3 HPLC-MS analysis

Mass spectra were obtained at Vioryl S.A. according to Kouri et al. (2006) via liquid chromatographic introduction into a 1100 Series LC/MSD Trap SL mass spectrometer (Agilent Technologies, USA), equipped with a HP 1100 Series gradient HPLC system (Agilent Technologies). The chromatographic separation of the compounds was achieved using a Zorbax Eclipse XDB-C18 column (250 x 4.6 mm, 5 mm) and the analysis was performed by the aforementioned elution system. The MS system was operated in electrospray ionisation (ESI) mode. The typical operating parameters were as follows: nebuliser, 50 psi; dry temperature, 350°C; dry gas, 12 L/min. Mass spectra were acquired in an m/z range of 50–1000 with a target mass of 300 m/z. It was possible to acquire data in positive and negative modes in a single LC run, using the continuous polarity switching ability of the mass spectrometer.

4.12 Statistical analysis

The oxidation experiments were carried out in duplicate or triplicate. The results were averaged and standard deviation was obtained using Excel. To analyze results statistically, the significant difference between variable data obtained the two-way or one-way ANOVA test (p, 0.05) was used, according to Statistica 7.0 statistical programme by comparison between means according to Duncan's multiple range test. One-way anova was used to analyze significant difference between oxidation factors.

References

Ancerewicz J., Migliavacca E, Carrupt P., Testa B., Bree F., Zini R., Tilliment J., Labidalle S., Guyot D., Chauvet-Monges A., Crevat A., Le Ridant A.: Structure-property relationships of trimetazidine derivatives and model compounds as potential antioxidants. *Free Radic Biol Med.*, **25**, 113–120, 1998.

Brand-Williams W., Cuvelier M., Berset C.: Use of a free radical method to evaluate antioxidant activity. *Lebensm Wiss Technol.*, **28**, 25–30, 1995.

Tsimogiannis D., Stavrakaki M., Oreopoulou V.: Isolation and characterisation of antioxidant components from oregano (Origanum heracleoticum). *Int J Food Sci Technol.*, **41,(Suppl 1)**, 39–48, 2006.

IUPAC: *Standard Methods for the Analysis of Oils, Fats and Derivatives*. 7th Edn. Eds. C. Paquot, A. Hautfenne, Blackwell Scientific Publications, Oxford (UK) 1987.

Kiokias, S., Dimakou, C., Tsaprouni, I., & Oreopoulou, V.: Effect of compositional factors against the thermal oxidative deterioration of novel food emulsions. *Food Biophysics*, **1,** 115–123, 2006.

Kouri G., Tsimogiannis D., Bardouki H.,. Oreopoulou V: Extraction and analysis of antioxidant components from Origanum dictamnus. *Innov. Food Sci. Emerg. Technol.* 2006.

Lee, C., Trevino, B. & Caiyawat, M.. A simple and rapid solvent extraction method for determination total lipids in fish tissue. *Journal of AOAC International*, **79,** 487–492, 1996.

Mercken H., Beecher G.: Liquid chromatographic method for the separation and quantification of prominent flavonoid aglycones. *J Chromatogr A.*, **897**, 177–184, 2000.

McDonald, R. & Hultin, H. Some characteristics of the enzymic lipid peroxidation system in the microsomal fraction of flounder skeletal muscle. *J. of Food Sci.,* **52,** 15–21, 1987.

Chapter Five

Results and Discussions

5.1 Extraction Methods

5.1.1 Successive Extractions

Soxhlet extraction is frequently used to isolate flavonoids from solid samples using extraction solvents such as alcohols (methanol, ethanol, ethyl acetate, diethyl ether) (Stalikas, 2007), while the nonpolar parts can be extracted using nonpolar solvents. In this work, the extraction was done by four solvents of increasing polarity starting from less polar; petroleum ether (P), diethyl ether (D), ethyl acetate (EAc) and ethanol (E), as decribed in the experimental part (section 4.4). The yield of each extraction is presented in Table 1 and the overall yield (on dry basis) amounted to 23%. The petroleum ether recovered an appreciable amount of the extractable components of the plant, followed by ethanol, ethyl acetate and diethyl ether.

The comparison between means of the yeilds, according to one-way ANOVA analysis, revealed that there was no significant difference between D and EAc extracts, while lower yield was obtained by D.

Table1: Yield of successive extractions of *Majorana syriaca* with petroleum ether (P), diethyl ether (D), ethyl acetate (EAc), and ethanol (E) or one-step extraction.

Extract	Yield (%)±SD Dry basis
Petroleum ether (P)	9.0 ± 0.9^{a}
Diethyl ether (D)	3.4 ± 0.2^{b}
Ethyl acetate (EAc)	4.1 ± 0.3^{b}
Ethanol (E)	6.0 ± 0.3^{c}
One-step extraction (1-EAc) by ethyl acetate	12.6 ± 0.4^{d}

* Values bearing different letters are significantly different

The purpose of using successive extraction with different solvents for the extraction was to fractionate the components of the plant according to their polarity so as to investigate their activity and facilitate their identification. Petroleum ether was used to extract the less polar compounds including the constituents of the essential oil. The oil-free plant material was then treated with diethyl ether, a low-polarity solvent, which was found efficient for the extraction of low-polarity flavonoid aglycones in previous experimental works (Tsimogiannis et al., 2006; Kouri et al., 2007) while ethyl acetate was further used for the extraction of flavonoids with medium polarity, which might possess antioxidant activity, as was shown by Vekiari et al. (1993). The extraction with ethanol was performed to collect the residual, polar phenolics. Each extraction was conducted for at least 8 h, until the solvent leaving the extraction funnel was colourless.

5.1.2 One-step extraction using Ethyl Acetate

A second extraction was applied by one step using ethyl acetate solvent alone (1-EAc), since its polarity is moderate and it is a food-grade solvent. The objective was to use a simplified procedure to extract the active components of *M. syriaca* with a one-step extraction. Ethyl acetate is not expected to extract the most polar components extracted by ethanol, however these components show limited solubility in lipid foods and, therefore, limited use as antioxidants in these foods. The extraction procedure was done

twice and the average yield based on both experiments was 12.6 ± 0.4 % as shown in Table1 in section 5.1.1.

5.2 Analysis of extracts obtained from *M. syriaca*

5.2.1 Analysis of petroleum ether extract

The petroleum ether extract was analyzed by GC-MS as it contains the major components of the essential oil. The essential oil content in fresh *M. syriaca* leaves varies between 1.3 % and 1.8 %, according to Putievsky et al. (1996). The higher yield (9 %) obtained in our experiments with petroleum ether is expected, since it is referred to dry material, but might be also attributed to extraction of additional compounds, other than the essential oil components. The identified compounds are presented in Table 2. It is apparent that the main components of the extract are thymol amounting to 43.89 % of the determined compounds and carvacrol amounting to 42.72 %. *p*-Cymene and g-terpinene were also detected, in agreement with the results of other researches for *M. syriaca* species (Putievsky et al., 1996; Akguel & Bayrak., 1987; Baser, 1993).

Table 2: The analysis of components of petroleum ether extract of *M. syriaca* identified by GC-MS analysis.

Retention time (min.)	Compound	A^*_I (%)
9.13	p-cymene	3.30
9.47	gamma-terpinene	0.23
9.55	cis/trans-sabinene hydrate	0.96
9.84	cis-sabinene hydrate	0.36
10.53	1-4-terpinol	0.59
10.95	2-2,5-cyclohexandiene-1,4-dione	3.33
11.23	thymol	43.89
11.34	carvacrol	42.75
12.34	1,2 benzendiol	0.43
12.57	trans(beta)- caryophyllene	1.25
13.62	carbophyllene oxide	0.61

* Area percentage of each compound as determined by Chemstation integrator

5.2.2 Analysis of the extracts by HPLC

HPLC-DAD and HPLC-Mass Spectrometry were applied for the analysis of D, EAc and E extracts obtained from successive extraction. The identification of unknown flavonoids was based on the UV-Vis spectra, the fragments obtained in MS and the correlation with standard compounds.

The UV-Vis spectra has long been used for structural analysis of flavonoids, identified through UV spectral characteristics combined with retention time (R_t). Flavonoids show two main absorbance bands: band I with λ_{max} between 300 and 380 nm due to the B-ring, and band II with λ_{max} between 240 and 290 nm due to the A-ring (Santos-Buelga et al., 2003; Sakakibara et al., 2003). The spectral details for each flavonoid subgroup show that flavones have λ_{max} between 310 and 350 nm in band I, and flavonols between 350 and 385 nm. Band II of the aforementioned flavonoid subgroups is almost similar and shows a bathochromic shift as OH substituents on the A-ring increase (Sakakibara et al., 2003; Tsimogiannis & Oreopoulou, 2006). Due to lack of conjugation between the A- and B-rings, flavanols, flavanones and dihydroflavonols hardly show absorption at band I; they show one major band II between 277 and 295 nm and a shoulder between 300 and 330 nm. Flavan-3-ols show a small peak between 270 and 290 nm, whereas anthocyanins have a peak in band I between 460 and 560 nm (Sakakibara et al., 2003).

The fragmentation of each flavonoid and the dehydration and carbon monoxide losses of the $[M+H]^+$ produces specific fragments by the members of each subgroup, thus allowing the definition of the flavonoid subgroup. Moreover fragments resulting through fission of the C-ring are typical of each subgroup and can reveal the substitution of A- and B-rings, as presented in Figure 1, which shows the specific fragments of major compounds, that are detected by HPLC-MS.

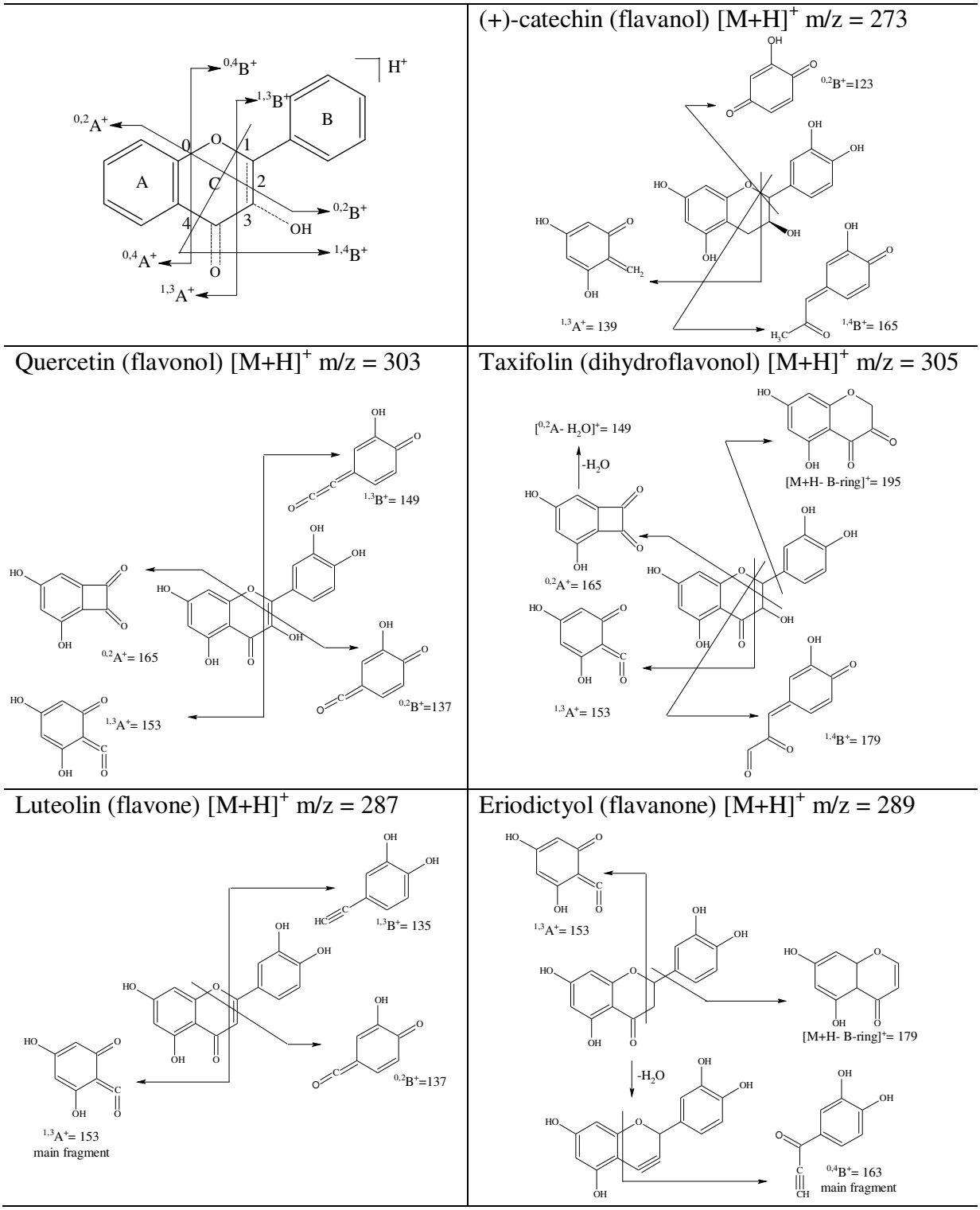


Figure 1: Specific diagnostic peaks of flavonoids resulting from cleavage in their C-ring and the mass *m/z* of their characterizing fragments in mass spectroscopic analysis. (Tsimogiannis et al., 2007)

5.2.2 a Analysis of the diethyl ether extract

Seven major peaks and several minor ones were observed in the HPLC chromatogram of the D extract (Figure 2). The characterization was based on retention times of the peaks (Rt), the UV spectra with combination of *m/z* of fragments in HPLC-MS analysis as presented in Table 3. Peak 1 amounted to 2% of the extract components according to % area. It was eluted at Rt =27.5 min with λ_{max} =288 nm and shoulder at 330 nm (Figure 1a in Annex, p 172) which is a characteristic spectra for dihydroflavonol that shows only one peak at band II due to the lack of conjugation between A and B ring. MS spectra showed a fragment corresponding to $[M+H]^+$ = 305 and several fragments due to fragmentation at C ring as explained in Figure 1. The detailed MS spectra is shown in Figure 1b of Annex, p 172). All these data were in accordance with the data obtained by the standard taxifolin, analysed in our laboratory (Tsimogiannis et al., 2007, Kouri et al., 2007), therefore peak 1 was identified as taxifolin. Peak 2 at Rt=36.6 min showed UV-spectra with λ_{max} =292 nm and shoulder 335 nm (Figure 2a of the Annex, p 173), which is characteristic for flavanone or dihydroflavanol, and main MS fragement $[M-H]^{2-}$=303 (Figure 2b of the Annex, 173). Peak 3 eluted at Rt= 41.4, as the standard eriodictyol. UV-spectra showed λ_{max} =288 nm at band II since it is a flavanone, while the MS spectra showed the main fragment with $[M-H]^-$=287 which is characteristic for eriodictyol (Figure 1). The MS spectra is shown in Figure 3b in Annex, p 174. Peak 4, according to its MS spectrum was identified to be carnosol as it was conbfirmed by internal standard in MS (Figure 4 in Annex). Peak 6 has the characteristic spectrum of flavones; m/z of the main fragment ions $[M+H]^+$=270 & $[M-H]^-$=269 correspond to apigenin but UV maxima are shifted to higher wavelengths (Figures 5a & b in Annex, p 176) could be due to increase of oxygentation on A-ring. Peak 5 did not present a clear UV spectrum due to poor separation, while peak 7 presented UV spectrum that did not resemble to any of the aforementioned flavonoid subgroups. A similar study done by Kouri et al. (2007) identified in the ethyl ether extract of *Origanum dictamnus* the existence of taxifolin at Rt=28.7 min and eriodictyol at 40.7 min which is close to Rt of same identidfied compounds by our study (m/z=303 for taxifolin m/z =287 for eriodictyol). Taxifolin,

eriodictyol and apogenin were reported in diethyl ether extract of *Origanum heracleoticum* by Tsimogiannis et al. (2006).

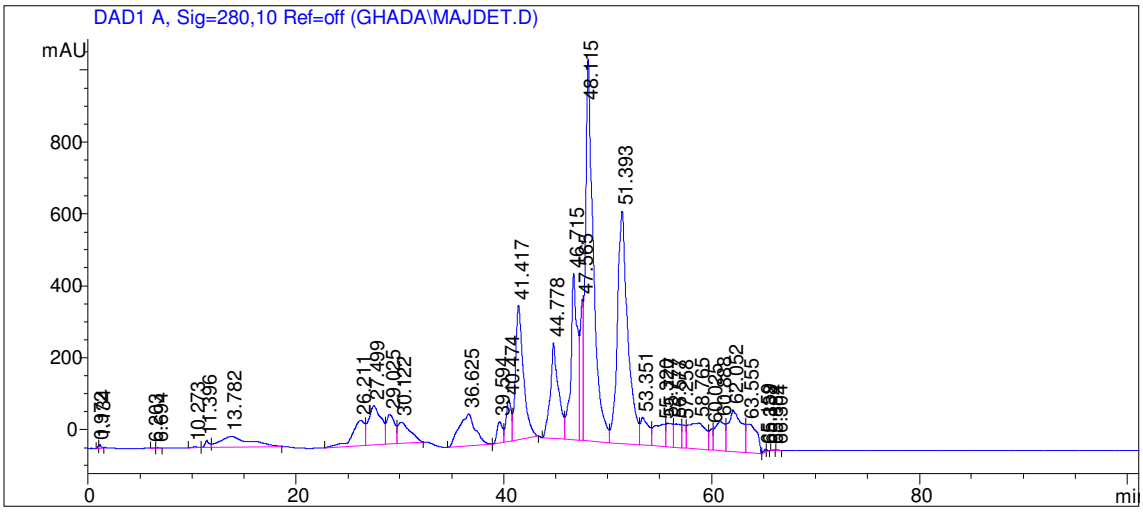


Figure 2 : The UV-spectra of diethyl ether extract at 280nm

Table 3: the analysis of diethyl ether extract (D) by HPLC-DAD and HPLC-MS

Peak	Rt (min)	% area	UV-max (nm)	m/z		Proposed structures
				$[M+H]^+$	$[M-H]^-$	
Diethyl ether extract						
1	27.5	2.1	288, 330 sh	305, 327	303	Taxifolin
2	36.6	3.5	292, 335sh		303†	Dihydroflavonol or Flavanone
3	41.4	9.9	288, 330sh		287	Eriodictyol
4	44.8	7.3	228, 292, 335sh		329	Carnosol
5	46.7	8.9	255, 305, 350	271, 331	287, 329	Poor separation
6	48.1	23.4	258, 346	270	269	Flavone
7	51.4	18.7	222, 278, 328sh		380	Unknown

† $[M-2H]^{2-}$

5.2.2b Analysis of ethyl acetate extract

The major peaks of the HPLC analysis of the EAc extract, together with their UV and MS characteristics are presented in Figure 3 and Table 4. Peak 4 at Rt =37.1 was identified as rosmarinic acid, which was confirmed by m/z =359 as it was used as a standard (Figures 8 a & b in the Annex, p179). Similarly peak 8 was identified as apigenin with maxima at 338 nm with m/z =271 (Figures 10 a & b in the Annex, p 181). Also in the ethyl acetate extract obtained from *Origanum dictamnus* apigenin was identified at Rt = 47.6 (Kouri et al., 2007). Peaks 2, 3, and 5 (Figures 6, 7 & 9 a & b in the Annex, p 177-180) have the characteristic spectra of flavanones or dihydroflavonols with λ_{max}=290 nm and shoulder 330 nm, while the short retention times and the high m/z of the first two indicate probably a glucoside structure (m/z=429 & 473 respectively). Glycosylation at position 7, 4′ or 3′ has no effect on the wavelength maximum or the spectrum shape (Mercken & Beecher, 2000). The rest peaks of the extract could not be classified to any subgroup due to poor separation or unknown spectra.

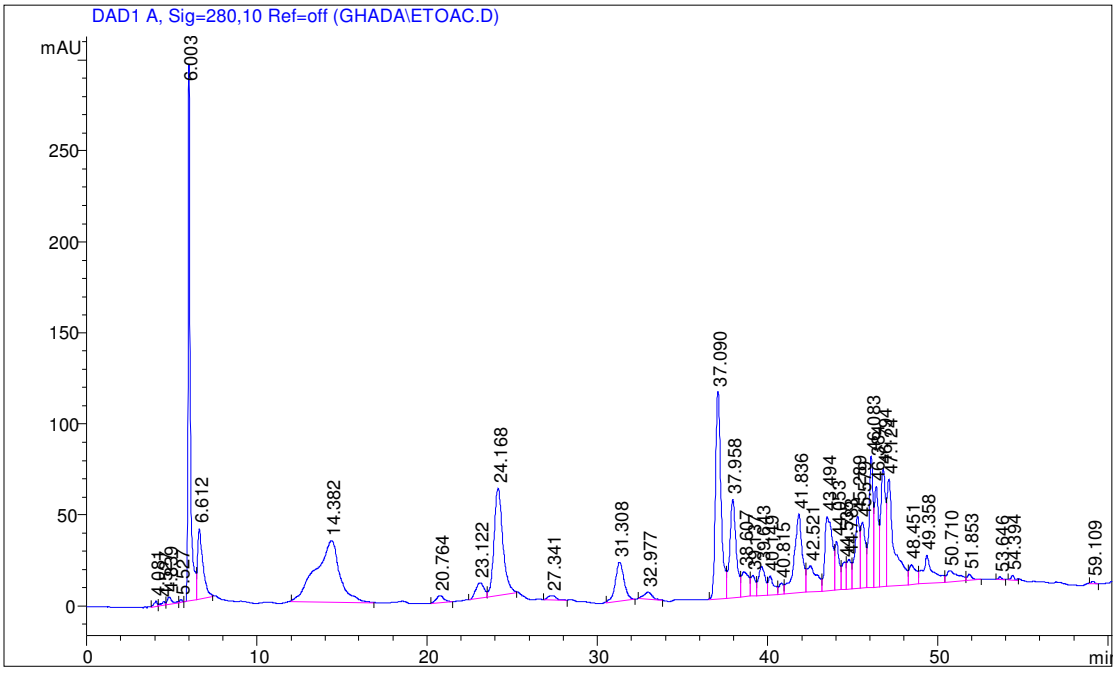


Figure 3: The UV-spectra of ethyl acetate extract

Table 4: The analysis of EAc extract from HPLC-DAD and HPLC–MS.

Peak	Rt (min)	% area	UV-max (nm)	m/z		Proposed structures
				$[M+H]^+$	$[M-H]^-$	
1	6.0	9.5	230, 298	311, 395	199, 273	Unknown
2	24.2	9.4	290, 330sh		429†	Flavanone or dihydroflavonol glucoside
3	31.3	3.00	290, 336sh		473	Flavanone or dihydroflavonol glucoside
4	37.1	9.3	290sh, 330		359	Rosmarinic acid
5	38.0	5.1	290, 330sh		288, 393	Flavanone or dihydroflavonol
6	41.8	6.2		291, 331	287	Poor separation
7	43.5	6.1		271	387	Poor separation
8	46.1	4.8	268, 338	271		Apigenin

† $[M-2H]^{2-}$

5.2.2c Analysis of ethanol extract

The detailed analysis of E components is presented in Table 5 and the UV chromatogram at 280 nm is presented in Figure 4. Peaks 1 and 2 at Rt =25.5 and 26.1 have the characteristic spectra of flavones (λ_{max}=272, 336), and the *m/z* of the main fragment ions indicate glucosidic structures (m/z 305, 387 & 593) (Figures 11 and 12 b in the Annex, p 182-183). Peak 3 indicated rosmarinic acid as a major component, amounted to 20.8% according to calculations of % area with maxima at 330 nm, a shoulder at 290 nm and m/z=359 (Figures 13 a &b). Peak 5 has a characteristic spectrum similar to that of rosmarinic acid and probably corresponds to a phenolic acid with maxima at 328 and shoulder at 294 and m/z =318 and 364 as shown in Figures 14a & b in Annex, p 185)

(Tsimogiannis et al. (2006) reorted rosmarinic acid at Rt=38.3 and apigenin at Rt=47.0 in the ethanolic extract of *Origanum heracleoticum*.

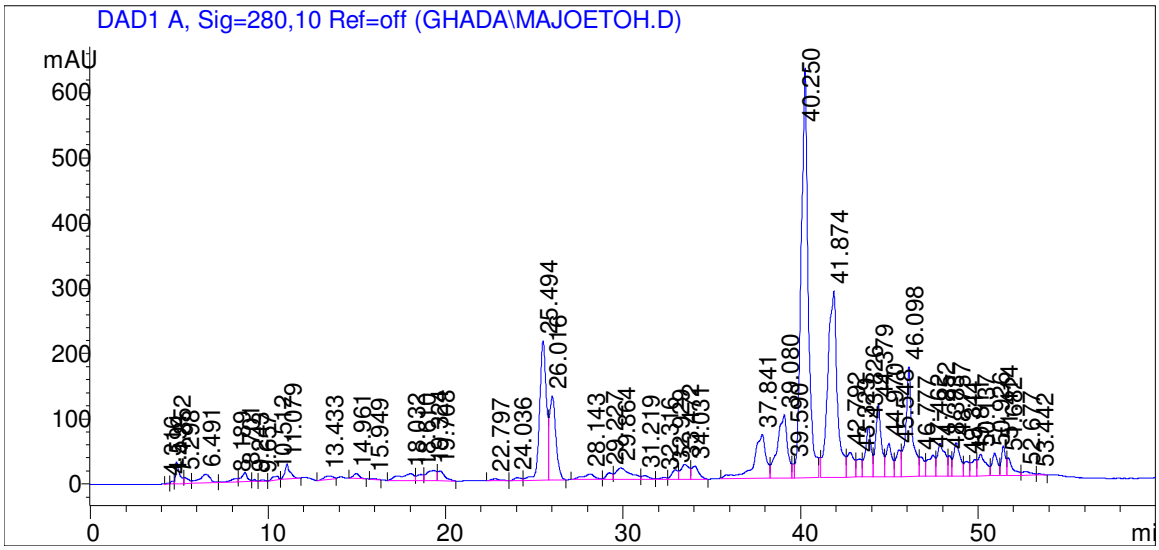


Figure 4 : UV-spectra of ethanol extract at 280nm

Table 5: The analysis of E extract from HPLC-DAD and HPLC–MS

Peak	Rt (min)	% area	UV-max (nm)	m/z		Proposed structures
				$[M+H]^+$	$[M-H]^-$	
Ethanol extract						
1	25.5	6.6	272, 336		305†, 387, 593	Flavone glucoside
2	26.1	4.1	272, 336		305†, 387, 593	Flavone glucoside
3	40.2	20.8	290sh, 330		359	Rosmarinic acid
4	41.9	12.8	268, 338		565, 392	Poor separation
5	46.1	5.6	294sh, 328	318, 364		Phenolic acid

† $[M-2H]^{2-}$

5.3 Estimation of Antiradical Efficiency against DPPH

The radical scavenging activity towards DPPH radical was used to evaluate the applicability of the extracts to donate hydrogen ions and consequently scavenge free radicals. The efficiency against DPPH radical has been successfully correlated with the activity of antioxidants against bulk oil oxidation (Tsimogiannis & Oreopoulou, 2004)
The antiradical efficiency of the each extract against DPPH radical was tested in methanol and in ethyl acetate. The reason of using two different solvents is that P was not soluble in methanol, while the other extracts were soluble in both solvents, and also to compare between activities of the extracts in both DPPH/EAc and DPPH/methanol.

Different concentrations of the extracts were added to DPPH solution and the absorbance of the remaining DPPH was measured for 90 minutes continuously. As concentrations of the extracts increased there was higher depletion of DPPH, which means higher quenching of the radical and so stronger antiradical effect. The effect of adding variable concentrations of D extract on depletion of DPPH/methanol is presented in Figure 5 The depletion in concentration of DPPH was plotted against the ratio of the mass (g) of the extracts to mass (kg) of DPPH, and from the plot line, the amount of extract needed to reduce the concentration of DPPH to half of its initial amount, i.e. the EC_{50} value, was calculated from the equation obtained from trendline (Figure 6).

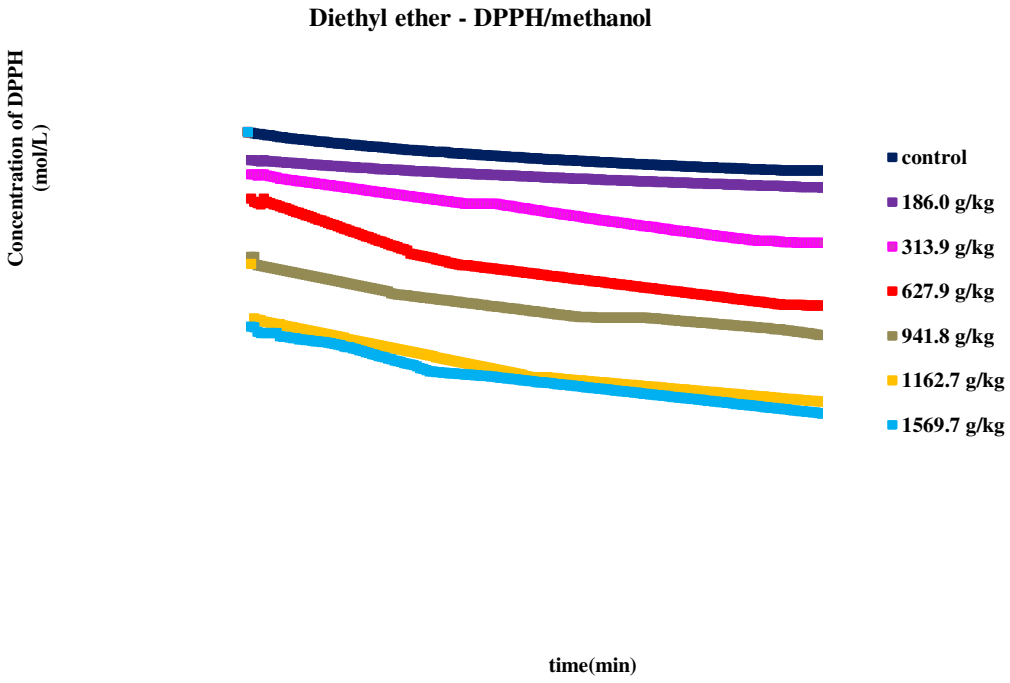


Figure 5: The depletion of DPPH at various concentrations of D extract in DPP/methanol

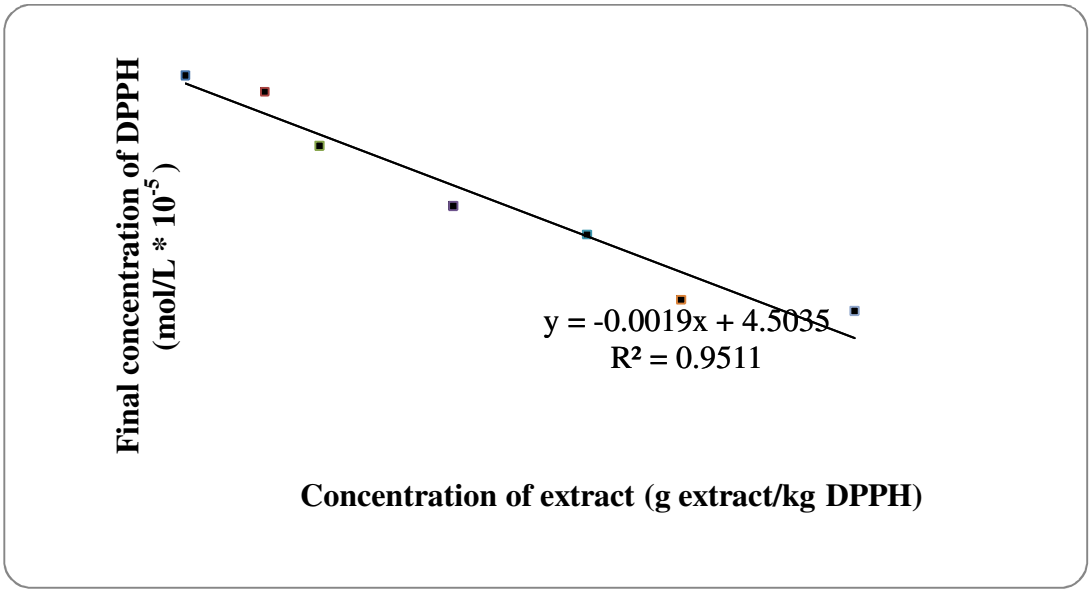


Figure 6: Calculation of EC_{50} on D extract in DPPH/methanol

The four extracts obtained from successive extraction showed variable levels of reduction of DPPH. The depletion of DPPH curves and calculation of EC_{50} for each of the extracts in both DPPH/methanol and ethyl acetate is shown in Figures 15-21 a & b in Annex, p186-192. A higher activity was indicated by the D extract which had EC_{50} = 1030 and 1000 ($g_{extract}/kg_{DPPH}$) in ethyl acetate and methanol, respectively. The other extracts showed a lower ability of radical scavenging, following the order D> E> EAc> P as presented in Table 6.

Table 6: The antiradical efficiency, represented as EC_{50} value, for extracts from both successive and one-step extraction in DPPH/ethyl acetate and DPPH/methanol

Extract	EC_{50} [$g_{antioxidant}/kg_{DPPH}$]	
	In ethyl acetate	In methanol
Petroleum ether (P)	6000 [3]	
Diethyl ether (D)	1030 [a1]	1000[a2]
Ethyl acetate (EAc)	2739[f2]	1422[b3]
Ethanol (E)	1500[b4]	1018[c2]
One-step extraction by ethyl acetate	-	1000[2]

*values of the same row bearing different letter are significantly different

** values of the same column bearing different numbers are significantly different

According to ANOVA one-way analysis comparing between EC_{50} values of extracts in DPPH/Ethyl acetate in table 6, it was found that there is significant difference between each of extracts in DPPH/ethyl acetate, while in DPPH/methanol, there was no significant difference between D and E, However, in DPPH/ethyl acetate the EC_{50} values of all the extracts increased, compared with the respective values in methanol. This fact is explained by the differences in mechanism due to the change of the solvent. Alcohols form an H-bond complex with the nitrogen N_2 of DPPH, thus resulting in increasing

electron localization in the nitrogen N_1 and, consequently, the reactivity of the radical with phenolic antioxidants (Valgimigli, 1995; Tsimogiannis & Oreopoulou, 2004).

According to the above-presented results of the extract analysis, a correlation of their antiradical activity with their composition can be attempted. The low efficiency of the P extract against the DPPH radical is well explained by its composition. The phenolic monoterpenes carvacrol and thymol exhibit low hydrogen-donating ability (Vekiari et al., 1993; Dorman et al., 2004). Carvacrol has been reported to have an EC_{50}= 17000 g/kg DPPH (Kulisic et al., 2004), while the structures of the other identified compounds do not justify such ability, either. The activity of the other extracts in DPPH scavenging is higher because they comprise mainly flavonoids and phenolic acids. The identified compounds in this study are known to possess antiradical activity. Tsimogiannis and Oreopoulou (2006) reported EC_{50} for taxifolin to be 93 mg/kg DPPH and 107 mg/kg DPPH for eriodictyol. Apigenin seems to have low activity when tested alone with DPPH (Hotta et al., 2002). In a study by Lo et al. (2002) carnosol and rosmarinic acid (major components of rosmary) exhibited antiradical activity, concentration dependant, and more potent than vitamin C & E.

On the other hand the antiradical efficiency of the one-step extraction done with ethyl acetate alone was noticeable and the EC_{50} value was 1000 g/kg, as shown in Table 6, which is logical since it is expected to contain most of the extracted components by diethyl ether and ethyl acetate. It is also noticed that EC_{50} of EAc is closer to the EC_{50} value of D extract (Table 6 and Figure 22 a & b in Annex, p 193).

5.4 The Antioxidant Activity of Extracts Against Corn oil Oxidation

The antioxidant activity of the extracts against corn oil was tested in accelerated oxidation experiments run in a ventilated oven at 70°C. The extracts were added to corn oil at concentrations of 200, 500, and 1000 ppm (on dry basis), and the solvents were removed through purging with nitrogen. Oxidation of the samples was followed by measurement of PV and CD over a period of two weeks.

5.4.1 Antioxidant activity of extracts from successive extraction

5.4.1a Peroxide value (PV)

The first parameter used to test the antioxidant activity of the extracts of successive extraction was measuring the peroxide value. The results of PV for the concentration of 200 ppm are plotted against time in Figure 7. The induction period, at which the PV remain low, was approximately estimated, through the change of slope of the respective curves, equal to 100 h for the control sample, 140 h for the P extract and 190 h for the remaining extracts, as shown in Figure 7

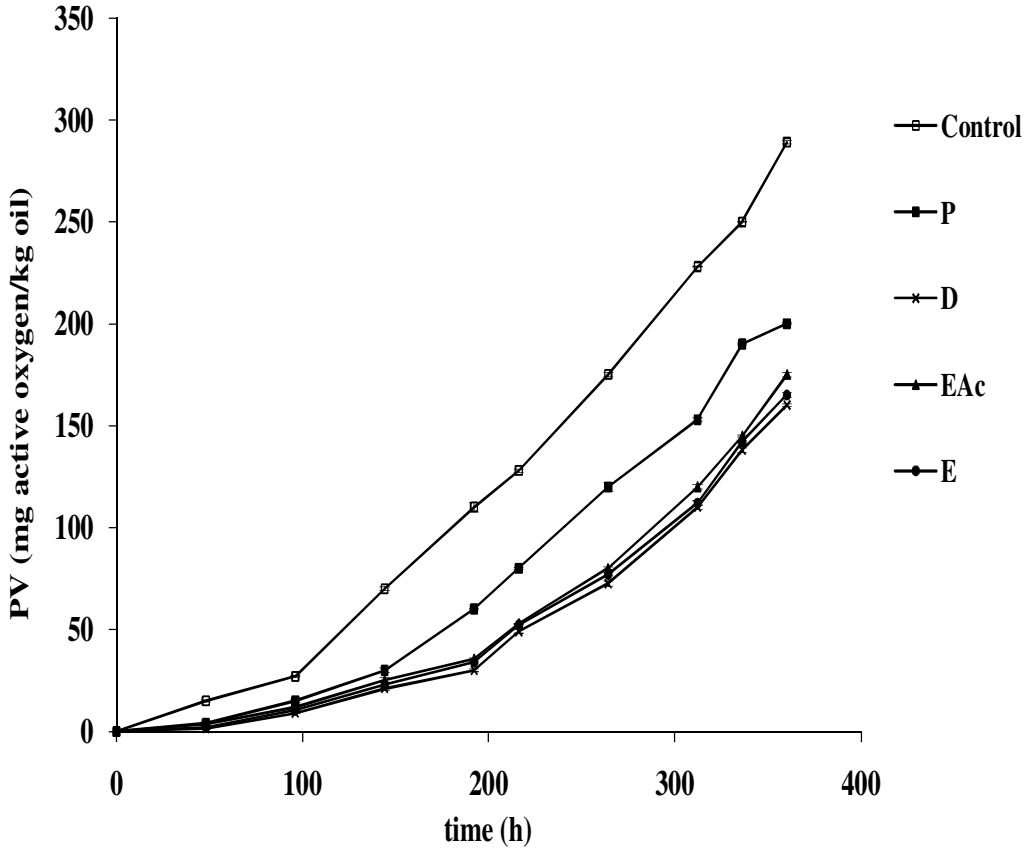


Figure7: Effect of the extracts (200 ppm) obtained by successive extractions with petroleum ether (P), diethyl ether (D), ethyl acetate (EAc), and ethanol (E) on PV of corn oil stored at 70 °C.

According to ANOVA two way analysis, the results indicated that there was no signifant difference between D and E extracts, while both presented a small, although significant difference with the EAc extract. The P extract showed remarkably lower protection compared to the aforementioned extracts. Thus, the order of the rate of oxidation was P>

EAc > E ≥ D, which is in rough agreement with the above-mentioned results of the DPPH.

The effect of increasing the concentration of ethanol extract is shown in Figure 8, and it was found that the antioxidant effect was higher as concentration increased in the order 200ppm > 500ppm ≥1000ppm. The same experiment was repeated with other extracts that followed the same order (Figures 23a, b & c in Annex, p194). However, in petroleum ether extract and ethanol extract there was no significant difference between 500 and 1000 ppm while there was significant difference between 200 ppm and both 500 and 1000.

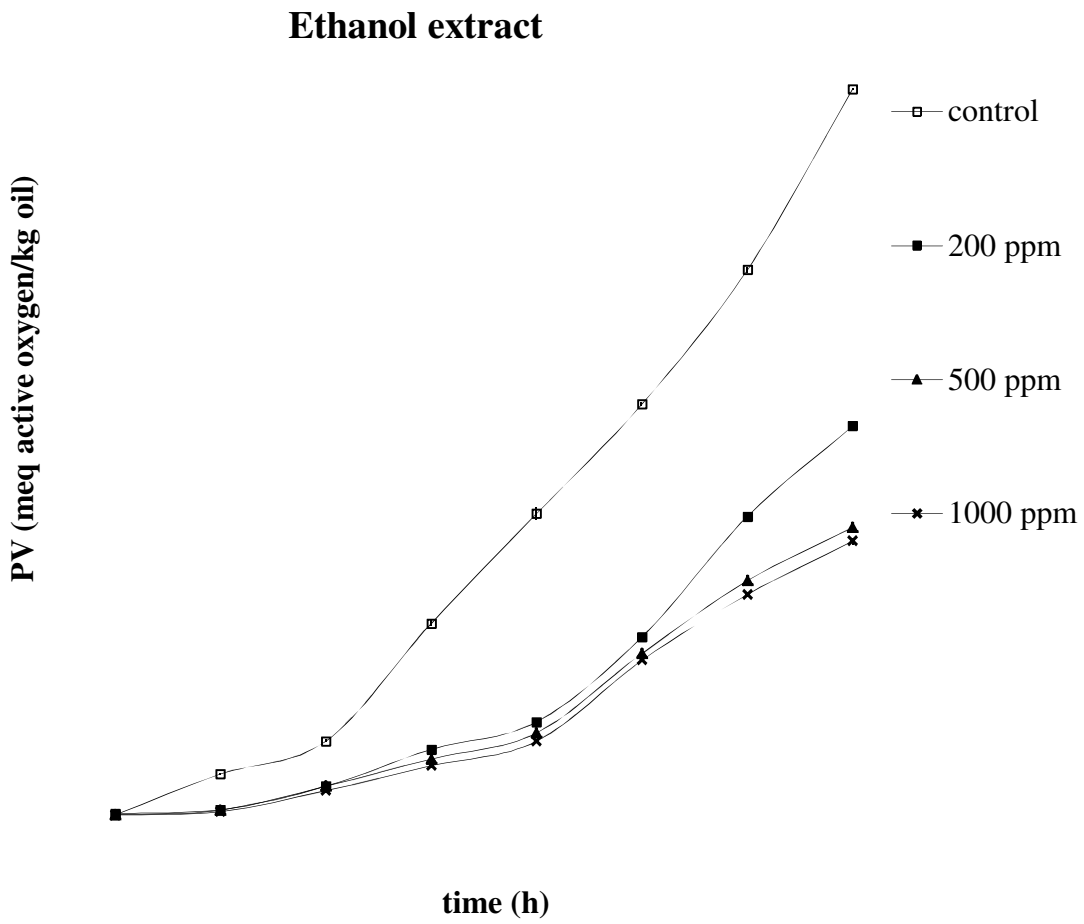


Figure 8:. Effect of concentration of ethanol extract on PV of corn oil stored at 70 °C.

5.4.1.b Conjugated dienes (CD)

As a second indicator of the level of oxidation and so of the antioxidant effect, the amount of CD was also calculated through the absorption at 232 nm. Figure 9 shows the increase of CD for the extracts from successive extraction at 200 ppm. Though the addition of D extract resulted in lower mean values of CD than the addition of the other extracts, the difference between extracts was not statistically significant due to higher standard deviations observed in duplicate experiments, as indicated by the error bars in Figure 9. The increase in CD in our experiments was moderate, and therefore differences among the extracts were too small to permit comparisons on their activities.

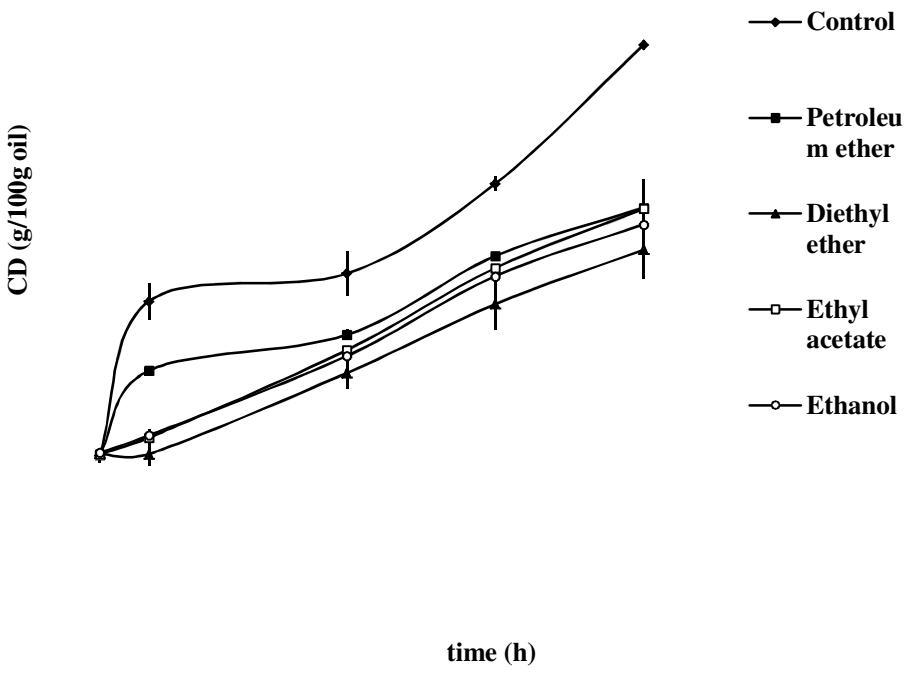


Figure 9: Effect of the extracts (200 ppm) obtained by successive extractions with petroleum ether (P), diethylether (D), ethyl acetate (EAc), and ethanol (E) on CD content of corn oil stored at 70 °C.

However, the antioxidant activity of these extracts in corn oil was in agreement with data from literature. In previous observations by Kulisic et al. (2004) and Tsimogiannis et al. (2006). The extracts obtained from oregano species were found to have antioxidant activity in oils. The antioxidant activity of D and EAc extract should be mainly attributed

to flavonoids and carnosol, while that of the E extract to rosmarinic acid, which is the most abundant component of the extract (Table 5). Rosmarinic acid possesses free –OH groups and is known to have antioxidant potentiality. Ordoudi et al. (2009) found that rosmarinic acid exhibited strong antioxidant efficiency in all essays used for examination.

5.4.1c Estimation of Antioxidant Efficiency through oxidative factors

The oxidation factor OF described in 4.7.6 that indicates the change of peroxide values and conjugated dienes (OF_{PV}, OF_{CD}) observed in the sample with the additive during the oxidation experiments are calculated according to equations 4 and 5 expressed in section 4.7.6. Statistical analysis based on ANOVA two-way test comparing the differences between extracts and at the same time differences between different concentrations is presented in table 7. The results showed that OF_{PV} values of the samples with P and EAc extracts differed significantly from all the rest, while there was no statistically significant difference between OF_{PV} values of the samples with D and E extracts.

Table 7

Effect of different concentrations of petroleum ether (*P*), diethyl ether (*D*), ethyl acetate (*EAc*), and ethanol (*E*) extracts of *M. syriaca* on oxidative factors of corn oil, based on PV (OF_{PV}) and CD content (OF_{CD}).

Extract	OF_{PV}			OF_{CD}		
	Extract concentration in oil (ppm)			Extract concentration in oil (ppm)		
	200	500	1000	200	500	1000
P	0.692^{a1}±0.002	0.547^{a2}±0.001	0.518^{a2}±0.002	0.565^{a1}±0.052	0.451^{a2}±0.126	0.476^{a2}± 0.011
D	0.553^{c1}±0.002	0.374^{c2}±0.003	0.363^{c2}±0.002	0.500^{b1}±0.006	0.479^{a1}±0.097	0.508^{b1}± 0.134
EAc	0.606^{b1}±0.004	0.434^{b2}±0.004	0.422^{b2}±0.002	0.597^{a1}±0.001	0.544^{b2}±0.005	0.536^{b2} ±0.016
E	0.571^{c1}±0.004	0.385^{c2}±0.002	0.380^{c2}±0.002	0.602^{a1}±0.037	0.475^{a2}±0.100	0.476^{a2} ±0.275

Values in the same column bearing a different letter, or in the same row bearing a different number are significantly different ($p<0.05$, Duncan's multiple range test).

The statistical analysis of OF_{CD} data indicated that there was no significant difference between samples with different extracts or different concentrations of any of the extracts (partly attributed to high discrepancies of duplicate measurements of UV absorbance).

On the other hand the increase of concentration from 200 to 500 ppm induced a statistically significant difference to OF_{PV}, but a further increase to 1000 ppm had no effect. A low effect of concentration was also observed when other plant extracts were added as antioxidants into vegetable oils (Kouri et al, 2007). In fact, the activity of antioxidants does not increase linearly with concentration, and some of them, e.g. tocopherols, may even exhibit pro-oxidant activity at elevated concentrations.

According to the results presented in Table 7, it is evident that the obtained with diethyl ether, ethyl acetate, and ethanol are potent antioxidants in vegetable oils. More specifically, they approximately double the induction period and markedly depress the formation of peroxides throughout oxidation (with a kind of extract- and concentration-dependent effect) (Figure 8), resulting in a reduction of OF_{PV} values to approximately 0.38 when D or E extracts, at a concentration of 500 ppm, are used. Also, all the above extracts depress CD formation and reduce OF_{CD} values to almost half, when added to the oil at a concentration of 200 ppm or higher. According to the above results, the concentration of 500 ppm is considered a suitable amount of extract to be added in oils, since it showed satisfactory effect for all indexes. The extract obtained with petroleum ether has a remarkably lower effect concerning both the induction period and the retardation of peroxide formation after that period.

5.4.2 Antioxidant activity of one-step ethyl acetate extract

5.4.2a Peroxide value

The extract with ethyl acetate only was also applied to accelerated oxidation test in which samples of oil containing 200, 500 and 1000 ppm were tested in series of experiments. The results of measurement of PV showed that by increasing concentration from 200 to 500 there is slightly higher, but statistically significant, inhibition of PV, as presented in Figure 10, noting that there was no significant difference between 500 and 1000 ppm. This is in accordance with the results obtained previously.

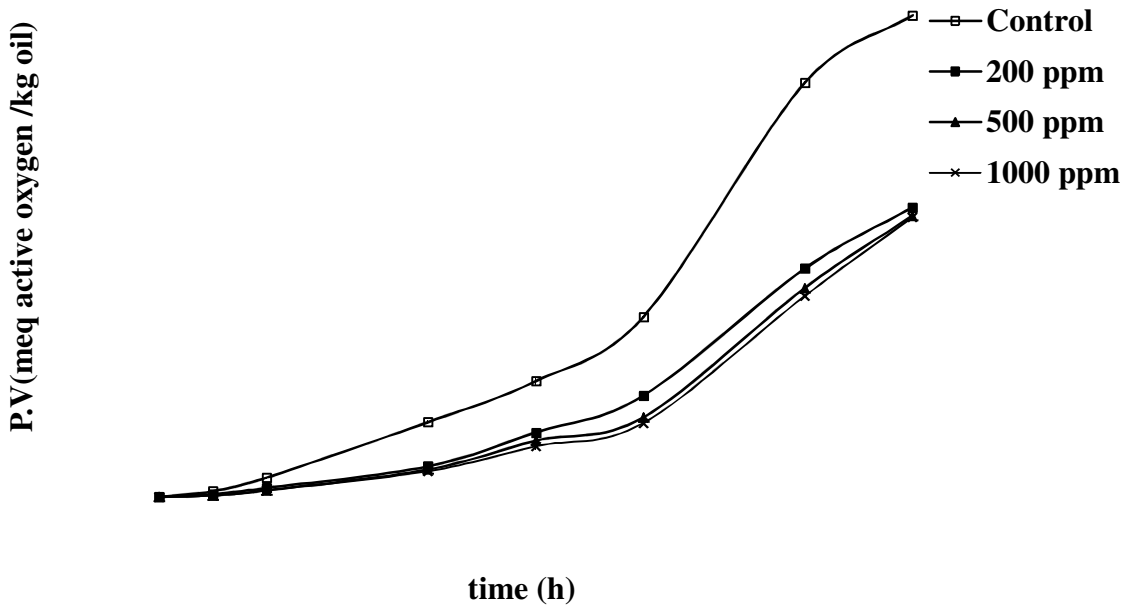


Figure 10: Effect of concentration of the extract obtained by a single extraction with ethyl acetate on PV of corn oil stored at 70 °C.

5.4.2.b Conjugated dienes

The increase of CD for the one-step extraction with ethyl acetate is presented in Figure 11 It is also noticed that there is no significant difference between 500 and 1000 ppm. On the other hand, 200 ppm was significant different from 500 and 1000 ppm, which supports the previous found evidence, that 500 ppm can be the upper amount for further experimental applications in oils.

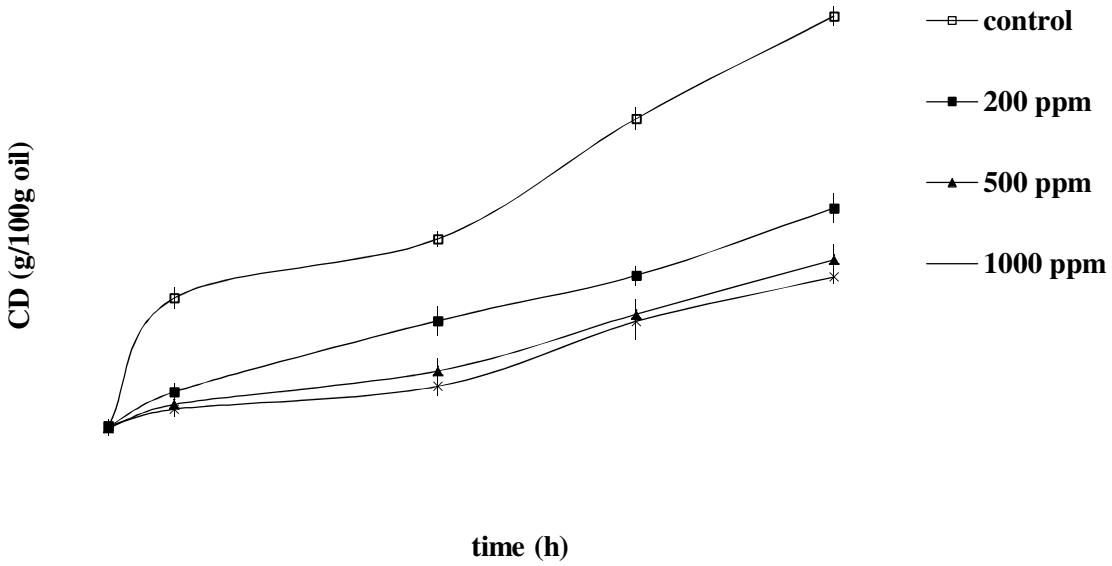


Figure 11: The effect of adding ethyl acetate extract of the one-step extraction at concentration at 200, 500, 1000 ppm on reducing CD of corn oil during accelerated oxidation at 70°C.

5.4.2c Antioxidant efficiency of one-step ethyl acetate extract

The antioxidant efficiency of 1-EAc extract against oil oxidation was also estimated through calculating oxidation factors of CD and PV (OF_{CD} & OF_{PV}) from the results obtained previously during accelerated oxidation period presented in sections 5.4.2a and 5.4.2b. The oxidation factors for 1-EAc presented in Table 8 showed an appreciable antioxidant efficiency of the 1-EAc extract, which is comparable to the oxidation factors obtained from successive extract's oxidation (Table 7). For example at 500 ppm, the OF_{CD} of 1-EAc = 0.524 was comparable to OF_{CD} of extracts obtained by successive extractions that varied from 0.479 to 0.544 (Table 7). The OF_{PV} of 1-EAc at 200 ppm was almost equal to OF_{PV} of EAc-successive (0.606), while at 500 ppm and 1000 ppm the EAc-successive was slightly more effective than 1-EAc (Table 7 & 8).

From Table 8 it is recognizable that there was no significant difference between 500 and 1000 ppm between both OF_{CD} & OF_{PV}, while 200 ppm was significant different from 500 and 1000 ppm values of OF_{CD}.

Table 8: Values of OF_{CD} and OF_{PV} of one-step ethyl acetate extract

Concentration (1-EAc)	OF_{CD}	OF_{PV}
200	$0.674^{a} \pm 0.090$	$0.600^{a} \pm 0.080$
500	$0.524^{b} \pm 0.081$	$0.584^{ab} \pm 0.100$
1000	$0.491^{b} \pm 0.010$	$0.581^{b} \pm 0.120$

*Columns bearing different letters are significantly different.

5.5 An Approach to apply *M. syriaca* in oil-based systems

As shown in the previous sections ethyl the acetate extract obtained by a single extraction (1-EAc) proved to have valuable antioxidant effect against corn oil and antiradical efficiency against DPPH. The extraction is less time-consuming than successive extracts, and by one step extraction, it is expected to recover the majority of medium-polarity antioxidants, since it has moderate polarity, and it is a food-grade solvent. So according to that, the further experimental procedures for testing the antioxidant activity of *M. syriaca* in oil-based food systems were performed by using one-step extraction with ethyl acetate.

5.5.1 Effect of adding *M. syriaca* extract on emulsions

As shown earlier in the analysis of the extracts of *M. syriaca*, they were rich in flavonoids. From the technologic point of view, recent research interest has been devoted to the in vitro antioxidant activity of flavonoids, which is due to their ability to act as free radicals scavengers and terminate the radical chain reactions that occur during the oxidation of triglycerides (Rice Evans et al., 1996). Therefore they present antioxidative efficiency in oils, fats and emulsions (Kiokias, 2008, Roedig-Penman and Gordon, 1998). Oil samples enriched with 200 ppm of ethyl acetate extract of *M. syriaca* were used in preparation of 20 % oil-in water emulsions using Tween 20 as an homogenizer (as detailed in 4.5.2). Two types of enriched emulsions were compared to control samples (not enriched); one type was enriched with EAc of successive extractions, and the other one was enriched with 1-EAc of one-step extraction. All the samples were left for

accelerated oxidation in a shaking water-bath for about 12 days at 40 °C. The oxidation was monitored periodically by measuring the increase in CD (as indicator of primary oxidation products) and TBARs (as indicator of secondary oxidation products). Figures 12a and b shows the increase in CD and TBARs for enriched samples compared to control. The OF_{CD} of EAc-successive was 0.71 which means the EAc-successive reduced the oxidation of emulsion by 29% of the control sample, while the 1-EAc reduced the oxidation by 48% regarding the control. The 1-EAc showed a remarkable antioxidant effect almost inhibited emulsion oxidation to the half amount of control and presented double effect of EAc-successive as shown in Figures 12a and 12b..

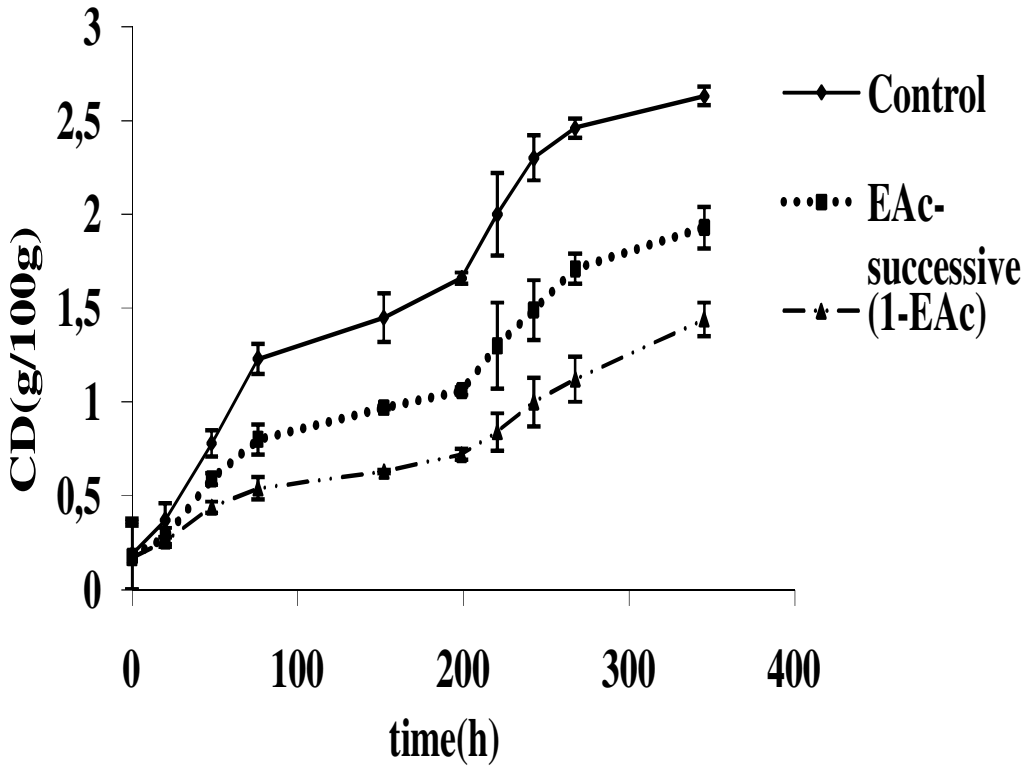


Figure 12a: The effect of ethyl acetate extract (200 ppm) obtained by single extraction (1-EAc) or successive extractions (EAc-successive) on the increase of CD of 20% o/w emulsion, subjected to accelerated oxidation at 40°C.

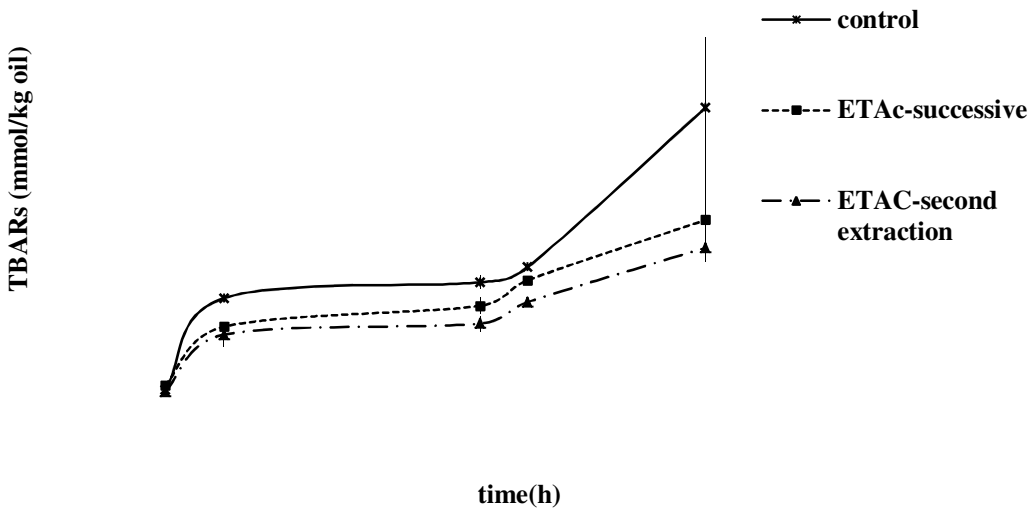


Figure 12b: The effect of ethyl acetate extract (200 ppm) obtained by single extraction (1-EAc) or successive extractions (EAc-successive) on the increase of TBARs of 20% o/w emulsion, subjected to accelerated oxidation at 40°C.

Comparing between EAc-successive and 1-EAc in Figures 12a and b there significant difference between them during the oxidation period. For the rest of experiments, the 1-EAc extract was used. The accelerated oxidation in shaking water-bath was repeated with 1-EAc but the temperature of shaking water-bath was increased to 60 °C during the accelerated oxidation test. The extract of *M. syriaca* possessed antioxidant activity at higher temperature and reduced the oxidation rate as shown in Figures 13a and b that show the values of TBARs and CD during the period of oxidation.

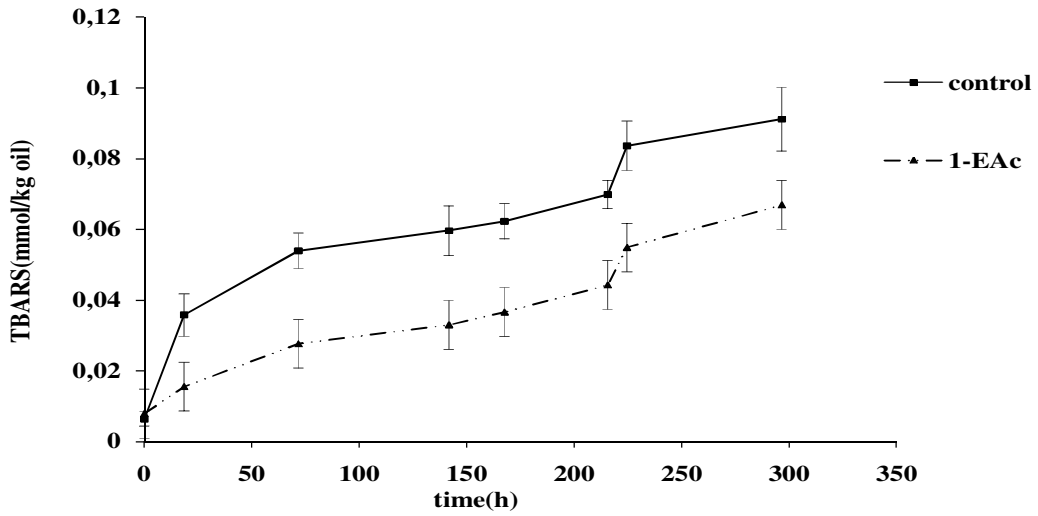


Figure 13a : The effect of ethyl acetate extract (200 ppm) obtained by single extraction (1-EAc) on the increase of TBARs of 20% o/w emulsion subjected to accelerated oxidation at 60°C

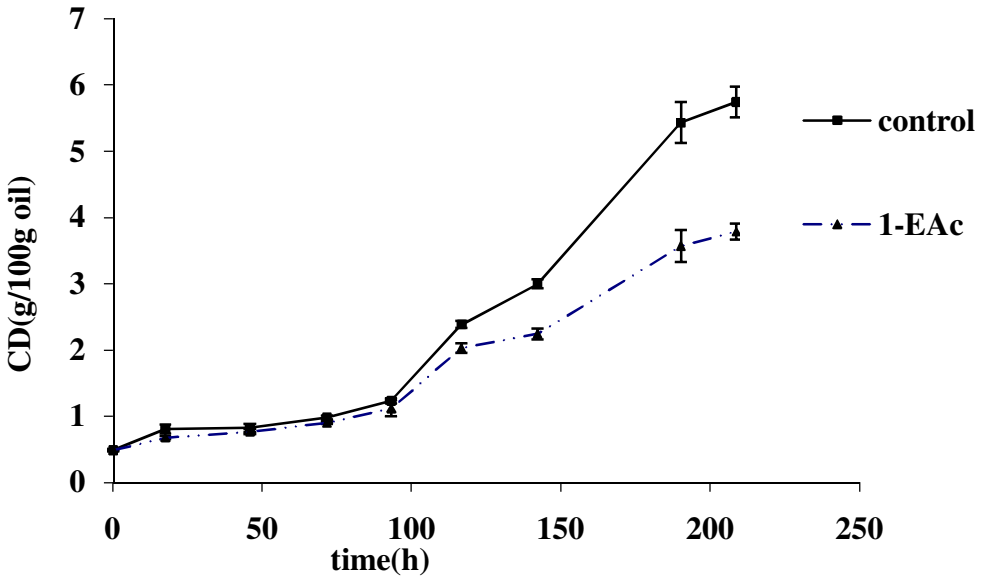


Figure 13b: The effect of acetate extract (200 ppm) obtained by single extraction (1-EAc) on the increase of CD of 20% o/w emulsion subjected to accelerated oxidation at 60°C.

The 1-EAc keep protecting the emulsion and delaying the oxidation despite of increase in temperature, with OF_{TBARs} = 0.696 and OF_{CD} = 0.63, although the OF_{CD} at 40°C was 0.52. The antioxidant effect at higher temperature was less but still satisfactory. Finally it can be clearly concluded from this experiment that *M. syriaca* extract can be added to emulsion with noticeable antioxidant effect as it reduced emulsion oxidation strongly.

These results are in accordance with findings from literature, which emphasize that the plants of Labiatae family are promising for the recovery of antioxidants. Both rosemary and sage extracts have been tested in bulk oil or emulsified fat systems and found to present a strong antioxidant character (Coppen, 1994). Similarly, Abdalla & Roozen (1999) reported that thyme and lemon balm extracts clearly inhibited the generation of volatile aldehydes during oxidation of sunflower oil and emulsions.

5.5.2 Antioxidant effect of *M.syriaca* in bulk oil and emulsion subjected to HHP

High hydrostatic pressure (HHP) processing has been introduced as an alternative non-thermal technology that causes inactivation of microorganisms and denaturation of several enzymes while minimally affecting quality and sensory characteristics (Rastogi, Raghavarao, Balasubramaniam, Niranjan & Knorr, 2007). Over the last two decades, it has attracted considerable research attention related to the extension of shelf life of food products such as fruits, fruit juices, milk and milk products, meat and meat products (Farkas & Hoover, 2001). The application of HHP, instead of thermal processing, could be beneficial for the sensory characteristics of emulsified foods like salad dressings or fresh-cheese type products, which are susceptible to thermal deterioration. However, since these products have a high fat content, the effect of HHP treatment on their oxidative stability should be first examined.

Therefore, in the first series of experiments we examined the oxidative stability during storage of emulsions and bulk oil samples that had been previously subjected to HHP treatment,so as to permit comparisons and beter interpretation of the results. The aim was to examine the effect of pressure on the oxidation rate of bulk oil and emulsion during processing and storage, and correlate it with oxygen concentration or physical changes of the emulsion. In the second series of experiments 1-EAc extract of *M. syriaca* was added

to the samples, so as to examine if the extract can still retard the oxidation caused by HHP treatment.

5.5.2a Effect of HHP on emulsion and bulk oil oxidation

Samples of bulk oil or emulsions (20% o/w) were prepared, packaged, and subjected to high hydrostatic pressure of 200 or 650 MPa. The samples were left to autoxidize for 2 weeks under accelerated conditions (70 °C), and the oxidative status was estimated through measurements of PV and CD values for oil, and CD and TBARs values for emulsions. According to our results, the effect of pressure is already evident at the initial point just after pressurization and before applying samples to further accelerated oxidation in the oven. For example the initial CD value for non HHP-treated emulsion samples was 0.129±0.01 g/100 g oil while it was increased to values 0.194±0.01 and 0.346±0.02 g/100 g oil after HHP treatment at 200 and 650 MPa, respectively. The application of high pressure during the treatment cause an increase of oxygen partial pressure in the headspace of the samples and therefore an increase of dissolved oxygen in the emulsion according to Henry's law. This results in acceleration of the initiation stage of oxidation and consequently in acceleration of the following propagation stage.

In Table 9, values for dissolved oxygen of o/w emulsion and oil samples are presented. The results indicate an increase in dissolved oxygen content as pressure increased from 200 to 650 MPa. Statistical analysis (Duncan's multiple range test) revealed that there was a significant difference in dissolved oxygen among the emulsions subjected to different treatments. The same was observed for bulk oil, although the samples treated at the lowest pressure did not present significant difference compared to the non-treated sample.

Another critical point of HHP processing is the effect of applied high pressures on the structural properties of emulsions. Droplet sizes of emulsions, before and after each HHP treatment were measured. Results are shown in Table 9. It is clear that the application of HHP at 200 and 650 MPa did not lead to change of average droplet size of emulsions indicating that the emulsion structure was not affected by pressure. However it should be noted that any change in droplet size would not be expected to affect the oxidation rate of the emulsion as several researchers (Dimakou et al., 2007; Osborn & Akoh, 2004) found

no effect of droplets size on lipid oxidation of sunflower, soybean and structured lipid-based o/w emulsions.

Table 9

Dissolved oxygen content in oil and o/w emulsion and oil droplet size in o/w emulsion before and after each HHP treatment (pressure, 200, and 650 MPa; time, 6 min; temperature, ambient) (Average values ± Standard deviation).

HHP treatment (Pressure, MPa)	O/W Emulsion		Oil
	Dissolved oxygen (mg/ml)	Droplet size	Dissolved oxygen (mg/ml)
_	$0.014^{a} \pm 0.002$	$0.74^{a} \pm 0.01$	$0.024^{a} \pm 0.002$
200	$0.020^{b} \pm 0.003$	$0.72^{a} \pm 0.02$	$0.026^{ab} \pm 0.002$
650	$0.029^{c} \pm 0.002$	$0.73^{a} \pm 0.01$	$0.029^{b} \pm 0.002$

*Values in the same column bearing a different letter are significantly different ($p<0.05$)

The oxidation of emulsion and oil samples subjected to HHP treatment, compare to non-treated samples is presented in Figures 14 and 15. It is clear that as pressure increased the oxidation rate during storage increased. The fact is well explained by the increase of dissolved oxygen, described above, that causes an increase in the initiation and also propagation reaction that take place during storage of the products. More specifically, concerning the emulsions, TBARs values increased with pressure and time showing agreement with primary oxidation products increase, though, TBARs of the samples subjected to 650 MPa presented a sharp increase after 150 h of oxidation. Comparing between samples of emulsions and oil, which were enriched and the one non-enriched with extract, based on ANOVA analysis, there was significant difference between all enriched and non-enriched samples at 200 MPa and 650 MPa (Figures14 & 15).

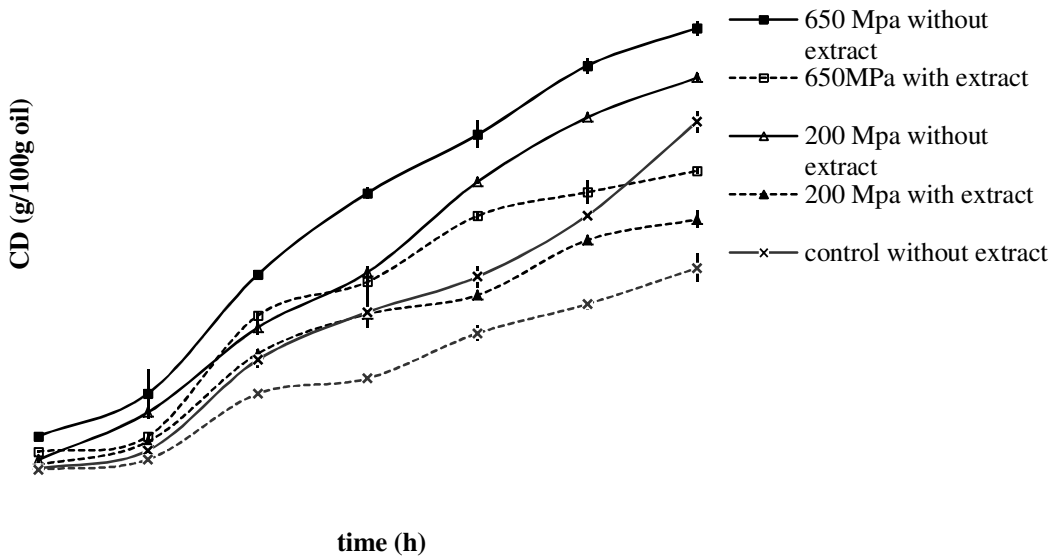


Figure 14a: Increase of CD during accelerated oxidation of emulsion samples (with and without 200 ppm of 1EAc extract) after being subjected to HHP.

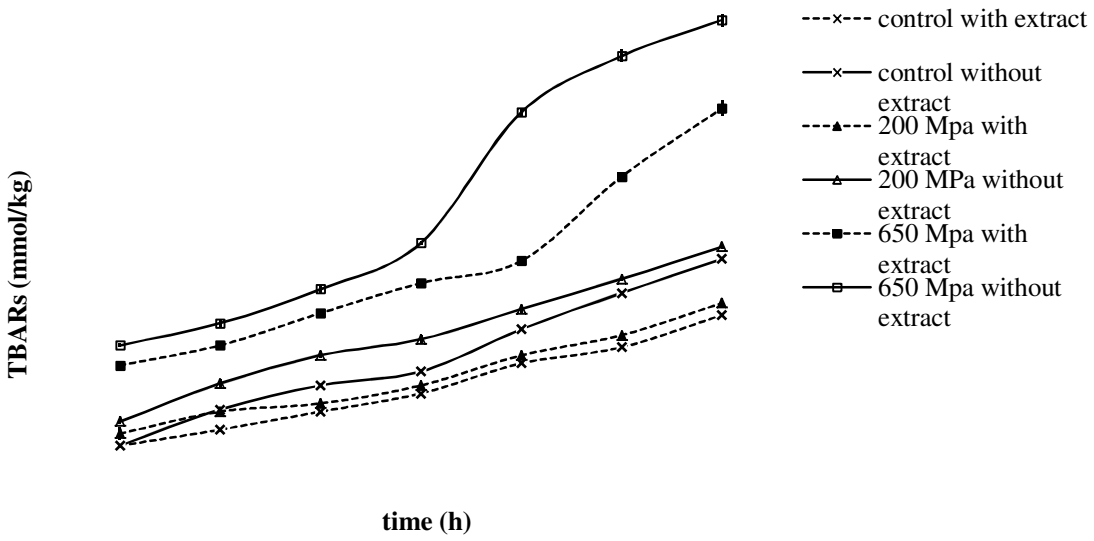


Figure 14b: Increase of TBARs during accelerated oxidation of emulsion samples (with and without 200 ppm of 1EAc extract) after being subjected subjected to HHP.

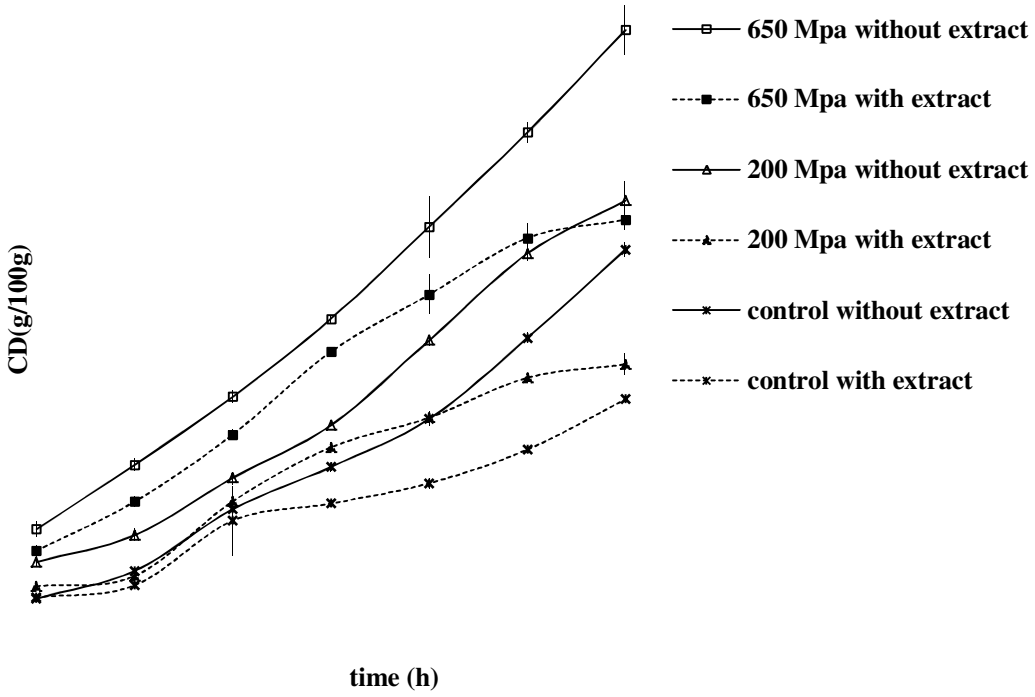


Figure 15a: Increase of CD during accelerated oxidation of bulk oil samples (with and without 200 ppm of 1EAc extract) after being subjected to HHP.

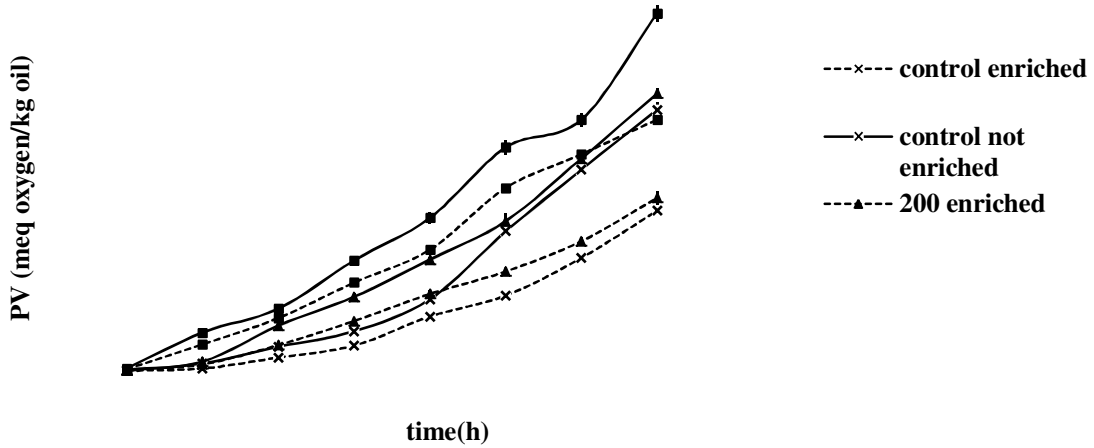


Figure 15b: Increase of PV during accelerated oxidation of bulk oil samples (with and without 200 ppm of 1-EAc extract) after being subjected subjected to HHP.

5.5.2b Effect of *M. syriaca* extract

Oil and emulsions samples were prepared as in the previous series but enriched with 1-EAc extract, packaged, and subjected to high hydrostatic pressure (HHP). The extract was added at a concentration of 200 ppm to the oil, regarding to the synthetic antioxidants in which the highest permitted level for the addition is 200 ppm, and therefore it is a good reference value that would allow comparison of efficacy at the same addition level.

The effect of HHP treatment (200, 650 MPa) on the oxidation rates was studied by leaving the samples to autoxidize for 2 weeks under accelerated conditions (70 °C). The oxidative status was periodically estimated as in the previous series.

Figure 14a shows the effect of HHP on the oxidative stability of the treated emulsions and the protective action of *M. syriaca* extract. These results are representing mainly the primary oxidation products that are followed by the formation of secondary oxidation products at the propagation stage as was tested by TBARs. It is clear that the extract depressed the formation of both primary and secondary oxidation products in treated and non-treated samples.

To analyze the effect of *M. Syriaca* ethyl acetate extract (1-EAc) on lipid oxidation of the studied emulsions and oils under the selected experimental conditions the oxidation factor was calculated based on the change of each oxidative indicator of samples enriched with the extract compared to values of non-enriched samples (Equations 4, 5 and 6 in section 4.7.6) and is presented in Table 10.

Table 10

Effect of the extract of *M.Syriaca* on oxidative factors (CD, TBARs, or PV) of bulk oil and o/w emulsion samples under different treatment pressures (no treatment, and treatment at pressures, 200, and 650 MPa) based on oxidation factors (OF) (Average values ± Standard deviation).

	Sample			
HHP treatment	**O/W Emulsion**		**Oil**	
(Pressure, MPa)	**OF based on Equations 1 and 2**			
	CD	**TBARs**	**CD**	**PV**
_	0.582^{a}±0.042	0.711^{a}±0.005	0.568^{a}±0.008	0.614^{a}±0.010
200	0.642^{b}±0.023	0.740^{a}±0.033	0.613^{b}±0.020	0.624^{ab}±0.007
650	0.688^{c}±0.008	0.791^{b}±0.021	0.663^{c}±0.026	0.703^{c}±0.014

Values in the same row bearing a different letter are significantly different ($p<0.05$).

Based on statistical analysis of the results, there were generally significant differences between OF values of samples after applying 200 and 650 MPa. As HHP treatment pressure increased from 200 to 650 MPa the antioxidant effect of *M. Syriaca* extract is decreased, for o/w emulsion samples as well as oil samples However, the addition of 1-EAc extract reduced the formation of CD in treated emulsions up to 32.2 %, compared to 42.8 % for the non-treated samples and the formation of TBARS up to 20.9 % compared to 28.9 % for the non-treated samples. Similar differences were also observed in the protection of treated and non-trated oil samples, as can be seen from Table10. The lower protection in treated samples can be explained by the higher rate of the initiation and propagation reactions that are caused by the increase of oxygen concentration. When oxidation proceeds faster the antioxidants are faster consumed in reactions with peroxides and can no longer protect the lipids.

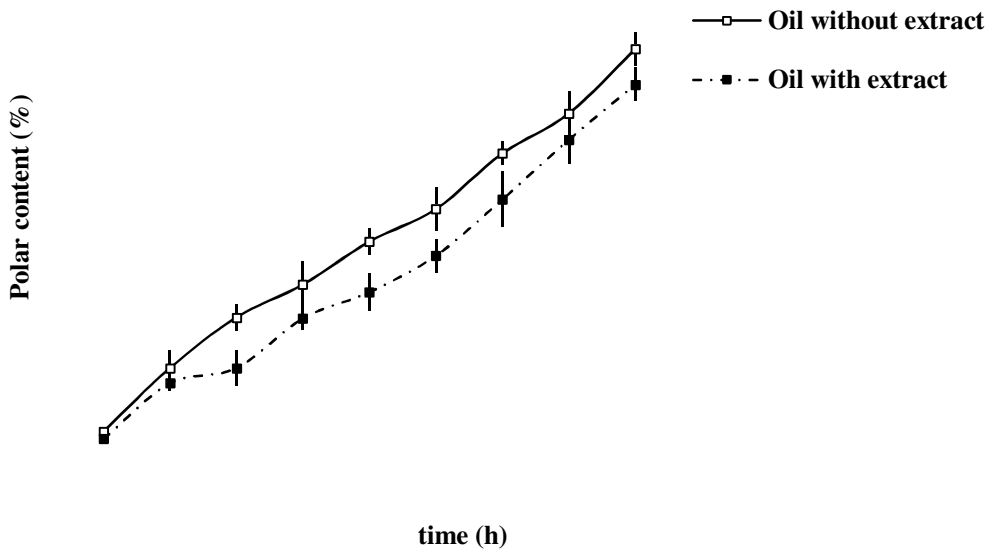
From the results, it can be clearly concluded that the alternative non-thermal treatment of HHP enhanced lipid oxidation of both oils and emulsions, but the antioxidative effect of *M. syriaca* is still noticed.

5.5.3 Effect of *M. Syriaca* in deep frying and storage of potato chips

During frying, new compounds are formed due to the joint action of high temperature and the presence of air and moisture. The most abundant compounds formed possess higher polarity than their parent triacylglycerols and low volatility. Hence column chromatography is the most appropriate technique to determine the alteration level in used frying oils and fats by measuring the amount of total polar compounds. Advantages of this method is that values obtained provide a direct measurement of the degradation, and determination is independent of the type of oil used in frying since initial values of polar compounds are similar in fresh oils. Most of the present regulations limiting the degradation of used frying fats and oils for human consumption have established a maximum level of polar compounds of around 25% (Firestone, 2007). Several attempts have been made to correlate the measurement of polar compounds with rapid and simpler measurements of oxidation. Among them, conjugated dienes content is adequately correlated to polar content and a reliable index of oxidation of the oil Houhoula, 2004. The polyunsaturated fatty acid oxidation occurs with the formation of hydroperoxides and the double bond displacement followed by the formation of consequent conjugated dienes which remain in the frying oil and can be detected by absorption at 232nm (Cella et al., 2002). Therefore, the polar content and CD were used to estimate the oxidation status of the frying oil in the experiments with the addition of *M. Syriaca* extract.

The ethyl acetate extract obtained by a single extraction was added to the oil before frying at a concentration of 500 ppm on dry basis, as the activity of the extract in corn oil increased with concentration up to this limit (as presented in 5.4.2), and frying is an intense thermal treatment where oxidation proceeds at a high rate, therefore demanding the higher effective concentration of antioxidant. Frying was continued for 16 h, while samples of chips removed at 2, 4, 6, 8, 10, 12, 14 and 16 h were further subjected to accelerated oxidation to examine the effect of *M. Syriaca* extract on the fried product.

Figures 16a and b show the increase of polar content and CD, respectively, of the frying oil during frying time.



Figures 16a: Effect of adding *M.syriaca* extract on increase of polar content during deep-frying at 185°C

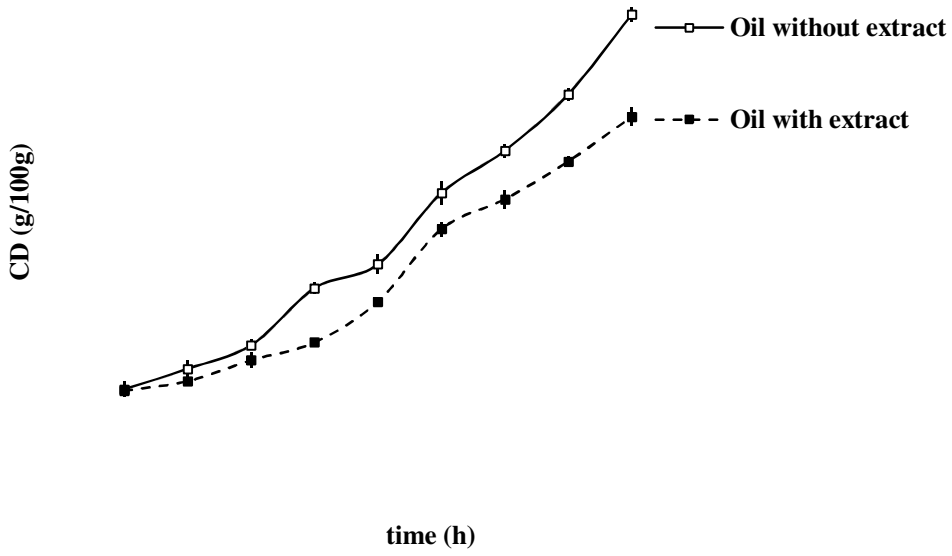


Figure 16b: Effect of adding *M.syriaca* on CD increase during deep-frying of potato chips at 185°C

As noticed, through the period of frying, there was a remarkable increase in polar content accompanied with an increased amount of CD. Comparing polar content values and amount of CD of the frying oil enriched with the extract to the one not containing the extract, there was a low but significant ($P < 0.05$) reduction in the oxidation rate. The polar content of the pure, fresh oil was 1.2 % and amounted to 27.2% after 14 h, while the polar content of the oil with the extract amounted to 25.0 % at the same time. Similarly, the CD content of the frying oil enriched with the extract was 2.0 g/100g compared to 2.5 g/100g for the non-enriched oil. Therefore, it is concluded that adding the extract of *M. syriaca* extended the stability of corn oil in frying.

The effect of the addition of *M. syriaca* to the frying oil was also examined regarding the oxidative stability of the fried potato chips during storage. Figure 17a and b shows the increase in CD and PV for samples fried with oil enriched and not enriched with the extract and removed from the fryer after 2 and 16 h of frying.

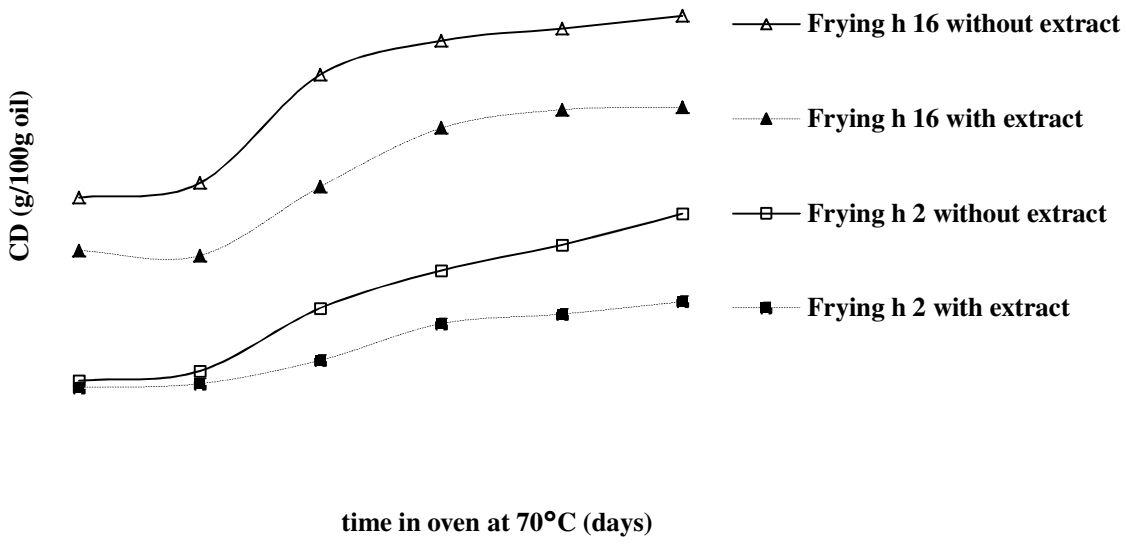


Figure 17a: Effect of adding *M.syriaca* in frying oil of increase of CD content of the oil extracted from potato chips of frying hour 2 and 16 and stored under accelerated oxidation at 70°C.

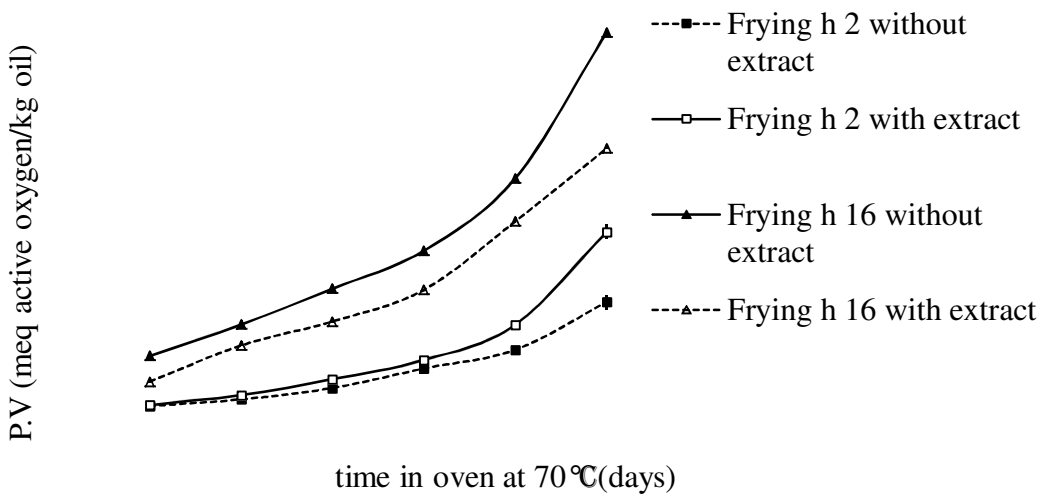


Figure 17b: Effect of adding *M.syriaca* in frying oil of increase of PV of the oil extracted from potato chips of frying hour 2 and 16 and stored under accelerated oxidation at 70°C

It is evident that the oxidation rate of the chips increases as the time of operation increases, as expected, since the frying oil that is absorbed in the chips is more oxidized (figures 17 a &b). The addition of the extract of *M. syriaca* to the frying oil significantly

($P < 0.05$) affected the stability of potato chips of both samples. For example, the PV of the sample removed from the fryer after 2 h was lowered from 106.3 meq/kg to 63.8 meq/kg after 10 days of storage, while that of the sample removed after 16 h from 227.1 meq/kg to 156.8 meq/kg. The results for the chips removed from the fryer at 4-14 h are p resented in Figures 24 a,b & c (for CD) and Figures 25 a,b & c (for PV) in Annex. In order to evaluate the antioxidant activity of the extract in samples removed from the fryer at different operation times, the oxidation factor (OF) was calculated according to equations 1 and 2 (sections 5.4.1.c).

The results of OF_{CD} and OF_{PV} are presented in Table 11. It is recognized from Figures 24 and 25 a,b & c of Annex, p 196-198, and from Table 11 that as frying proceeded, the antioxidant protection of the extract was less effective although it was still powerful. The decrease of protective action might be attributed to loss of antioxidant activity due to the prolonged application of high temperature, as well as to the oxidation status of the oil. It is well known that antioxidants can retard effectively the oxidative reactions at the early stages, while their effectiveness is reduced as propagation and termination reactions proceed at a high rate.

Table 11: The antioxidant effect of *M. syriaca* (expressed as Oxidation factors OF_{CD} & OF_{PV} ± coefficient of variance) obtained from oxidation of potato chips during accelerated oxidation in oven at 70°C.

	OF_{CD}	OF_{PV}
Fried potato chips removed from the fryer after operation time (h)		
0	0.51±0.12[a]	0.59±0.37[a]
2	0.51±0.30[a]	0.60±0.43[a]
4	0.55±0.20[a]	0.61±0.21[a]
6	0.56±0.30[a]	0.61±0.34[a]
8	0.56±0.23[a]	0.62±0.15[a]
10	0.71±0.09[b]	0.66±0.24[b]
12	0.73±0.07[b]	0.66±0.63[bc]
14	0.74±0.07[b]	0.70±0.31[c]
16	0.79±0.08[b]	0.72±0.14[c]

*Values in the same column bearing a different letter are significantly different ($p<0.05$)

Our results showed that *M. syriaca* extracts have a positive antioxidant effect in frying oil and fried chips. Similar results were obtained with extracts of other plants of the *Labiatae* family. More specifically, extracts from oregano enhanced the oxidative stability of cottonseed oil during frying and of produced potato chips (Houhoula *et al*., 2004). Similarly, rosemary and sage extracts retarded oil deterioration and prolonged the storage life of potato chips (Che Man & Jaswir, 2000; Che Man & Tan, 1999), while rosemary extracts were further examined and found effective in preventing oxidation during deep-fat frying of potato chips by Lalas & Dourtoglou (2003) or in thermoxidation by Ramalho & Jorge (2008). The incorporation of thyme and lavender in sunflower seed oil

improved its thermal stability according to Bensmira et al. (2007). Attempts to examine herbs other than those belonging to the Labiateae family were done. Nor et al. (2008) examined the anioxidative effect of extracts from *Pandanus amaryllifolius* in deep-frying and found that the extracts significantly retarded oil oxidation and deterioration.

5.5.4 Effect of adding *M. Syriaca* to stability of cookies

It is evident that oxidation of cookies is one of the major deteriorative factors that affect their shelf life, since fat is an important constituent of these products. Therefore, the addition of natural antioxidants in cookies is of high importance to assure quality. Recently, there is a high demand to find new sources of antioxidants in cookies because they remain in shelves for a long time and need to be protected by antioxidants to extend their shelf life.

Experiments with cookies were conducted using both packaged and unpackaged samples, because the latter represent the conditions that the product is often kept under domestic use. Following the oxidation of oil in cookies during storage at 70° C, a continuous increase in the CD was noticed, as shown in Figure 18a.

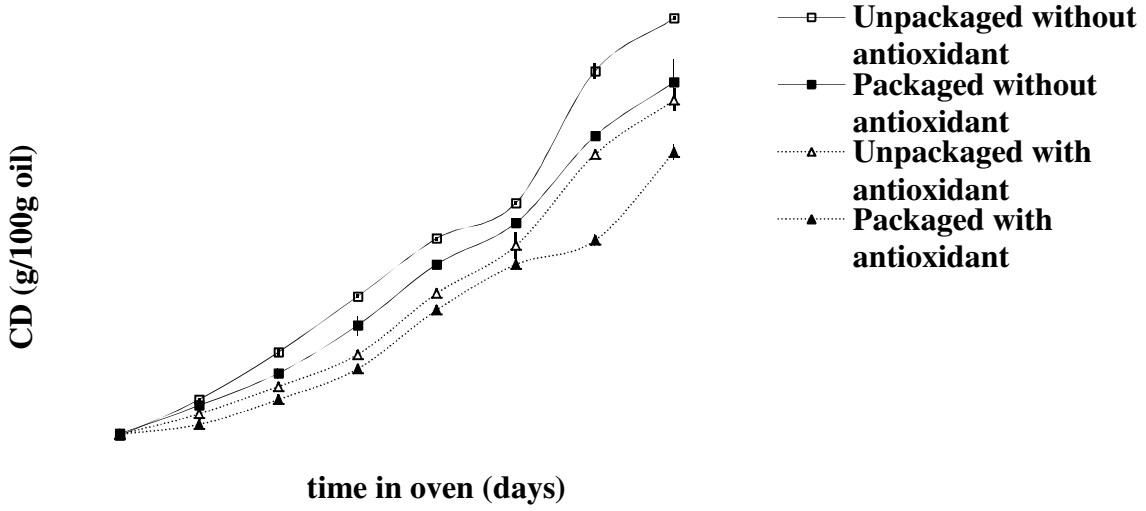


Figure 18a: Increase of CD for packaged and unpackaged cookies (with and without extract) during accelerated oxidation test of cookies at 70°C.

Packaged samples were oxidized at a lower rate than unpackaged samples as they were protected from air. In both packaged and unpackaged samples, the oxidation was depressed by the addition of the extract to the oil. The increase of PV during storage presented a similar trend as shown in Figure 18 b.The effect of adding the ethyl acetate extract from *M. Syriaca* was also estimated by calculating the oxidation factors as indicated in equations 1 and 2 section 5.4.1c The results are shown in Table 12. The packaged and unpackaged cookies were equally protected by the addition of the extract.

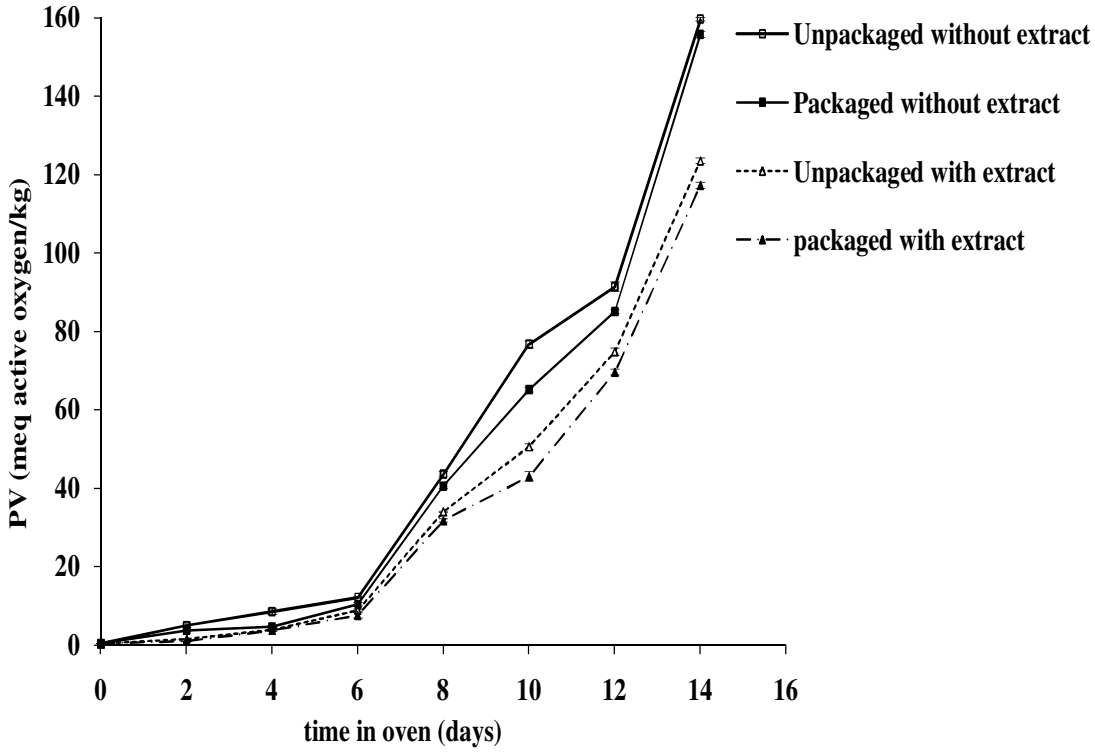


Figure 18b: Increase of PV for packaged and unpackaged cookies (with and without extract) during accelerated oxidation test of cookies at 70°C.

According to the aforementioned data, it is concluded that the addition of ethyl acetate extract from *M. syriaca* delayed the oxidation of cookies. However the oxidation factor calculations showed in revealed that it is more effective in frying, especially at the first few hours, while at both cookies and high hours of frying its effectiveness is affected slightly. For example OF_{CD} of frying hour 16 is 0.76 while for both packaged and unpackaged cookies it is 0.8 so it is closer to the potatoes which are fried in oil that was highly subjected to heat for a long time. The same thing with OF_{PV} of oil extracted from chips removed from the fryer at 16 hours is 0.72 while for packaged cookies is 0.75. This can be related to the application of high temperature, and probable interactions with components of the cookies.

Table 12:

The oxidation factors representing antioxidant effect of *M.syriaca* in baked cookies during storage in oven at 70°C.

	OF_{CD}	OF_{PV}
Baked cookies		
Packaged	0.80 ± 0.09^{b}	0.75 ± 0.10^{c}
Unpackaged	0.80 ± 0.04^{b}	0.77 ± 0.07^{c}

*Colums bearing different letters are significantly different.

5.5.5 Effect of adding *M.syriaca* extract on Tuna Yellowfin fish

The antimicrobial and antioxidant effect of *M. syriaca* extract, obtained by ethyl acetate, was also examined to Yellowfin tuna. The extract was added to refined corn oil at

different concentrations (0–3900 ppm). Minced tuna was mixed with corn oil containing the extract (tuna / oil: 3 /1 w/ w); the mixture was packed aerobically and stored at 0°C. Microbial growth and lipid oxidation were determined periodically.

5.5.5.a Antimicrobial activity of *M. syriaca* extract

The experimental data for microbial growth of different spoilage bacteria (total viable count, *Pseudomonas sp.* and lactic acid bacteria) on minced tuna with and without corn oil and *M. syriaca* extract are shown in Figures 19 a, b & c.

Pseudomonas sp. was the dominant organisms in all samples. This was in agreement with Gram and Huss (1996) who reported that *Pseudomonas sp.* can be the dominant spoilage microorganism in aerobic storage of fresh, chilled fish. The initial *Pseudomonas sp.* count was 3.34-3.95 logCFU/g and in the control treatment it reached around 8 logCFU/g after 10 days of storage at 0 °C. The use of *M. syriaca* extract as antimicrobial resulted in a maximum count of *Pseudomonas sp.* of 5.53 log CFU/g for 975 ppm, showing good antibacterial activity against *Pseudomonas sp.* growth (Figure 19b). Growth of total viable count and lactic acid bacteria was also inhibited by *M. syriaca* extract, with concentrations of 750 and 975 ppm showing the most obvious antimicrobial activity (Figure 19c).

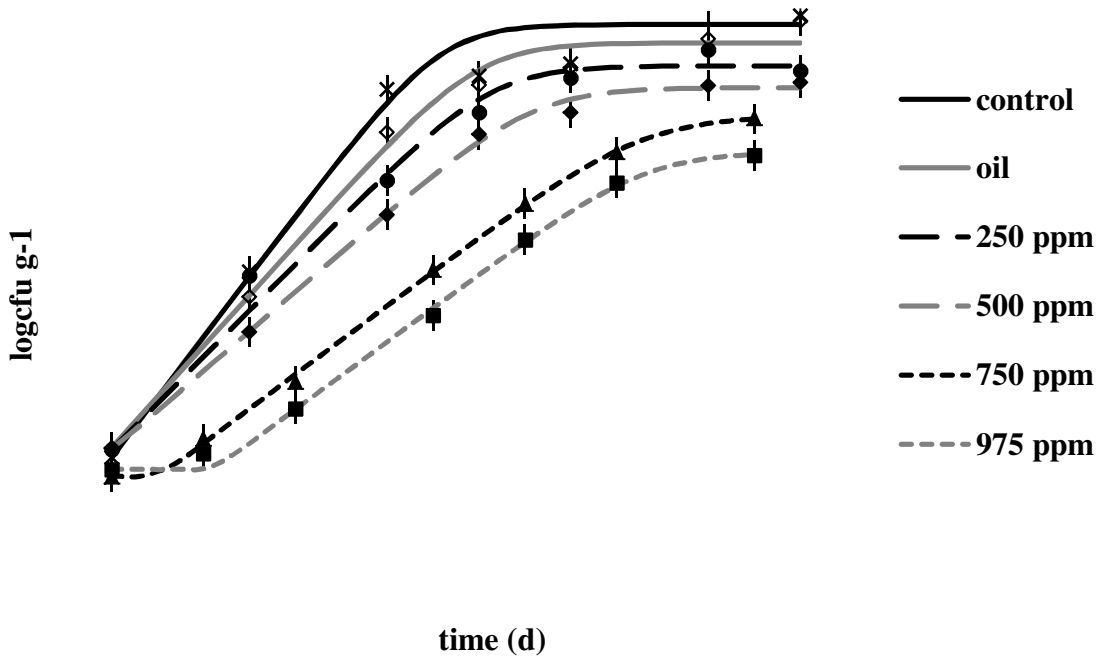


Figure 19a: Development of total viable count in minced tuna of different samples during aerobic storage at 0 °C for the following samples: (minced tuna alone-control), (mixed oil not enriched+ tuna sample, 3:1), and the enriched samples (.250, 500, 750 and 975 ppm).

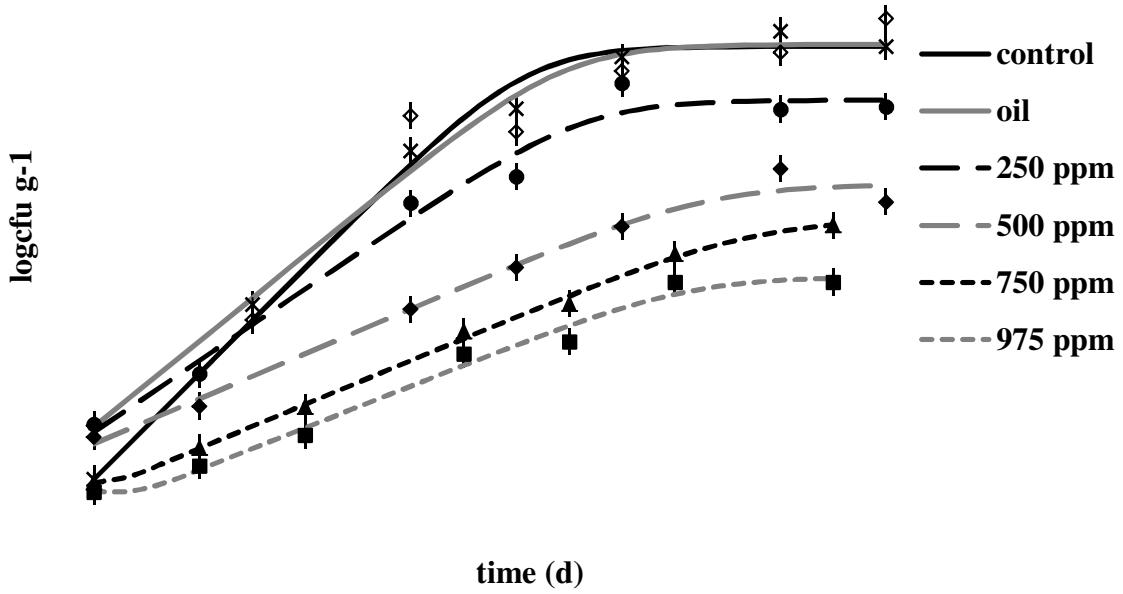


Figure 19b: Development of *Pseudomonas sp.* in minced tuna of different samples during aerobic storage at 0 °C for the following samples: (minced tuna alone-control), (mixed oil not enriched+ tuna sample, 3:1), and the enriched samples (.250, 500, 750 and 975 ppm).

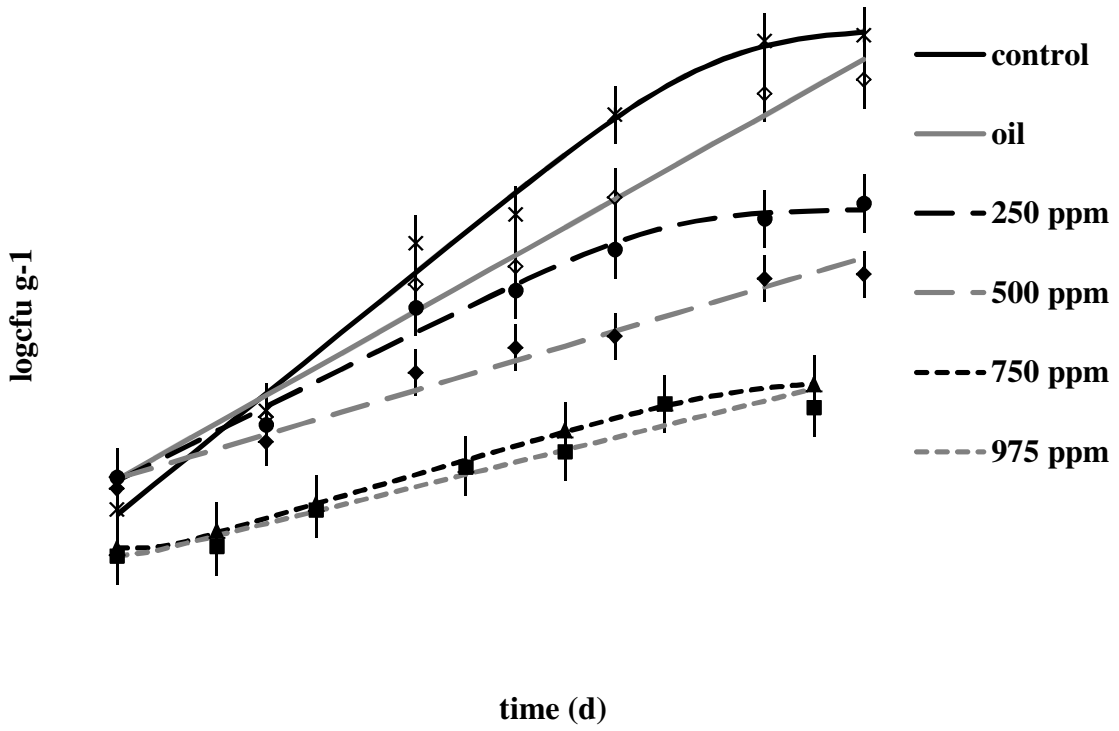


Figure 19c: Development of lactic acid bacteria in minced tuna of different samples during aerobic storage at 0 °C for the following samples: (minced tuna alone-control), (mixed oil not enriched+ tuna sample, 3:1), and the enriched samples (.250, 500, 750 and 975 ppm).

In Table 13 the reduction of specific growth rate (k) of *Pseudomonas sp*., lactic acid bacteria and total viable count in minced tuna by the use of *M. syriaca* extract is shown. All treatments containing *M. syriaca* extract, gave a significant reduction in microbial growth compared to the control samples ($P<0.001$). Treatment with corn oil alone gave also lower microbial growth than control samples ($P<0.05$).

Table 13:

Specific growth rates k (h^{-1}) with standard errors of total viable count, *pseudomonas sp*. and lactic acid bacteria on minced tuna and % reduction by the addition of corn oil and *M.*. syriaca extract at concentration of 250, 500, 750 and 975 ppm, stored at 0 °C.

	Total viable count		Pseudomonas		Lactic acid bacteria	
Treatment	k (d^{-1}) mean±SE	% reduction	k (d^{-1}) mean±SE	% reduction	k (d^{-1}) mean±SE	% reduction
Control	$0.61^{a} \pm 0.19$	-	$0.59^{a} \pm 0.08$	-	$0.21^{a} \pm 0.02$	-
Oil	$0.51^{b} \pm 0.07$	-15.9	$0.48^{b} \pm 0.08$	-18.9	$0.15^{b} \pm 0.02$	-30.7
250	$0.47^{c} \pm 0.08$	-23.2	$0.40^{c} \pm 0.04$	-32.3	$0.13^{b} \pm 0.01$	-38.8
500	$0.40^{d} \pm 0.02$	-34.2	$0.25^{d} \pm 0.02$	-57.4	$0.08^{c} \pm 0.01$	-63.6
750	$0.35^{e} \pm 0.01$	-43.0	$0.24^{d} \pm 0.01$	-58.5	$0.07^{c} \pm 0.002$	-64.1
975	$0.34^{e} \pm 0.02$	-43.9	$0.23^{d} \pm 0.02$	-60.6	$0.06^{c} \pm 0.01$	-69.7

Values in each column with different letters are significantly different ($P<0.05$).

There was an increased antimicrobial effect in samples supplemented with increasing concentrations of the extract. However, no significant differences in *Pseudomonas sp*. inhibition were observed among higher concentrations, as reduction of growth rates tended to reach a limit (approximately 58%) at concentrations above 500 ppm. The rate of growth of total viable count and lactic acid bacteria was also markedly reduced, up to 43% and 69%, respectively, with no significant difference observed above 750 and 500 ppm, respectively.

Under this context, *M. syriaca* extract could be used as a natural antimicrobial agent with the potential to extend the shelf life of seafood. Similar results have been reported by Mahmoud et al. (2004) who found that carvacrol and thymol, which are main

components of *M. syriaca* extract as presented in subchapter 5.2.1 can extend the shelf life of carp fillets by inhibiting bacterial flora. Furthermore, Harpaz et al. (2003) observed that the treatment with 0.05 % oregano and/or thyme, belonging to the Labiatae family as *M. syriaca*, increased the shelf life to 33 days at 0-2 °C, as compared to 12 days for the control. Mejlholm and Dalgaard (2002) found that oregano oil reduced the growth of *Photobacterium phosphoreum* and extended the shelf life of cod fillets kept in modified atmosphere packages. Tassou et al. (1996) reported also that treatment with olive oil, lemon juice and oregano inhibited the growth of *Pseudomonas sp.* in air-packed gilthead seabream fillets.

5.5.5.b Antioxidant activity of *M.syriaca* extract

For measurement of antioxidant activity, determination of TBARs is extensively used to estimate the secondary oxidation products related to off-flavours and therefore to deterioration of quality of fish tissues (Botta, 1995). The results of TBARs measurements of tuna samples with and without corn oil, as well as of samples enriched with *M. syriaca* extract at different concentrations are plotted in Figure 20a. It is evident that oxidation was seriously affected by the existence of the extracts. Statistical analysis revealed a significant difference ($P<0.05$) between all concentrations (0-975 ppm) of the extract.

The sample of tuna fish containing neither corn oil nor extracts showed an increase in TBARs until 9 days, which declined afterwards as shown in Figure 20a. As reported by Botta (1995), TABRs values during storage of frozen fish may be speculative as a substantial increase in may be caused by the break down (hydrolysis) of proteins rather than increase of oxidative rancidity. Additionally, the observed decrease in secondary

products of lipid oxidation (e.g. malonaldehyde) are at least partially caused by the reaction of malonaldehyde with protein resulting in compounds that fluoresce, although fluorescent products have also been reported to be formed through the reactions of many other biological compounds.

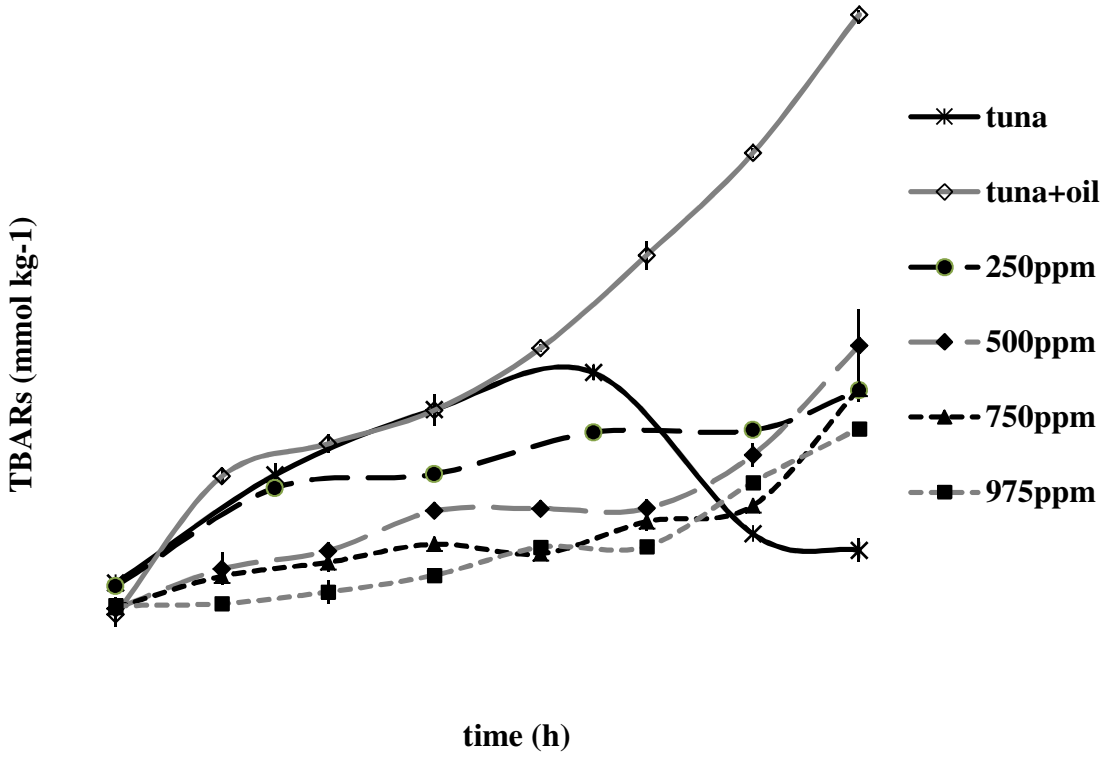


Figure 20a: Results of TBARs oxidation of the following samples: (minced tuna alone-control), (mixed oil not enriched+ tuna sample, 3:1), and the enriched samples (.250, 500, 750 and 975 ppm).

Therefore, PV was determined as another effective tool to measure the oxidation rate of tuna samples, especially since it provides an estimation of the peroxides formed as primary oxidation products. Lipids were quantitatively extracted from tuna fish samples and after being filtered and separated the PV were measured. The rate of oxidation of

tuna samples containing the extract was reduced noticeably compared to samples not enriched by extracts as shown in Figure20b. Comparing different concentrations according to Duncan's multiple range test ($P<0.05$) a significant difference was concluded between anyone of them, in accordance with the results of TBARs values. The oxidative rates of minced tuna or the homogenate with oil do not differ significantly, as can be also observed in Figure 20b. It can be assumed, consequently, that oxidation of both samples depends mainly on the composition and especially on the degree of unsaturation of tuna lipids. Fish lipids are known to be rich in polyunsaturated fatty acids (PUFAs) specially the ω3- PUFAs. The fatty acid composition of the lipid of most fish species consists mainly of C22:6ω3 (Docosahexaenoic acid, DHA), which is the major unsaturated fatty acid, C20:5ω3 (Icosapentaenoic acid, EPA), C22:5ω3 (Docosapentaenoic acid, DPA), and C20:4ω6 (arachiodonic acid, AA) that may be readily oxidized to form lipid hydroperoxides, while saturated fatty acids, C16:0 and C18:0, and mono-unsaturated ones, C16:1n-7 and C18:1n-9 are also encountered (Boran et al 2006; Njinkoue et al, 2002; Osman et al, 2001). Yellowfin tuna (Thunnus albacares) is also rich in PUFAs (Saito et al, 1995).

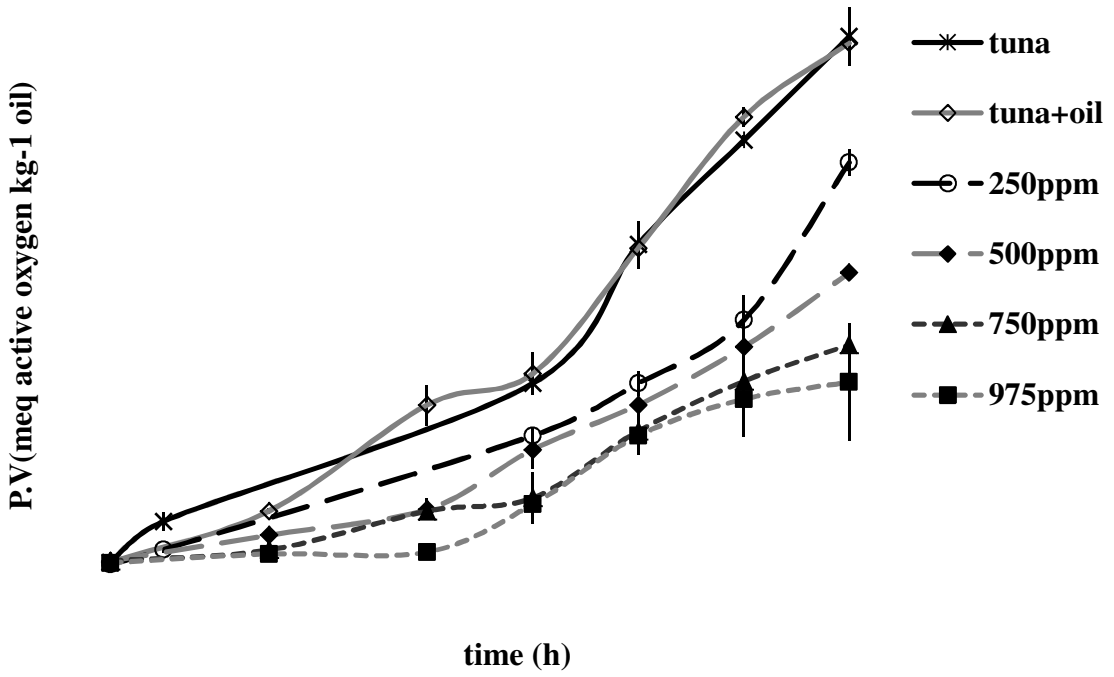


Figure 20b: Results of PV during oxidation of tuna samples during storage at 70 °C for control samples (minced tuna alone), oil sampl (minced tuna with oil 3:1 not enriched with extract), and enriched samples (.250, 500, 750 and 975 ppm).

The results of PV combined with TBARs confirmed that natural extracts of *M. syriaca* exhibit an antioxidative effect when added to tuna. This can be attributed to the presence of phenolic compounds and flavonoids as analyzed in sub-chapter 5.2 which revealed the existence of thymol, carvacrol, rosmarinic acid, taxifolin, eriodictyol, apigenin and several not identified flavonoids. Other studies, where extracts from plants were added to seafood model system and the effect on reducing lipid oxidation was estimated by measuring PV and TBARs, showed similar results (He and Shahidi, 1997; Sarkardei and Howell, 2006; Ramanathan and Das, 1992).

Abdalla, A. & Roozen, P. : Effect of plant extracts on the oxidative stability of sunflower oil and emulsion. *Food Chem.*, **64**:323–329, 1999.

Akguel A., Bayrak A.: Constituents of essential oils from *Origanum* species growing wild in Turkey. *Planta Med.* , **53**, 114-121, 1987.

Boros B., Jakabova S., Dornyei A., Horvathd G., Pluhare Z., Kilar F., Felingera, A., Determination of polyphenolic compounds by liquid chromatography–mass spectrometry in Thymus species, *J. of Chromatogr.* A, **1217,** 7972–7980, 2010.

Boué, S.; Carter-Wientjes, C.; Shih, B.; Cleveland, T.: Identification of flavones aglycones and glycosides in soybean pods by liquid chromatography-tandem mass spectroscopy, *J. of Chromatogr. A*, **991**(1), 61-68, 2003.

Baser K., Ozek T., Tumen G., Sezik E.: Composition of the essential oils of Turkish Origanum species with commercial importance. J Essential Oil Res., 5(6), 619-62, 1993.

Dorman H., Bachmayer O., Kosar M., Hiltunen R.: Antioxidant properties of aqueous extracts from selected Lamiaceae species grown in Turkey. *J Agric Food Chem.*, **52**, 762-770, 2004.

Coppen, P. J. (1994). In: Rancidity in Foods. 3rd edn. Allen, J. C., and Hamilton, R. J. Chapman and Hall, London.

Dorman H., Bachmayer O., Kosar M., Hiltunen R.: Antioxidant properties of aqueous extracts from selected Lamiaceae species grown in Turkey. *J Agric Food Chem.*, **52**, 762-770, 2004

Dimakou C,. Kiokias S. & Oreopoulou V.: Activity of natural carotenoid preparations against the autoxidative deterioration of sunflower oil-in-water emulsions, *Food Chem.* **114**, 1278–1284, 2009.

Dimakou C,. Kiokias S., Tsaprouni I., Oreopoulou V.: Effect of Processing and Storage Parameters on the Oxidative Deterioration of Oil-in-Water Emulsions. *Food Biophysics* **2**, 38–45,2007.

Dimakou C,. Kiokias S., Tsaprouni I., Oreopoulou V.: Effect of heat treatment and droplet size on the oxidative stability of whey protein emulsions, *Food Chem.*, **105** , 94–100, 2007.

Dimakou C,. Kiokias S., Tsaprouni I., Oreopoulou V.: Effect of Compositional Factors against the Thermal Oxidative Deterioration of Novel Food Emulsions, *FOBI*, **1,** 115–123, 2006.

Exarchou V., Nenadis N., Tsimidou M., Gerothanassis I., Troganis A., Boskou D.: Antioxidant activities and phenolic composition of extracts from Greek oregano, Greek sage and summer savory. *J Agric Food Chem.*, **50**, 5294-5299, 2002.

Kulisic T., Radonic A., Katalinic V., Milos M.: Use of different methods for testing antioxidative activity of oregano essential oil. *Food Chem.*, **85**, 633-640, 2004.

Kouri G., Tsimogiannis D., Bardouki H., Oreopoulou V.: Extraction and analysis of antioxidant components from Origanum dictamnus. *Innov Food Sci Emerg Technol.* **28** 155–162, 2007.

Kulisic T., Radonic A., Katalinic V., Milos M. : use of different methods for testingantioxidative activity of oregano essential oil. Food Chemistry, 85,633-640, 2004.

Li Z., Hua X., Wang Y., Fang J.: Apigenin inhibits proliferation of ovarian cancer A2780 cells through Id1 *FEBS Letters*, **583**, 1999–2003, 2009.

Lo A., Liang Y., Lin-Shiau S., Ho C., Lin J.: Carnosol, an antioxidant in rosemary, suppress inducible nitric oxide synthase through down-regulating nuclear factor kB in mouse macrophases, Carcinogenesis, **23,** 6, 983-991, 2002.

Litwinienko G., Ingold K.: Abnormal solvent effects on hydrogen atom abstraction. 1. The reaction of phenols with 2,2-diphenyl-1-picrylhydrazyl (dpph$^{\bullet}$) in alcohols. *J. Org Chem.* **68**, 3433-3438, 2003.

Lauerre, M., Lecomte J., Villeneuve, P.: Evaluation of the ability of antioxidants to counteract lipid oxidation: existing methods, new parameters, new trends and challenges, *Progress in Lipid Research.*, **46**, 244-282, 2007.

Ordoudi S., Befani C., Nenadis N., Koliakos G., Tsimidou M.: Further examination of Antiradical properties of Crocus sativus Stigmas extract Rich in Crocins, J Agric. Food Chem., **57**, 8, 3080-3086, 2009.

Putievsky E., Dudai N., Ravid U (1996): Cultivation, selection and conservation of oregano species in Israel. In: *Proceedings of the IPGRI International Workshop on Oregano*, 8–12 May, CIHEAM, Valenzano (Italy), pp. 102–109.

Roedig-Penman A., and Gordon, M. H. Antioxidant properties of myricetin and quercetin in oil and emulsions, *J. Amer. Oil. Chem. Soc.*, **75**, 169–180, 1998.

Sakakibara, Y. Honda, S. Nakagawa, H. Ashida, K. Kanazawa: Simultaneous determination of all polyphenols in vegetables, fruits, and teas. *J Agric Food Chem.* **51**, 571-581, 2003.

Tuberosoa C., Montoro P., Piacente S., Corona G., Deiana M., Dessi M., Pizza C., Paolo Cabras: Flavonoid characterization and antioxidant activity of hydroalcoholic extracts from *Achillea ligustica.*. *Journal of Pharm. and Biomed. Anal.* **50** , 440–448, 2009.

Tsimogiannis D., Oreopoulou V.: Free-radical scavenging and antioxidant activity of 5,7,3′,4′-hydroxy-substituted flavonoids. *Innov Food Sci Emerging Technol.* **5**, 523-528. 2004.

Tsimogiannis D., Stavrakaki M., Oreopoulou V.: Isolation and characterisation of antioxidant components from oregano (*Origanum heracleoticum*). *Int. J. Food Sci. Technol.*, **41**,(Suppl 1), 39–48. 2006.

Tsimogiannis D., Oreopoulou V.: The contribution of flavonoid C-ring on free radical scavenging efficiency. A kinetic approach for the 3′,4′-hydroxy-substituted members. *Innov Food Sci Emerging Technol.*, **7**, 140-146, 2006.

Tsimogiannis D., Samiotaki M., Panayotou G. & Oreopoulou V.: Characterization of Flavonoid Subgroups and Hydroxy Substitution by HPLC-MS/MS, *Molecules*, ***12***, 593-606, 2007.

Valgimigli L., Banks J. ., Ingold K., Lusztyk J.: Kinetic solvent effects on hydroxyl hydrogen atom abstraction are independent of the nature of the abstraction radical:Two extreme tests using vitamin E and phenol. *J Am Chem Soc.* **117**, 9966-9971, 1995.

Vekiari S., Tzia C., Oreopoulou V., Thomopoulos C.: Isolation of natural antioxidants from oregano. *Riv Ital Sost Grasse.*, **70**, 25–28, 1993.

Conclusions

The main target of this study was successfully achieved, by exploring *M. syriaca* as a new source of natural antioxidant for food technology, especially taking into consideration that this plant has not been studied previously in details. This goal was

approached through extraction and characterization of the components of the plant, followed by intensive study of its antioxidant activity in different food systems. Using successive extractions by soxhlet was efficient in obtaining natural extracts with antioxidant potency according to their polarity.

The solvents petroleum ether (P), diethyl ether (D), ethyl acetate (EAc), and ethanol (E), were used in order to fractionate the components of the plant according to their polarity so as to investigate their activity and facilitate their identification. P was used to extract the less polar compounds including the constituents of the essential oil. The oil-free plant material was then treated with D, a low-polarity solvent for the extraction of low-polarity flavonoid aglycones, and EAc, a medium polarity solvent for the extraction of flavonoids with medium polarity. The extraction with E was performed to collect the residual, polar phenolics. The yeild of extracts was 23%, in whic 9.04% obtained by P extraction, 3.40% by D, 4.10% by EAc, and 6.03% by E. Petroleum ether (P) collected the essential oil components (less polar), and according to GS-MS analysis the main constituents were thymol, 42.89 %, and carvacrol, 42.75 %., which is in accordance to the previous literature about the plant essential oil components.

On the other hand, the analysis of the D, E, EAc extracts of successive extraction was analyzed by HPLC-MS and HPLC-DAD revealed several flavonoids and phenolic acids. Seven major peaks and several minor ones were observed in the HPLC chromatogram of the D extract, the detected compounds were taxifolin (27.5%), eriodictyol (41%), carnosol (44%), while at peak 2 an unknown dihydroflavonol or flavones is expected and at peak 6 an unknown flavone is expected. In the EAc extract, apigenin was detected at peak 8, and rosmarinic acid at peak 4, while at peaks 2 and 3 an unknown flavanone or dihydroflavanol glycoside structure is expected.

The major component of E extract was rosmarinic acid (40%) detected at peak 4, while at peaks 1 and 2 an unknown flavones glucoside were found, and a phenolic acid is expected at peak 5. The activity of the extracts to scavenge the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$) was measured and found that they followed the order D > E > EAc > P. In order to evaluate the antiradical scavengablity, the term EC_{50} was calculated. EC_{50} represents the amount of dry extract that reduces DPPH concentration to half of its initial concentration. The EC_{50} values of extracts tested against DPPH showed

strong antiradical efficiency comparingly to well known antioxidants. The EC_{50}were 1000, 1018, 1422, and 6000 g extract/kg DPPH for the D, E, EAc, and P extracts respectively.

The obtained extracts of *M. Syriaca* with petroleum ether (P), diethyl ether (D), ethyl acetate (EAc), and ethanol (E) increased the stability of bulk corn oil in accelerated oxidation tests. They reduced peroxide values (PV) and conjugated dienes (CD) effectively as experessed by oxidation factor values (OF_{CD}, OF_{PV}). The oxidation factor represents the ratio between the differences in oxidation level of the extract to the oxidation level of the control (bulk oil without extract).

Moreover, the antioxidant activity in corn oil at concentrations of 200, 500 and 1000 ppm, where it was revealed that D and E extracts resulted in the highest protection followed by EAc and P extracts. It was also concluded from results that increasing the concentration from 200 to 500 ppm depressed peroxide formation, while a further increase had no effect.

The delay in oxidation caused by antioxidative effect of extracts is expressed in term of induction period, which is defined as the time that passes from initial point till the oxidation starts to get accelerated. The successive extracts, approximately double the induction period and markedly depress the formation of peroxides throughout oxidation (with a kind of extract- and concentration-dependent effect), resulting in a reduction of OF_{PV} values to approximately 0.38 for D or E extracts, at a concentration of 500 ppm. Also, all the above extracts depress CD formation and reduce OF_{CD} values to almost half, when added to the oil at a concentration of 200 ppm or higher. According to the above results, the concentration of 500 ppm is considered a suitable amount of extract to be added in oils, since it showed satisfactory effect for all indexes. The extract obtained with P had OF_{PV} at 200ppm equal to 0.69, had lower effect than other extracts (OF_{PV} for D, EAc, and E at 200ppm was 0.55, 0.60, 0.57 respectively).

While the purpose of the first part of successive extractions was to separate the components of the plant, the second part aimed at examining one-step extraction using ethyl actetate which is a food-grade solvent, while the procedure is a simple one suitable for possible commercial exploitation of the plant to recover antioxidants.

In the second series of experiments, *M. syriaca* was subjected to a simplified, one step extraction by ethyl acetate (1-EAc), to recover the main antioxidant components that are soluble in oil. The 1-EAc extract had strong antioxidant activity as found by the antiradical test and the accelerated oxidation test. The yield of extraction was 12.6% on dry basis. The antiradical efficiency of the extract against DPPH revealed an EC_{50} value of 1000 g extract/kg DPPH. The antioxidant activity was increased as follows; 200ppm> 500ppm≥1000ppm. The depression of PV was generally lower than that obtained by D, E and EAc extracts of successive extractions (OF_{PV} at 200 ppm was 0.60), while the depression of CD was comparable (OF_{CD} was 0.67).

The following approach was used to examine *M. syriaca* extract in various food systems: After examining the plant antioxidant activity in bulk oil, the experiment was extended to examine it in oil-in-water emulsions. The 1-EAc had antioxidative effect when used in oil-in-water emulsions, which were subjected to accelerated oxidation in shaking water bath, at 40°C and at 60°C. The results indicated, that 1-EAc extract protected the emulsion by delaying the oxidation, despite of increase in temperature, with OF_{TBARs} was 0.69 and OF_{CD} was 0.63, although the OF_{CD} at 40°C was 0.52. The antioxidant effect at higher temperature was less but still satisfactory. Finally it can be clearly concluded from this experiment that *M. syriaca* extract can be added to emulsion with noticeable antioxidant effect as it reduced emulsion oxidation strongly. These results are in accordance with findings from literature, which emphasize that the plants of *Labiatae* family are promising for the recovery of antioxidants.

In the second series, bulk corn oil and oil-in-water emulsions were subjected to high hydrostatic pressure (HHP) treatments at 200 and 650 MPa so as to estimate the effect of the applied pressures on lipid oxidation. According to the results, the effect of pressure is already evident at the initial point just after pressurization and before applying samples to further accelerated oxidation. The initial CD value of non HHP-treated emulsion samples was 0.129±0.01 g/100 g oil while it was increased to values 0.194±0.01 and 0.346±0.02 g/100 g oil after HHP treatment at 200 and 650 MPa, respectively. Similarly, the CD value of non HHP-treated oil samples was 0.57± 0.01 g/100g and amounted to 0.61± 0.02 g/100g and 0.66± 0.03 g/100g after HHP treatment at 200 and 650 MPa, respectively. Similar differences were also observed in the other oxidation indexes, i.e. TBARs and

PVs, and can be attributed to the increase of the concentration of dissolved oxygen caused by the HHP treatment (from 0.014 mg/mL in non-treated emulsion to 0.029 mg/mL in 650 MPa-treated emulsion and from 0.024 mg/mL in non-treated oil to 0.029 mg/mL in 650 MPa-treated oil). The addition of 1-EAc extract at 200 ppm led to protection against lipid oxidation during storage of the samples subjected to HHP treatment (200 and 650 MPa) at atmospheric conditions by 20.9-38.7, 28.9-43.2 %, respectively. According to comparison on PV, CD and TBARs values calculated for treated and non-treated samples of (bulk oil) and (oil-in-water emulsions) treated under HHP conditions, it can be deduced that the oxidation of lipids in oils and emulsions is favoured at higher pressures.

Although the essential oil of the *M.syriaca* has less antioxidant activity than other extracts, the literature shows that it possess an important antimicrobial activity. It was necessary also to study an example of meat products as they also contain lipids, for the antioxidant effect of *M. syriaca* extract. Yellowfin tuna was a useful choice since it was not studied by this plant previously, and it is widely used food of great importance. Examining the *M. syriaca* extract was more comprehensive in this part to include also antimicrobial activity, by testing the inhibitory effect against microbial growth. The results were optimistic, since it was found that mixing *M. syriaca* extract with chilled tuna fish was effective in inhibiting the respective natural spoilage flora and reducing oxidation when stored at 0 °C. From the results of this study, it was concluded that *M. syriaca* extract can be used to extend the shelf life and improve the commercial value of fresh fish products. The addition of *M. syriaca* in tuna increased its stabitlity against oxidation as results of its effect in depressing TBARs and PV during oxidation.

The extract of *M. syriaca* was also employed in other oil-based food systems that are used in daily life, mainly to study the antioxidative effect under thermal oxidation. It was added to oil, that was used in making cookies that were stored in oven at 70° C. The cocnlusion drawn from this experiment is that addition of the extract enhanced the protection against oxidation of cookies (both packaged and unpackaged samples) as the PV and CD of cookies decreased by 79% and 72%, respectively regarding the non-enriched samples.

In deep-frying of potato chips, the results were in accordance with previous findings and revealed an antioxidative and support protection against oxidation in deep frying system at 185° C. However, a remarkable improvement of the oxidative stability of the fried chips was observed through accelerated oxidation tests, where the (PV) and (CD) were decreased by 59 to72% and 51 to 79%, respectively, compared to samples with no additive. The above mentioned results claimed that *M.syriaca* extract increased oil stability during frying process and storage of potato chips. Both frying and baking are oil-based systems at which processing includes applying high temperature. The enrichment with *M. syriaca* proofed to be effective as well in stabilizing food models against thermal oxidation. According to the above mentioned results, it was concluded that adding 1-EAc extract of *M.syriaca* in different food systems at variable experimental conditions, exhibited appreciable antioxidant effect, because it is expected to extract majority of plant antioxidants, confirmed by the oxidation factors measured in every experiment (36%-80%), which supports using it for future researches.

Recommendations for future work

* It will be highly recommended to extend the study of the antioxidant activity *Majorana syriaca* in other food types, to meet the needs of industry and the trends in food technology, as it is showed to have a valuable antioxidant activity.

*It is also highly recommended to follow up the research so as to find if there are ways to optimize the extraction system, either by changing solvents, temperature, time or try new methods of extraction that also proved to be suitable for herbs from *lamiaceae*, such as supercritical carbon dioxide extraction.

*The analysis of the natural antioxidant components obtained in the extracts can be further extended to investigate the unknown flavonoid compounds that were found in the extracts.

*The usefulness of the plant as antimicrobial against tuna beside the antioxidant effect, makes it an attractive target for food preservation, that can be suggested for further development in food quality specially in meat foods, where there is a high need to protect against microbial growth, in addition to oxidation. It is also suggested that further studies may cover organoleptic qualites in fish and other food products as a part of commercial interest.

*It can be also suggested to study if there is a synergistic effect by adding other plants extracts with *M. syriaca* extracts, in order to maximize the efficiency against oxidation.

*It will be of great value, to estimate the antioxidant efficiency of the plant with other measurement tools to compare it with the results obtained in this search. For example, the antiradical efficiency can be measured using other radicals than DPPH, and the oil used in accelerated oxidation tests can be other vegetable oil.

*The antioxidant effect of *M.syriac*a can be applied in practical emulsion systems, such as mayonnaise, salad dressings. Moreover, the experiments with emulsion can be extended by changing emulsion conditions and oxidation effects such as pressure, emulsifier, and temperature.

*It will be also interesting to continue the search by studying not only the antioxidant effect of *M. syriaca*, but also to examine other effects on the food quality, sensory, and probably the nutritional value.

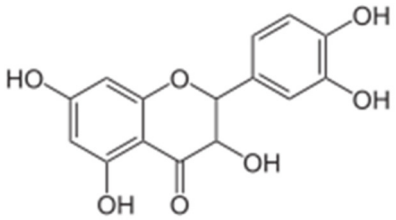
* Further search can be employed on studying the economic benefit of the extractions, and processes used so as to fit the commercial interest.

Analysis of Extracts

Diethethyl ether extract

Figure1a

Peak 1 Taxifolin at Rt 27.5 min, λ_{max} = 288 nm and λ_{min} =250 nm with shoulder at 328nm.



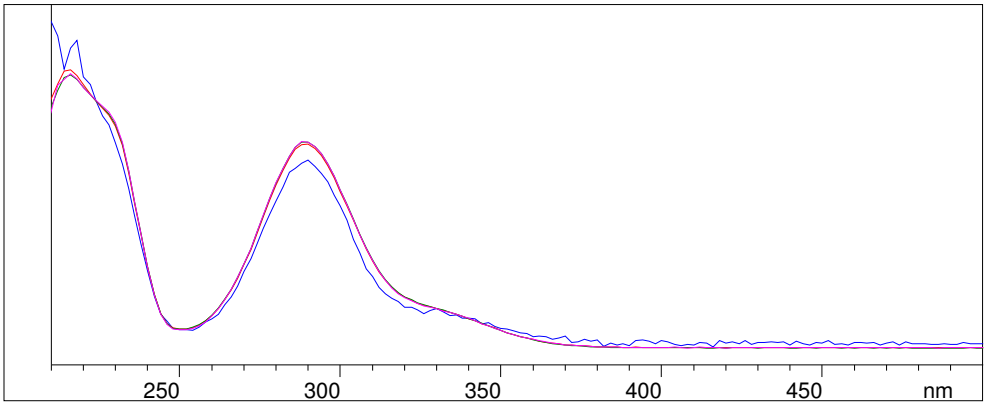


Figure 1b MS spectra $[M+H]^+$=305, 227

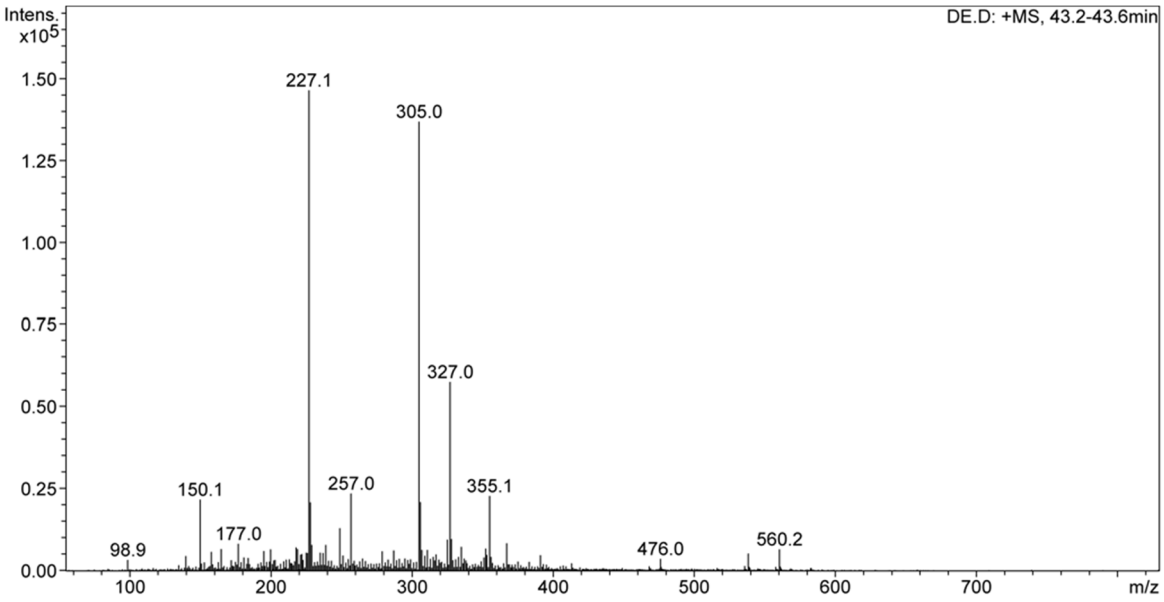
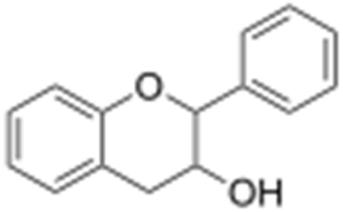
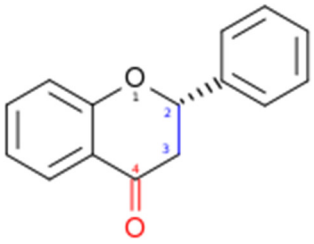


Figure 2a :

Peak 2, Dihydroflavonol or flavanone at Rt=36.6 min, λ_{max} =292 nm and shoulder at 335 nm.



dihydroflavonol



flavanone

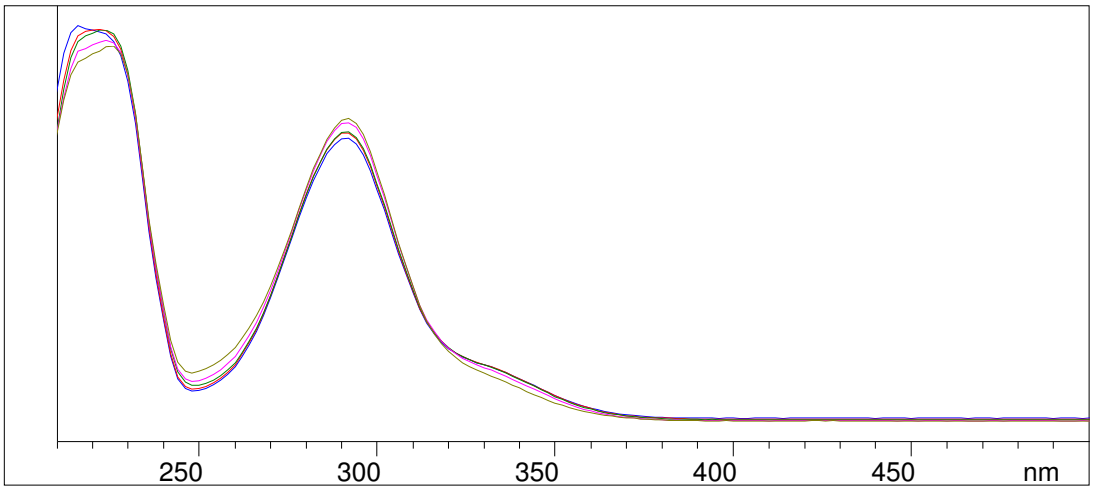


Figure 2b MS $[M-H]^{2-}$ =303

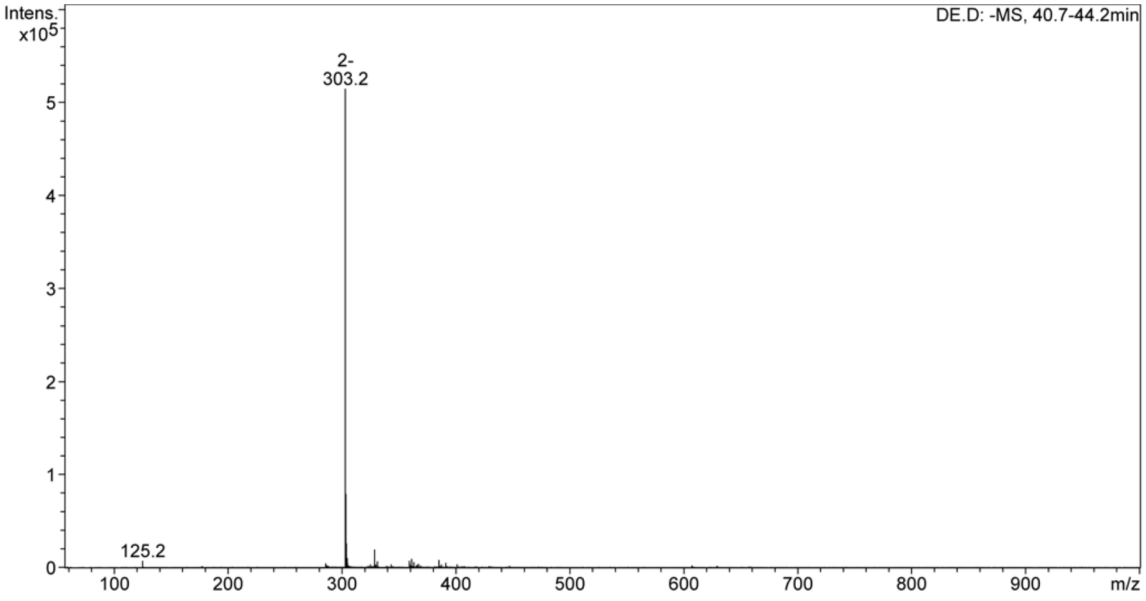
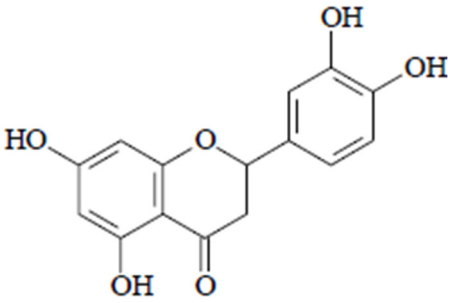


Figure 3a Eriodictyol, peak 3 Rt=41.4min, λ_{max}=288 min (flavanone).



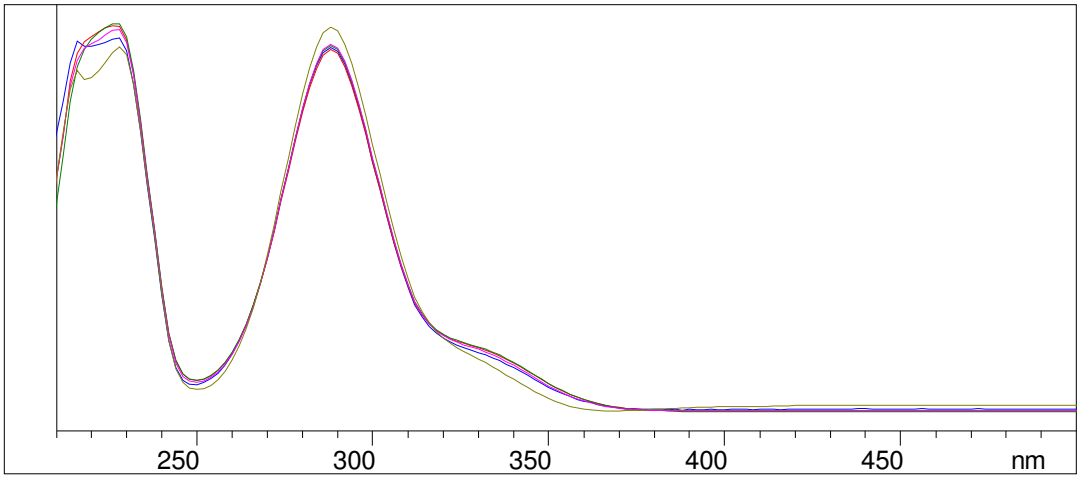


Figure 3b MS $[M-H]^{2-}$ =287

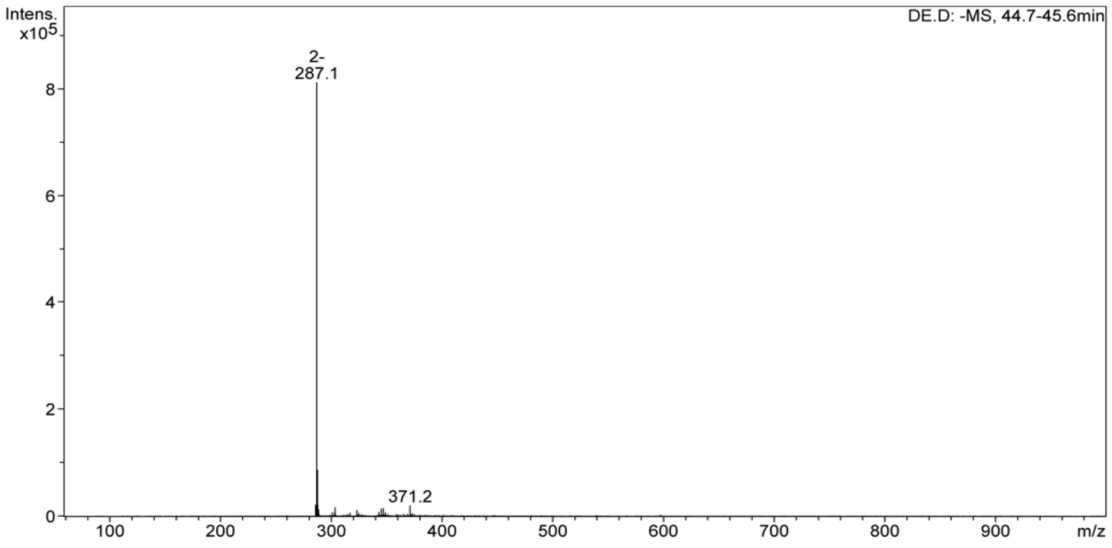


Figure 4a
Peak 4,Carnosol at Rt 44,8 min., and $\lambda_{max=}$228 &292 nm

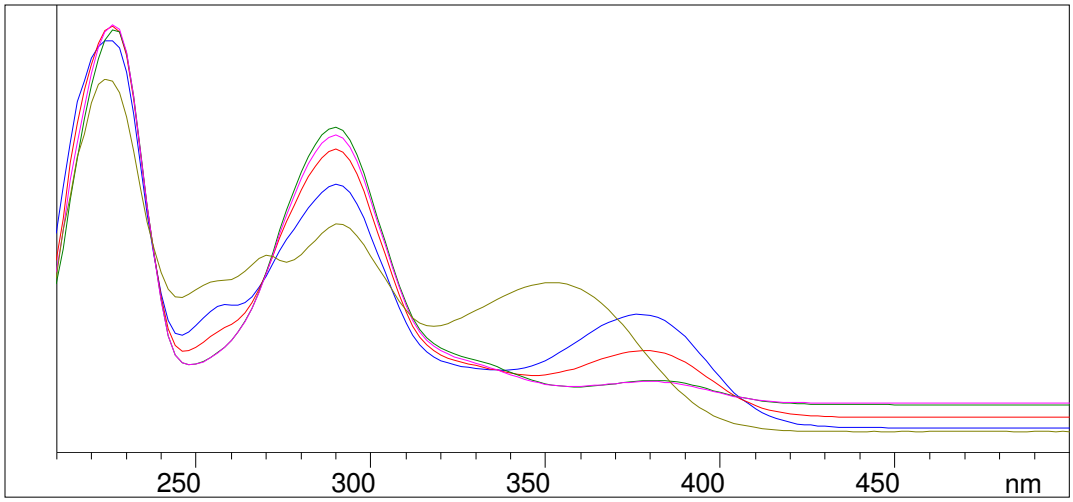


Figure 4b, in MS $[M-H]^-$ =329

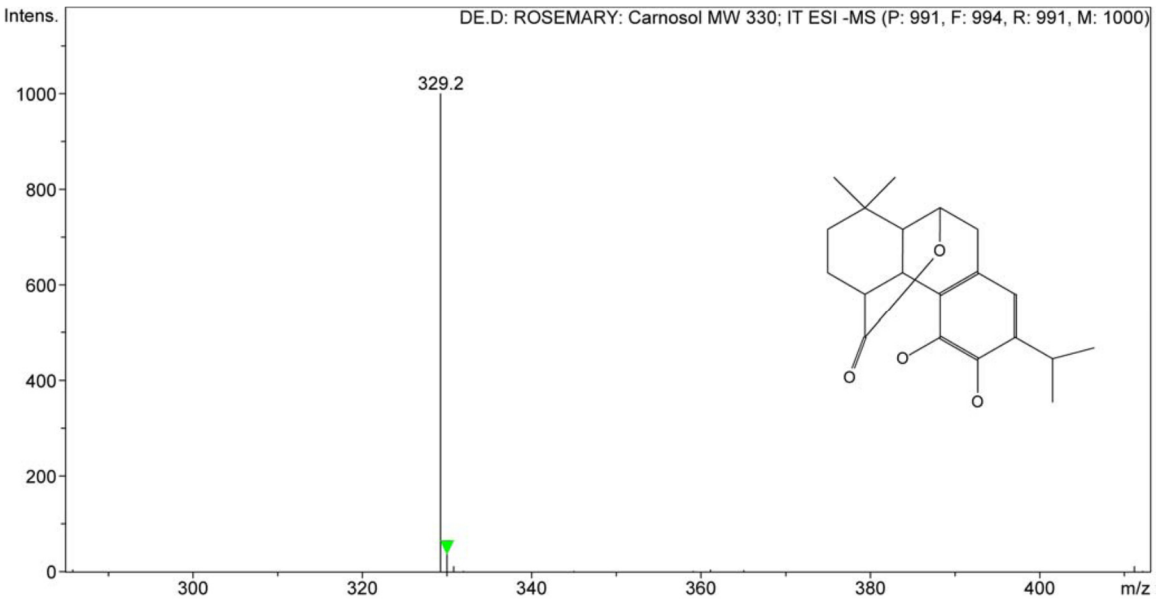
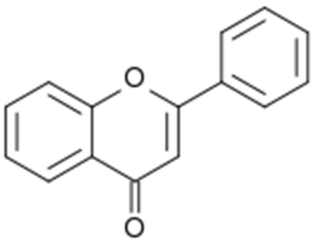


Figure 5a
Peak 6 at Rt=48.1min, flavone λ_{max}=258 nm (band II) ,346 nm (band I)



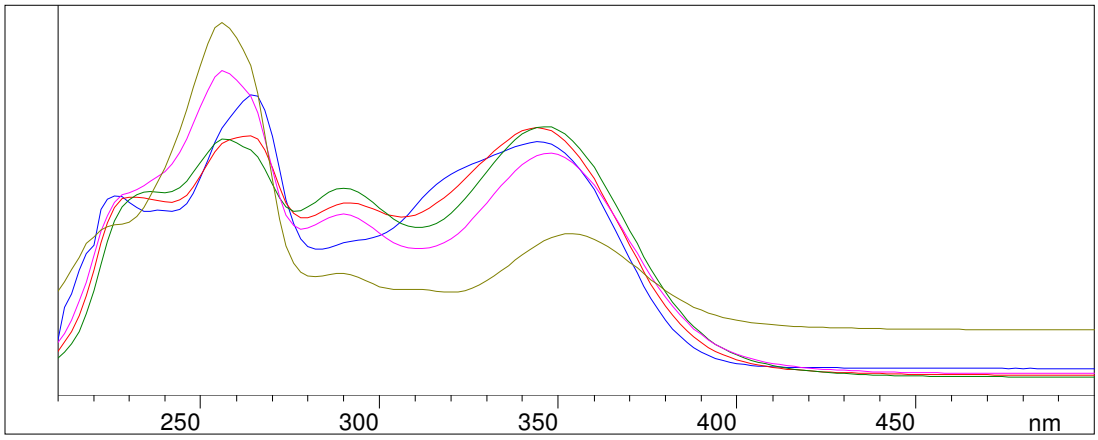
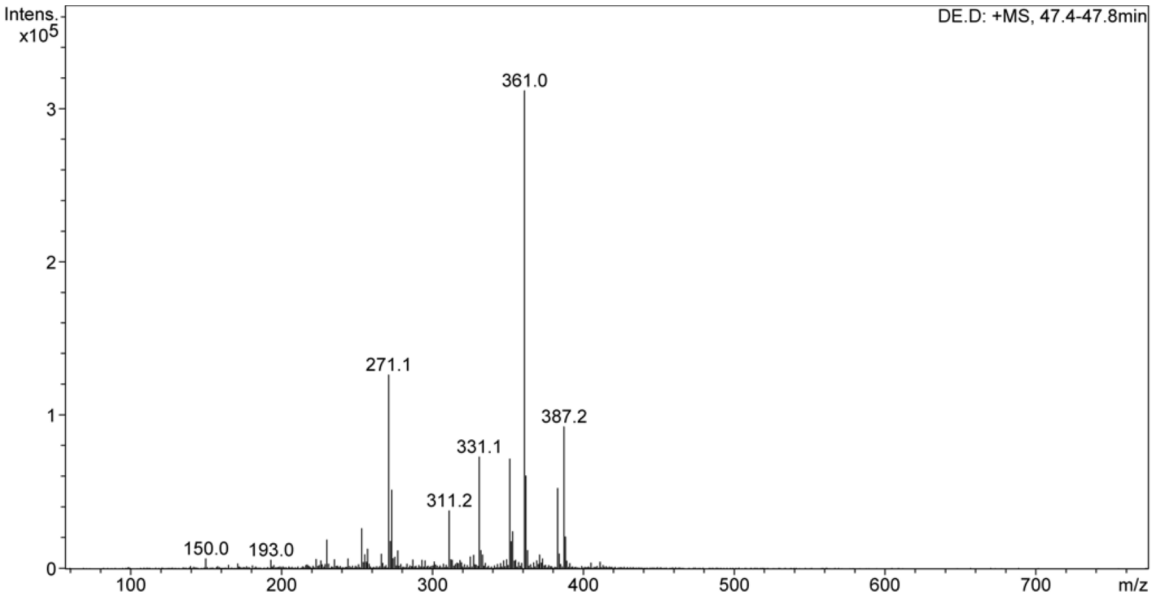


Figure 5b [M+H] =270, $[M-H]^-$=269



Ethyl acetate extract
Figure 6a
Peak 2 Flavanone and dihydroflavonol glucoside

Rt=24.2 min , λ_{max}= 290 nm and shoulder at 330 nm

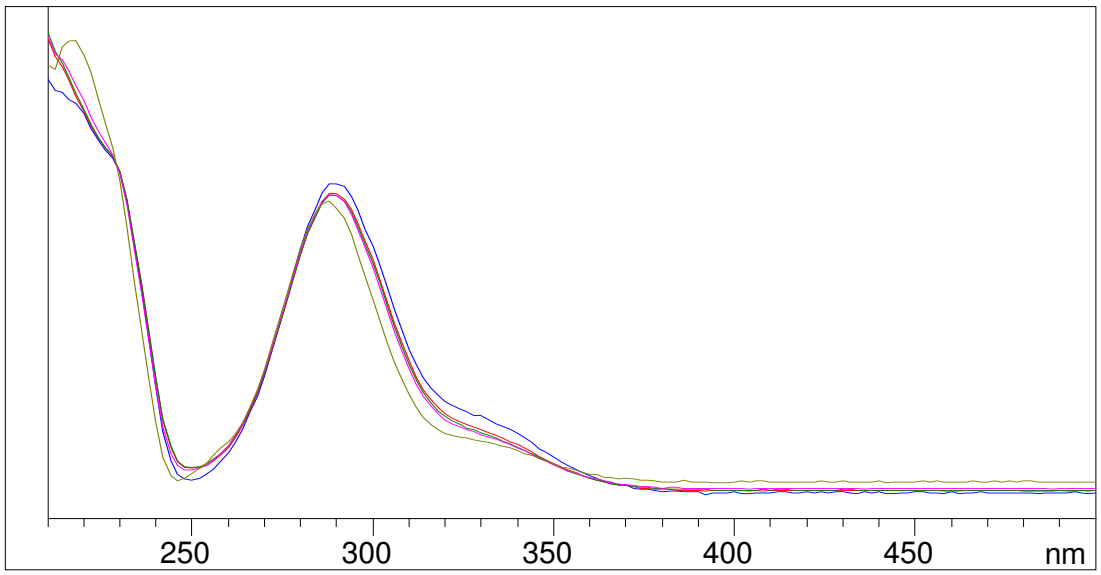


Figure 6b MS spectra at $[M-H]^{2-}$ =429

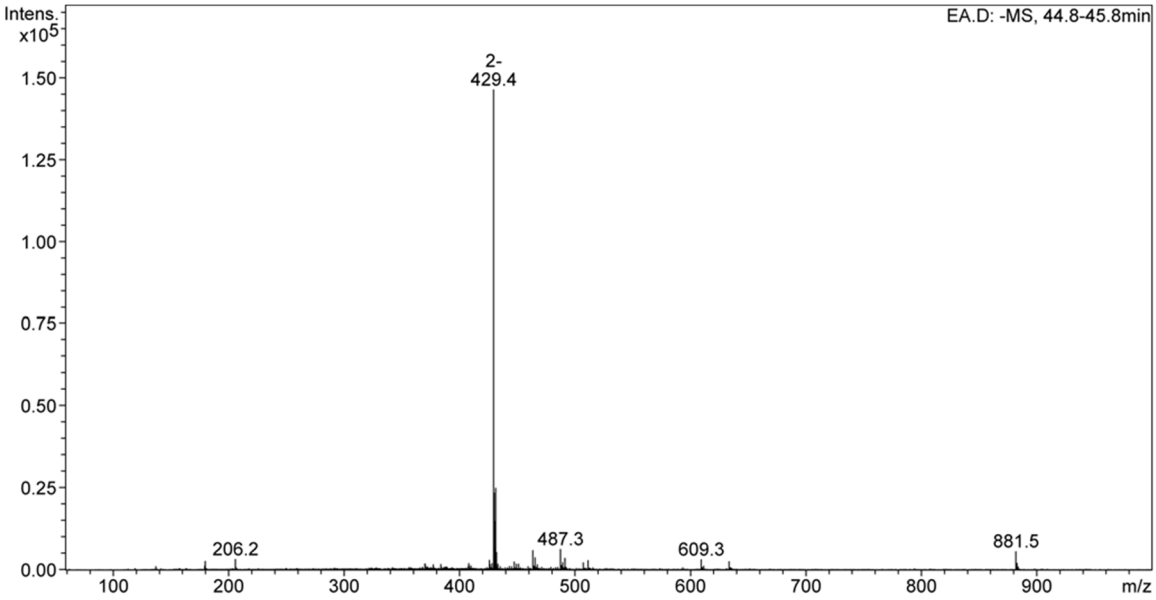


Figure 7a
Peak 3 Flavanone and dihydroflavonol glucoside
Rt=31.3min, λ_{max}= 290 nm and shoulder at 336 nm

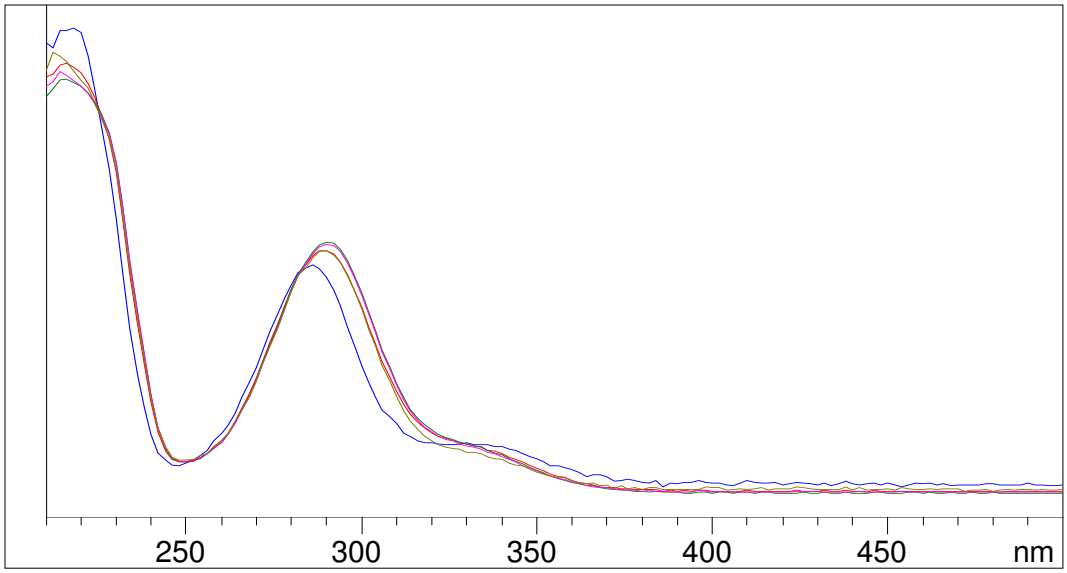


Figure 7b MS spectra at $[M-H]^-$ =473

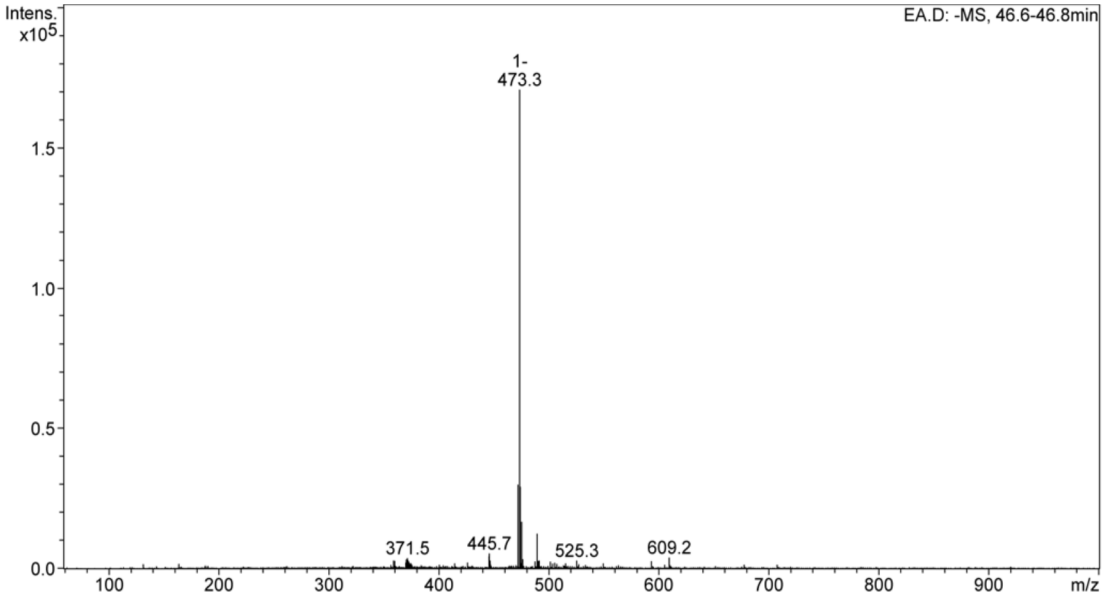


Figure 8a

Peak 4 Rosmarinic acid Rt=37.1min , λ_{max}=290 nm shoulder at 330 nm

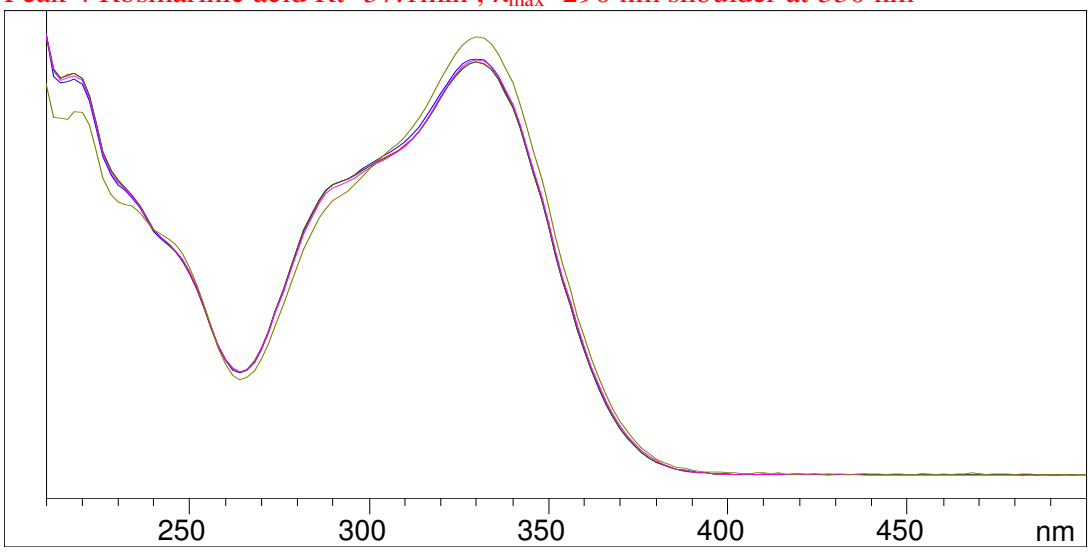


Figure 8b MS spectra $[M-H]^-$ =359

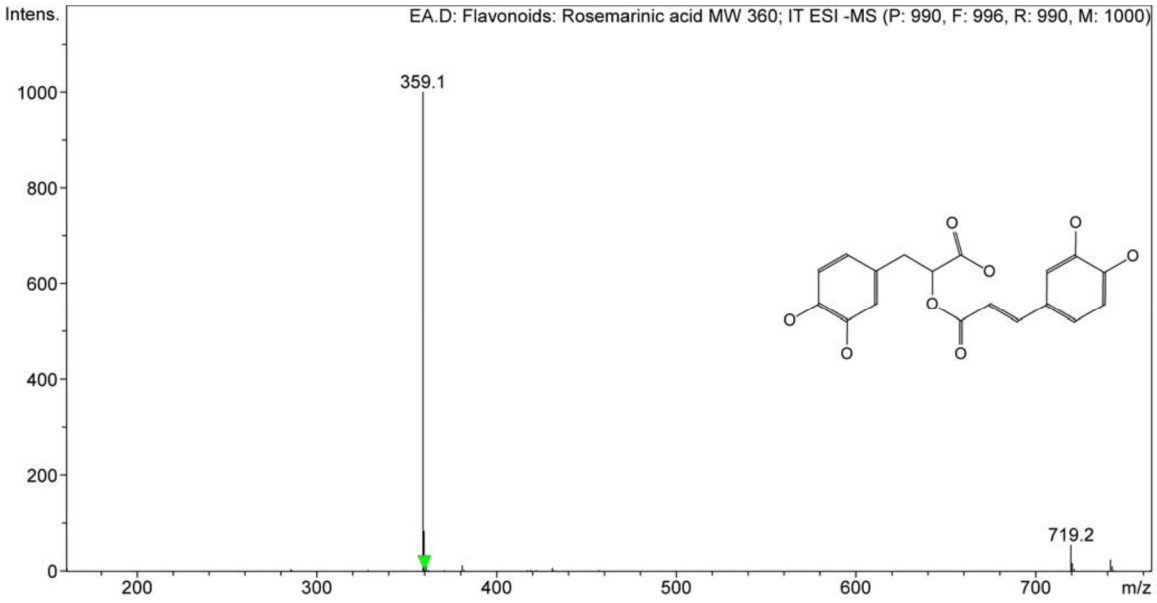


Figure 9a

Peak 5 Flavanone and dihydroflavonol Rt=38 min, λ_{max}=290nm and shoulder at

330nm

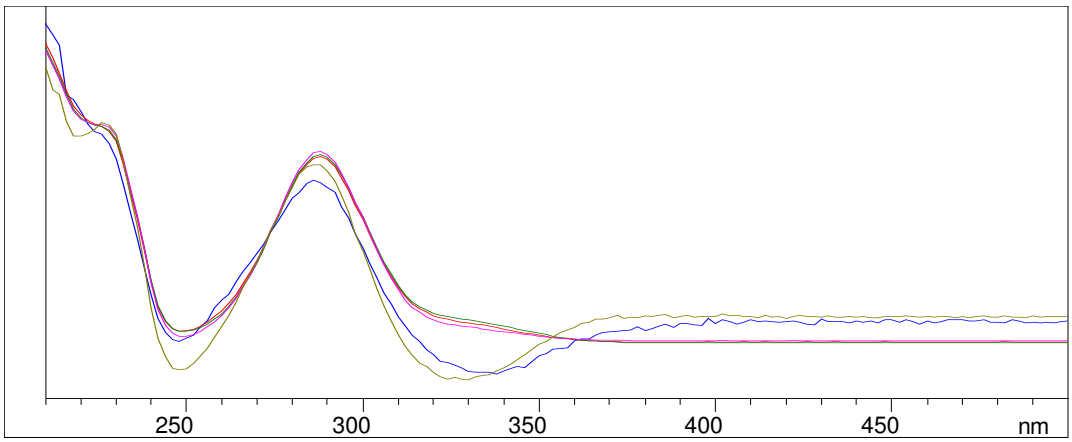


Figure 9b MS spectra $[M-H]^{-}$=393 and $[M-H]^{2-}$ =288.

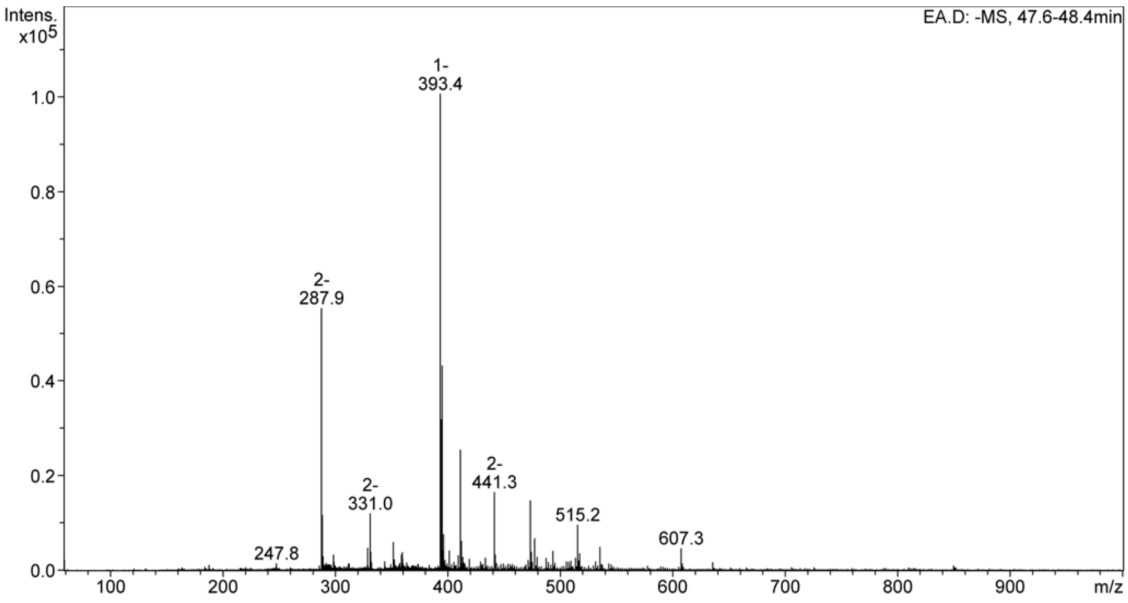
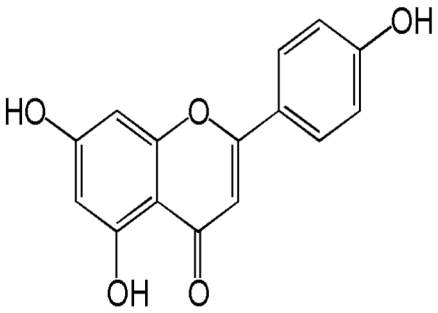


Figure 10a
Peak 8 Apigenin at Rt=46.1 min.λ_{max}= 268 & 338 nm at blue line (from UV-spectra of 280nm)



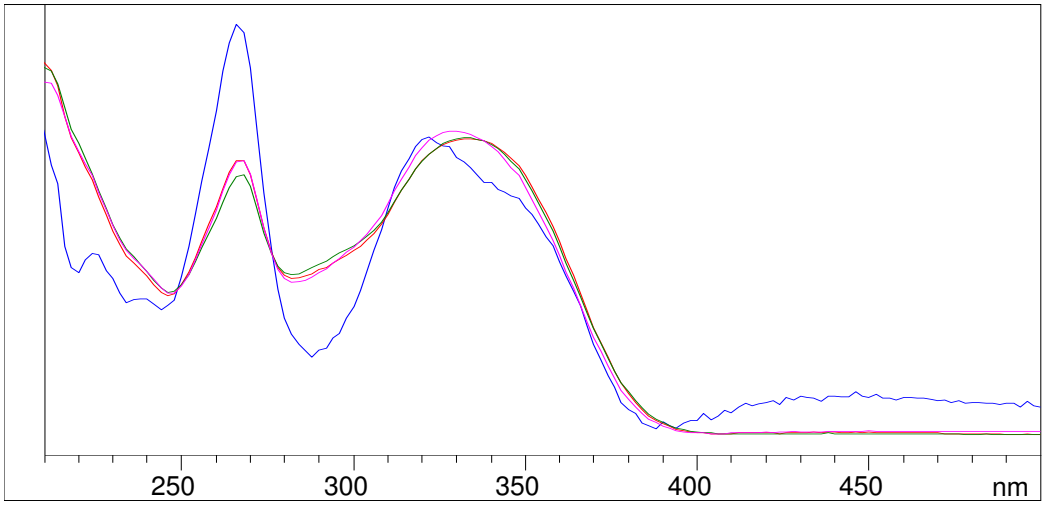
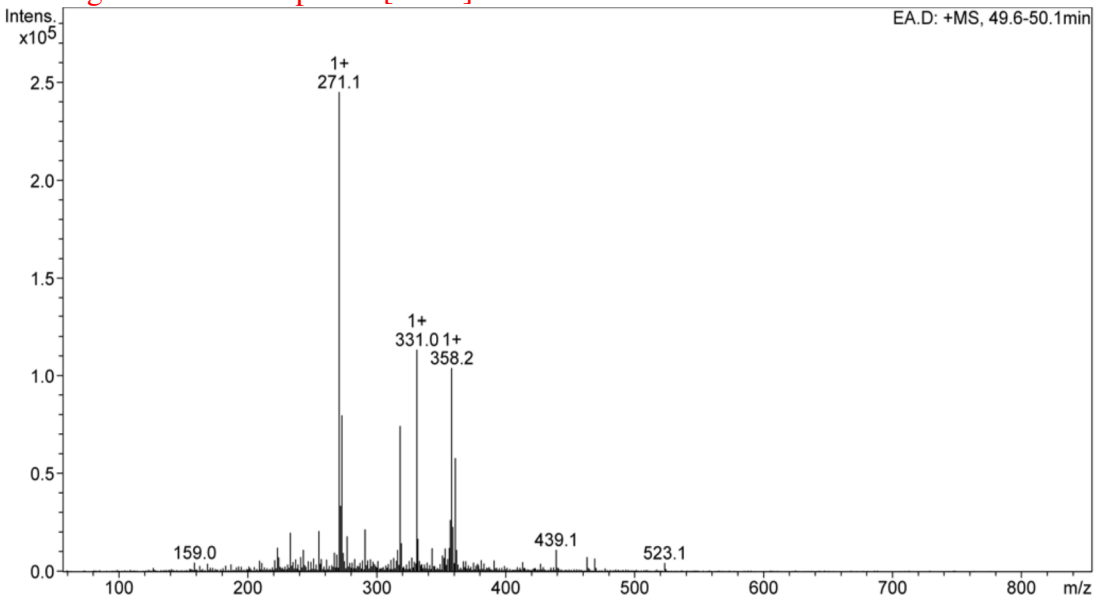


Figure 10b Mass spectra $[M+H]^{+}$=271



Ethanol extract

Figure 11a

Peak 1 Flavone glucoside at Rt=25.5 min, λ_{max}=272 &336 nm.

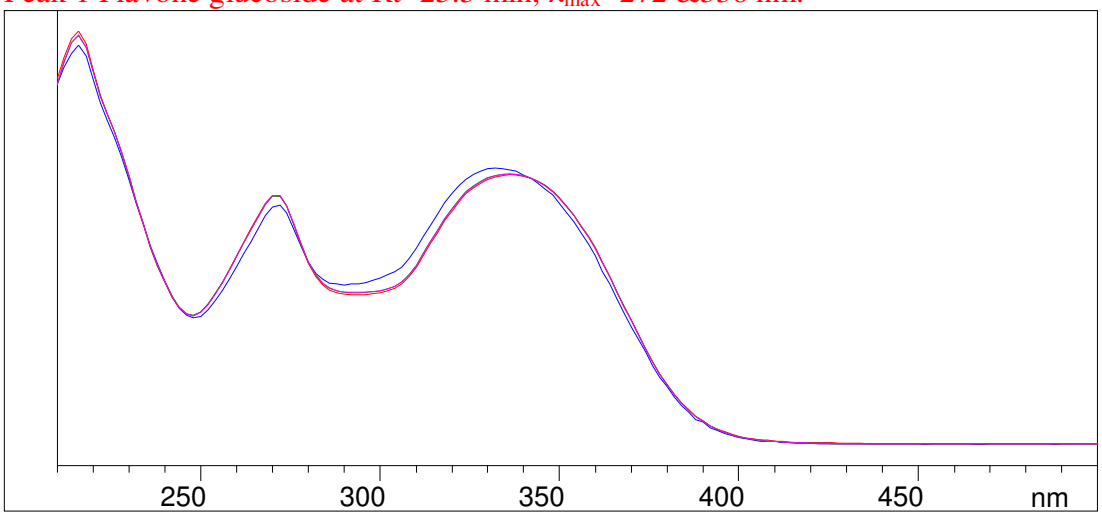


Figure 11b: MS $[M-H]^{-}$=387,593, $[M-H]^{2-}$ =305.

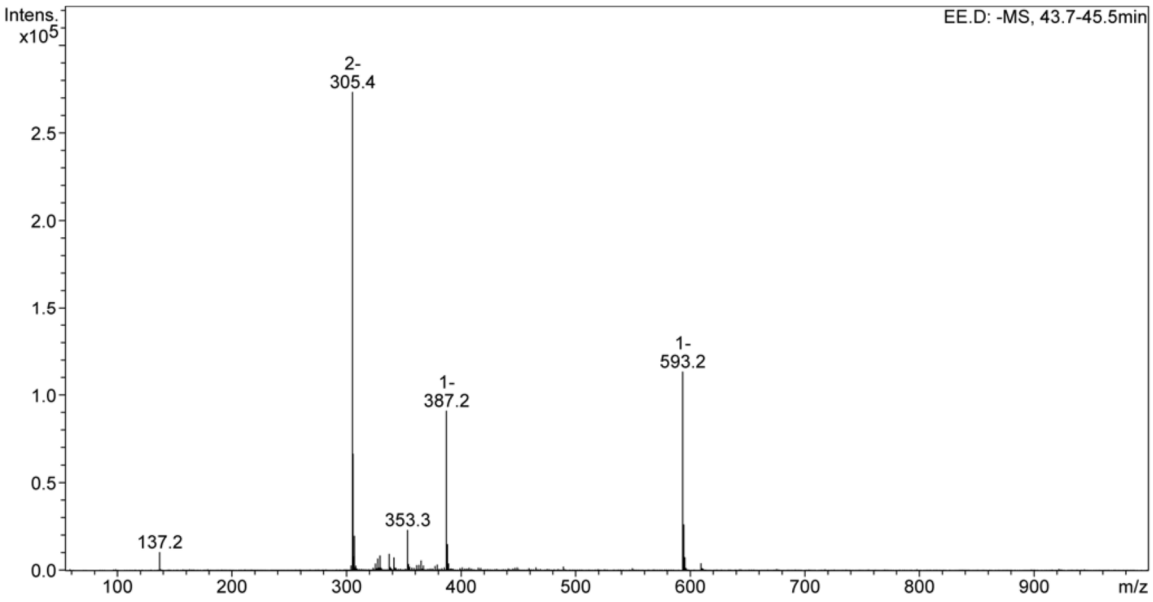


Figure 12 a

Peak 2 Flavone glucoside Rt=26.1 min, λ_{max}= 272 & 336 nm.

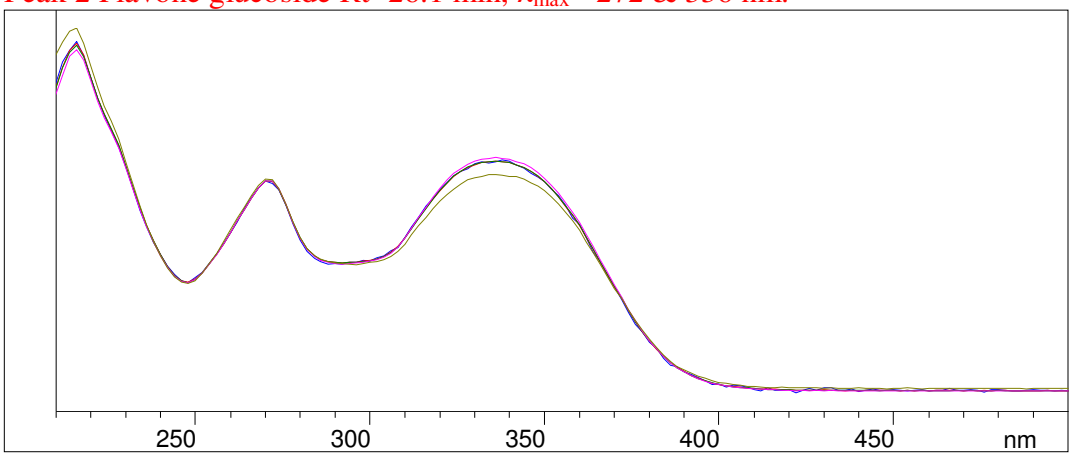


Figure 12b MS spectra at $[M-H]^{2-}$=305, $[M-H]^{-}$ =387 &593.

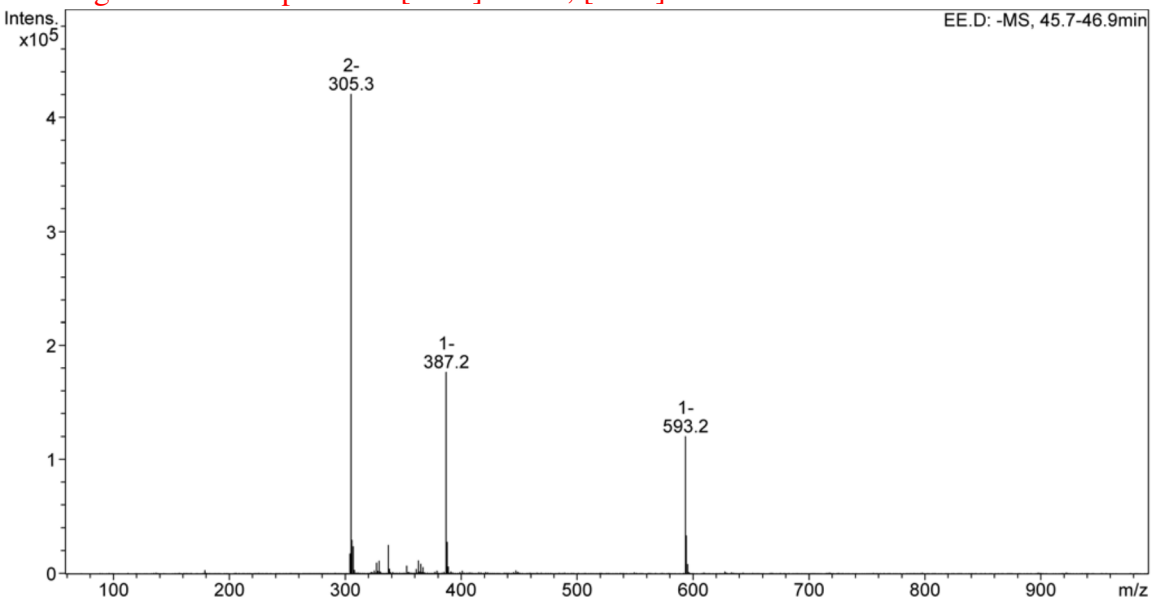


Figure 13a

Peak 3 Rosmarinic acid at Rt=40.2 min.,λ_{max} 330 and shoulder 290.

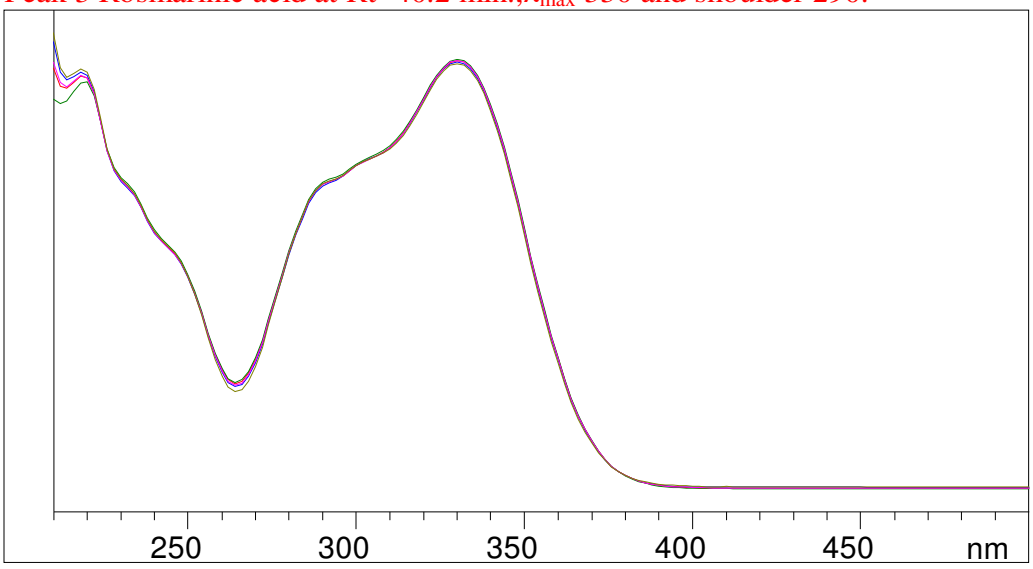


Figure 13b MS spectra $[M-H]^-$=359.

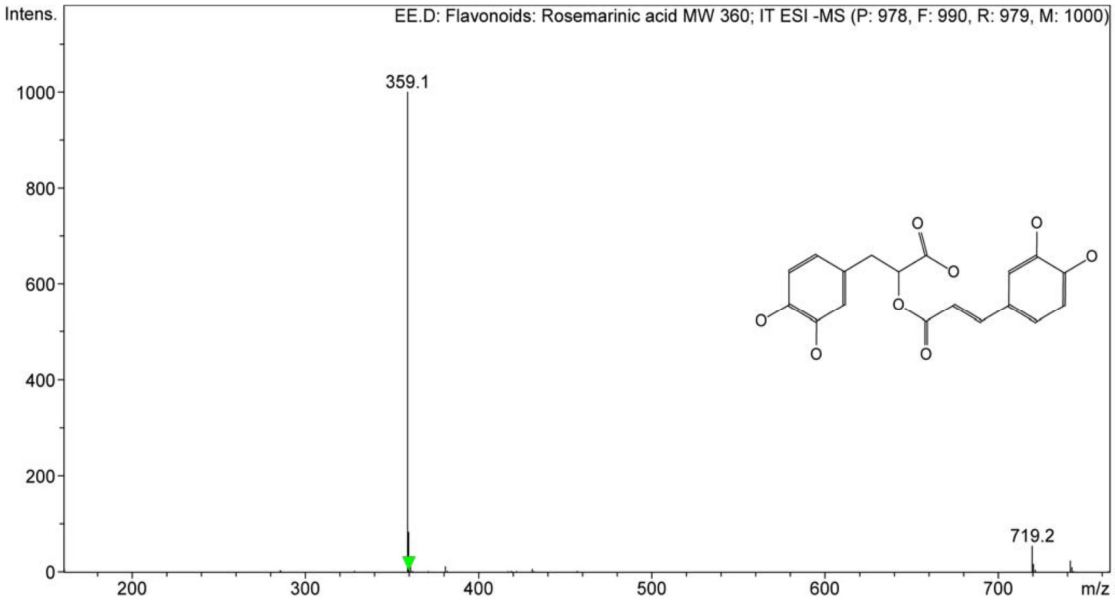


Figure 14a

Peak 5 Phenolic acid Rt=46.1min , λ_{max} = 328nm and shoulder at 294nm.

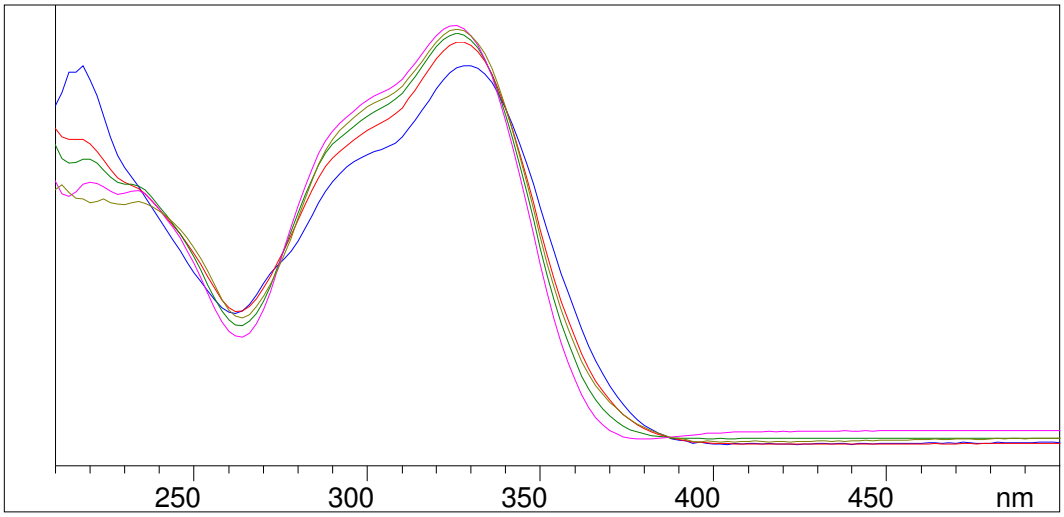
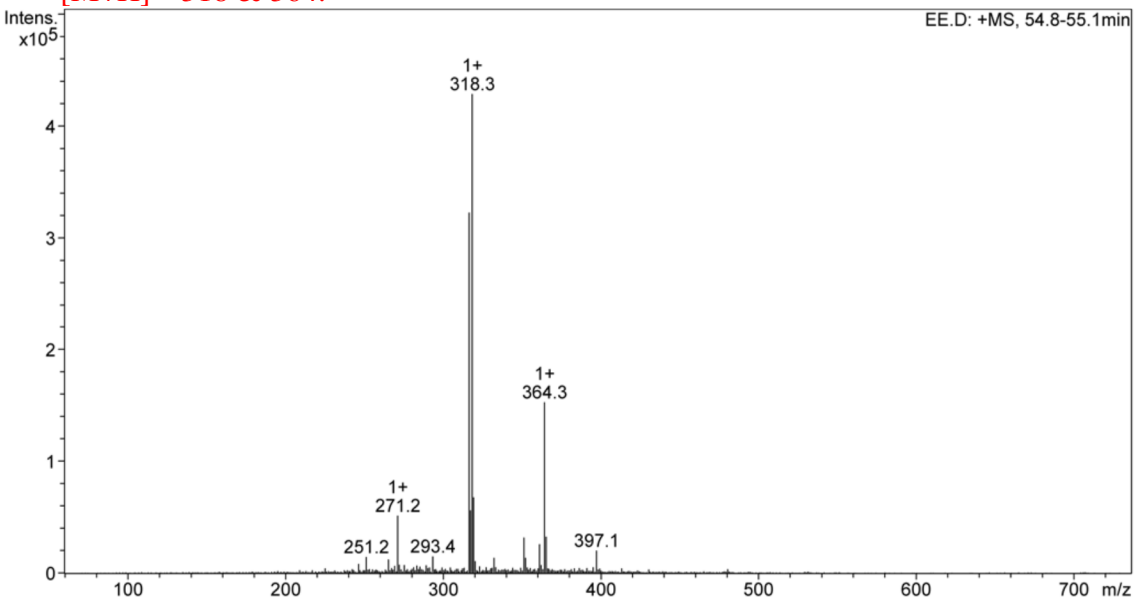


Figure 14b
$[M+H]^+$=318 & 364.



Dietyl ether extract - DPPH / ETAc

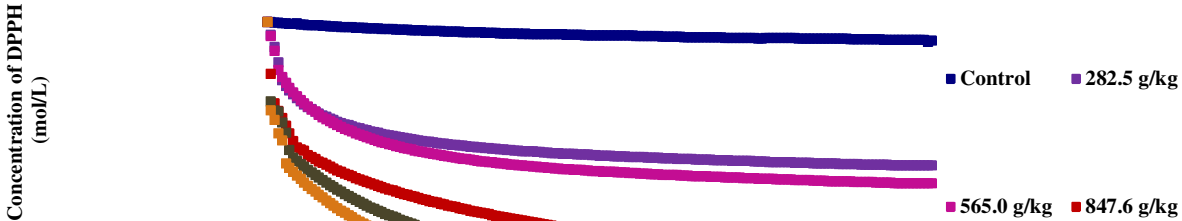
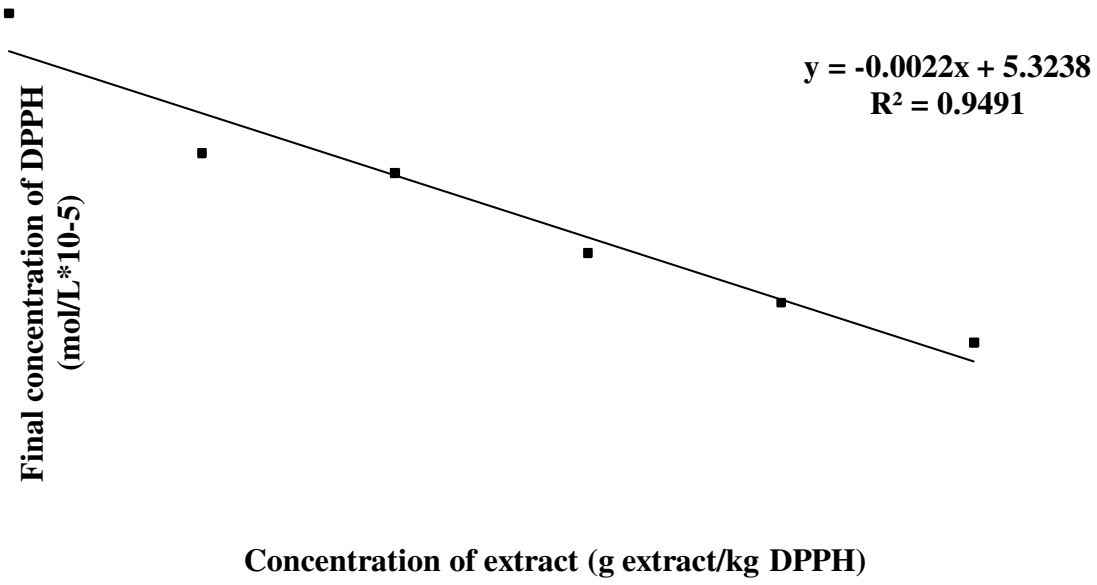


Figure 15a: Depletion of DPPH/EAc by D from successive extraction.



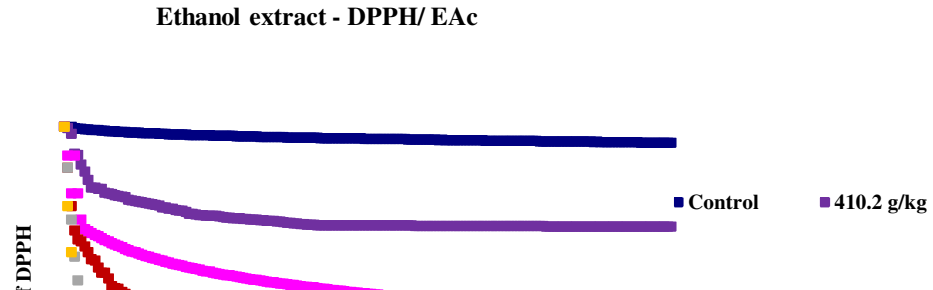


Figure 16a: Depletion of DPPH/EAc by E from successive extraction.

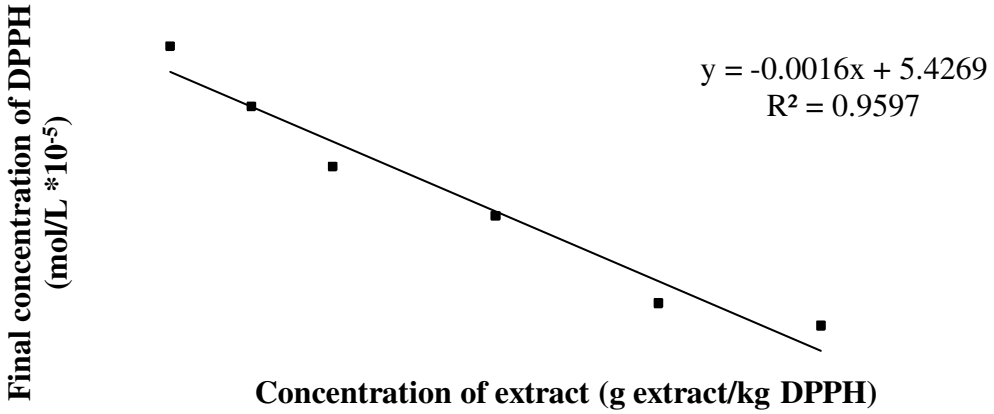


Figure 16b: Calculation of EC_{50}

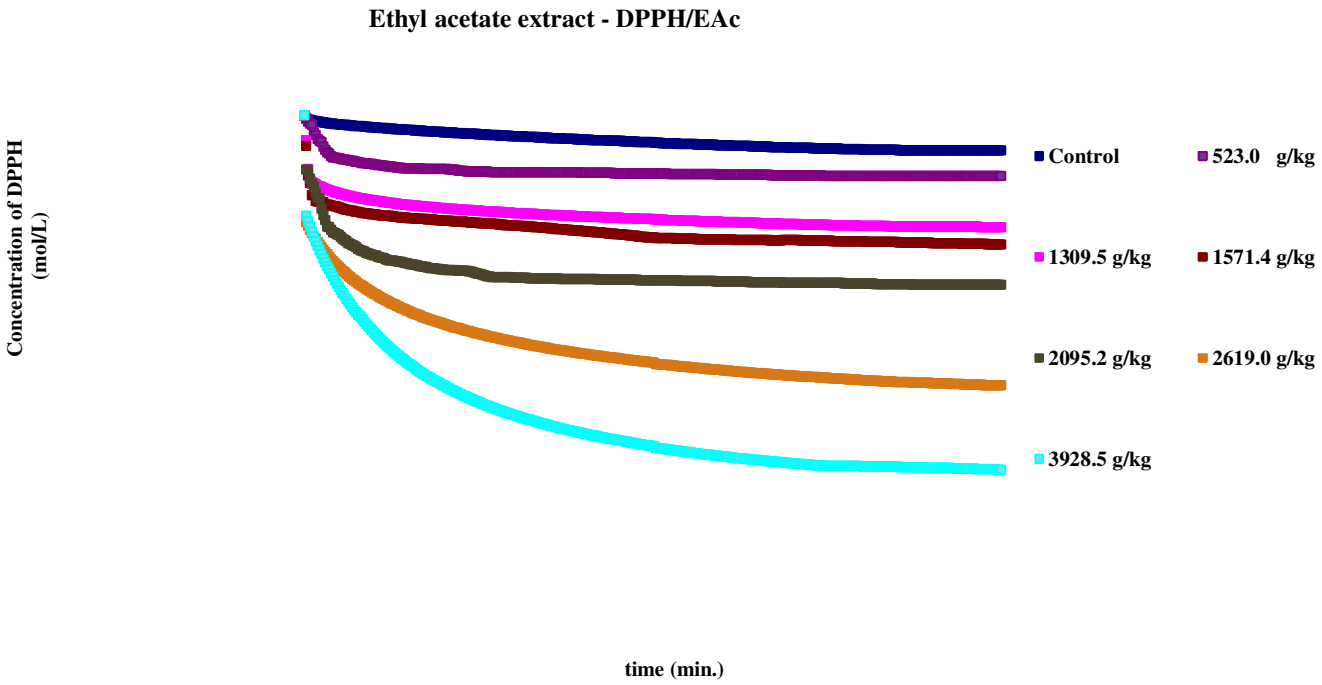


Figure 17a: Depletion of DPPH/EAc by EAc from successive extraction.

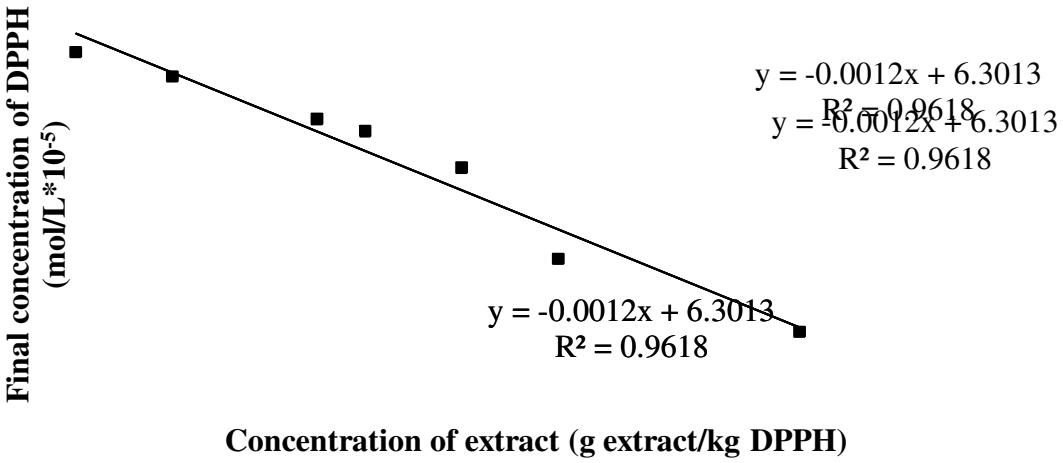


Figure 17b: Calculation of EC_{50}

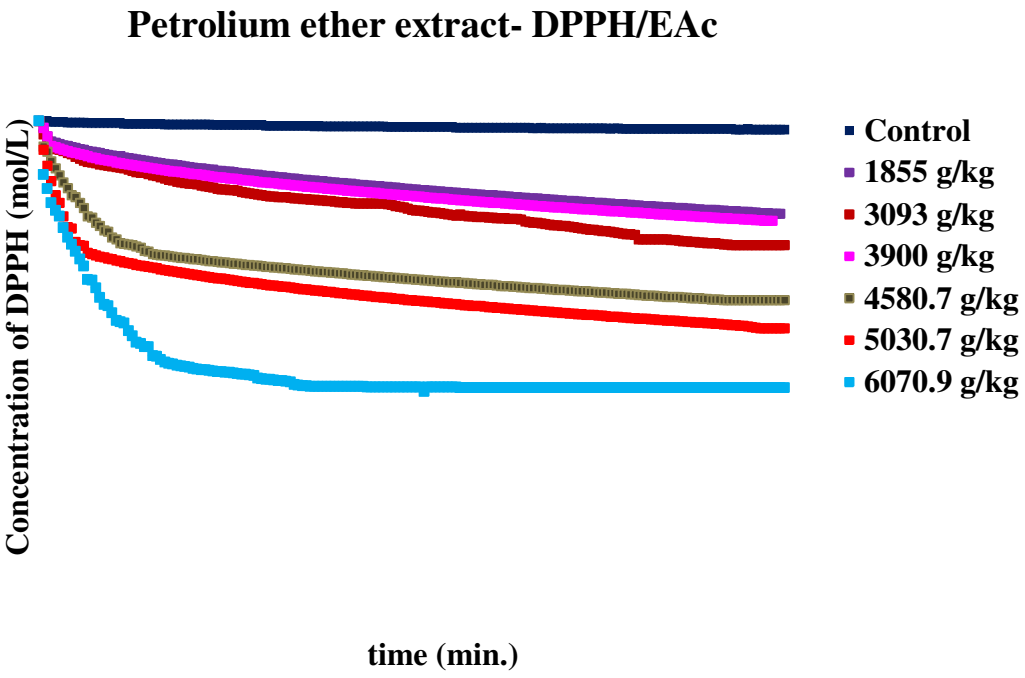


Figure 18a: Depletion of DPPH/EAc by P from successive extraction.

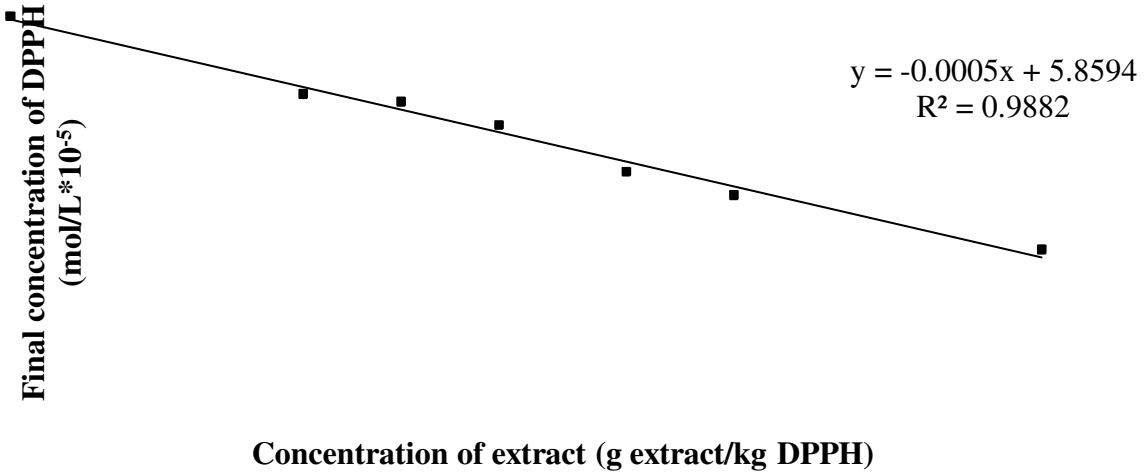


Figure 18b: Calculation of EC_{50}

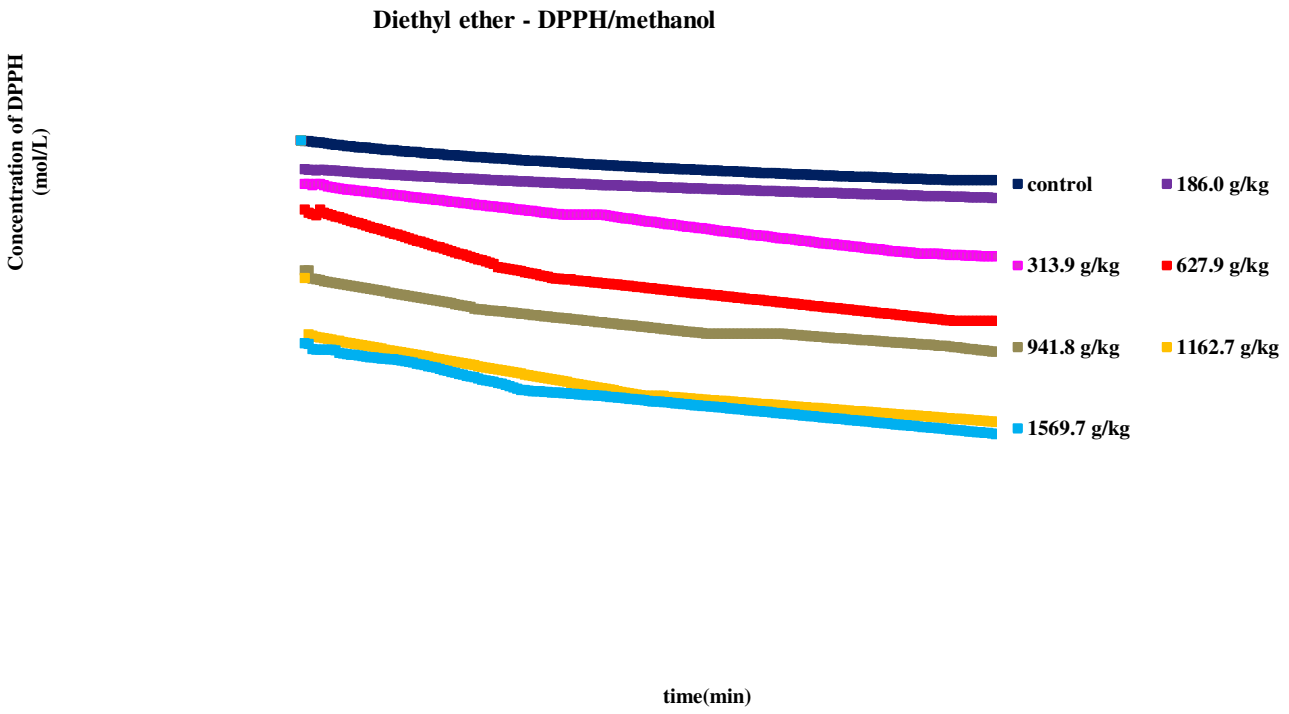


Figure 19a: Depletion of DPPH/methanol by D from successive extraction.

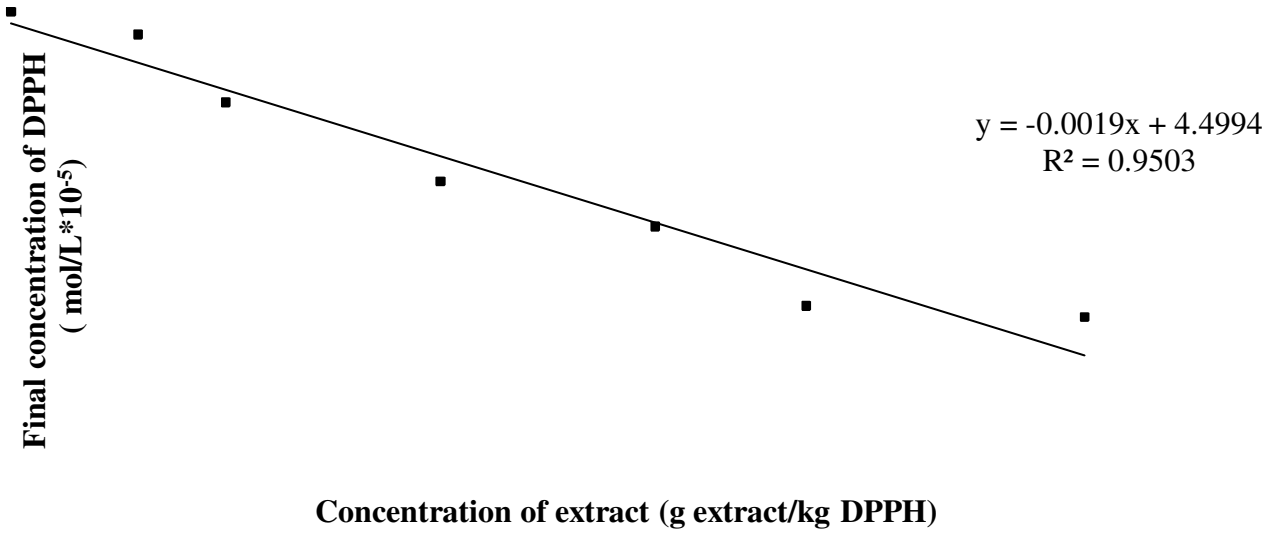


Figure 19b: Calculation of EC_{50}

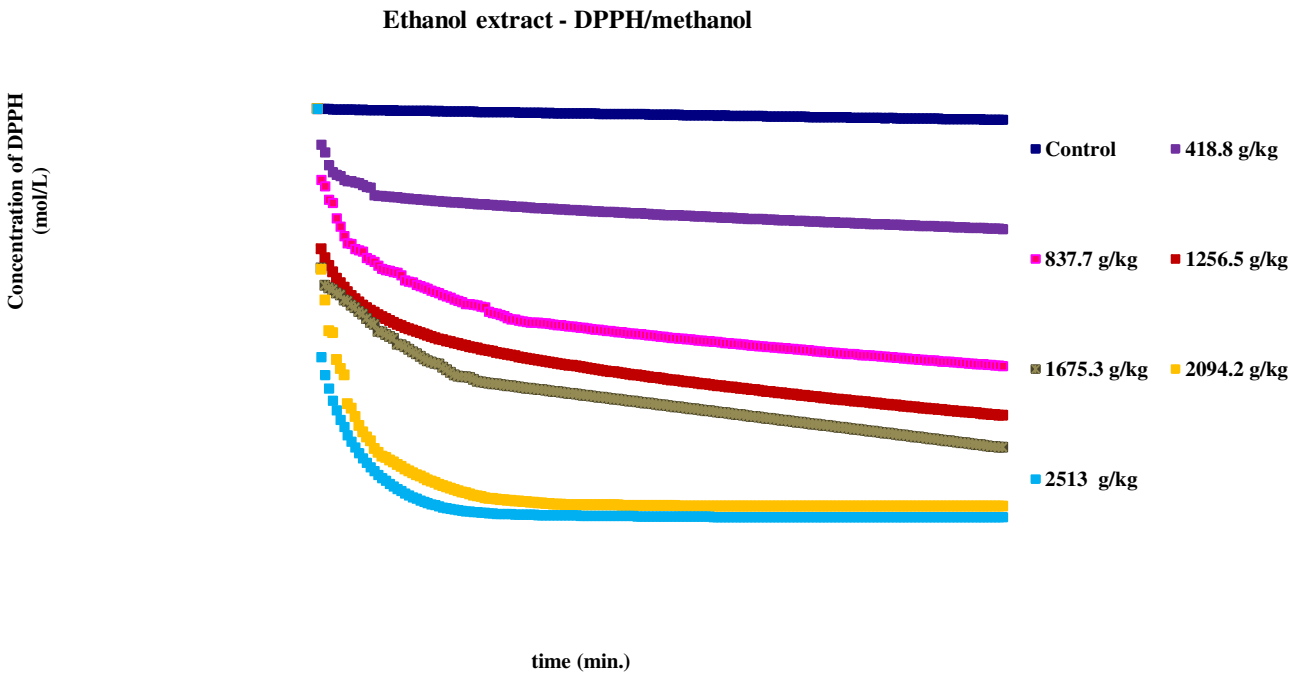


Figure 20a: Depletion of DPPH/methanol by E from successive extraction.

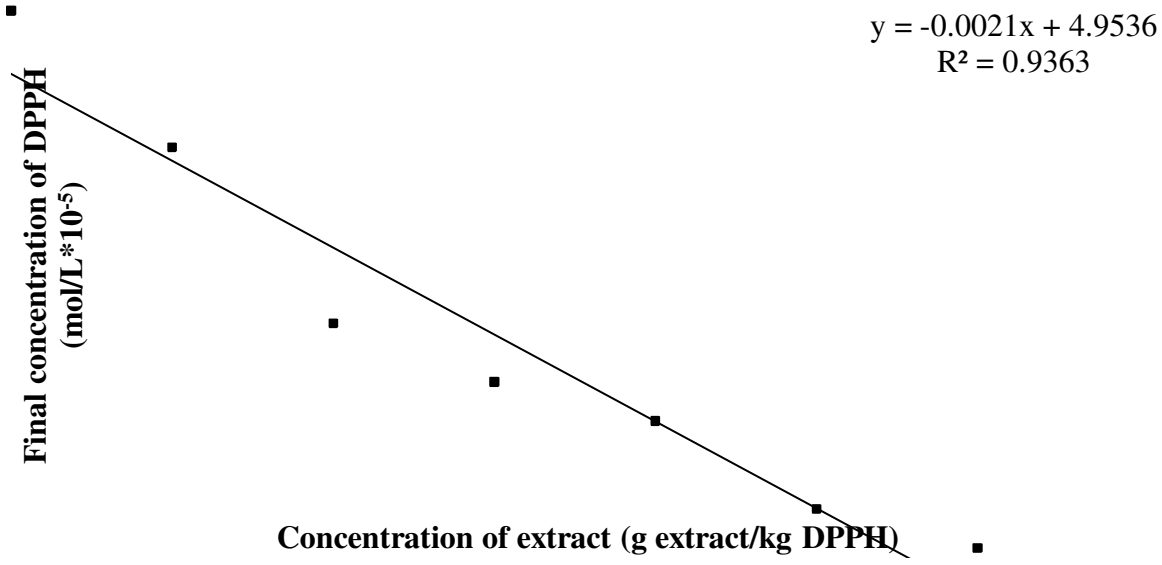


Figure 20: Calculation of EC_{50}

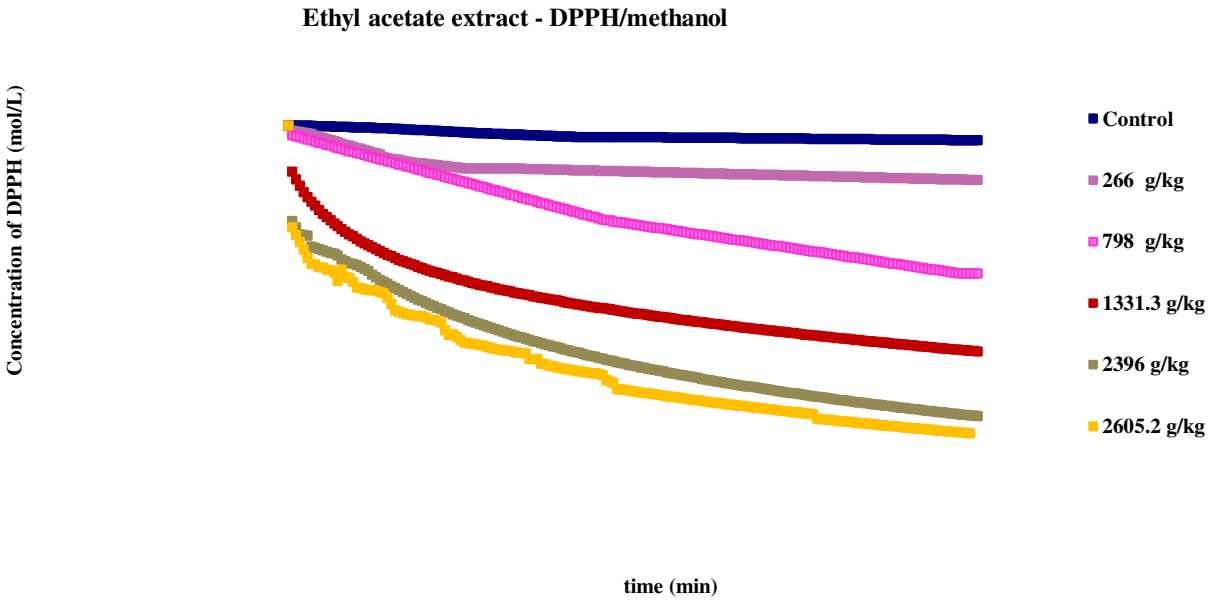


Figure 21a: Depletion of DPPH/methanol by EAc from successive extraction.

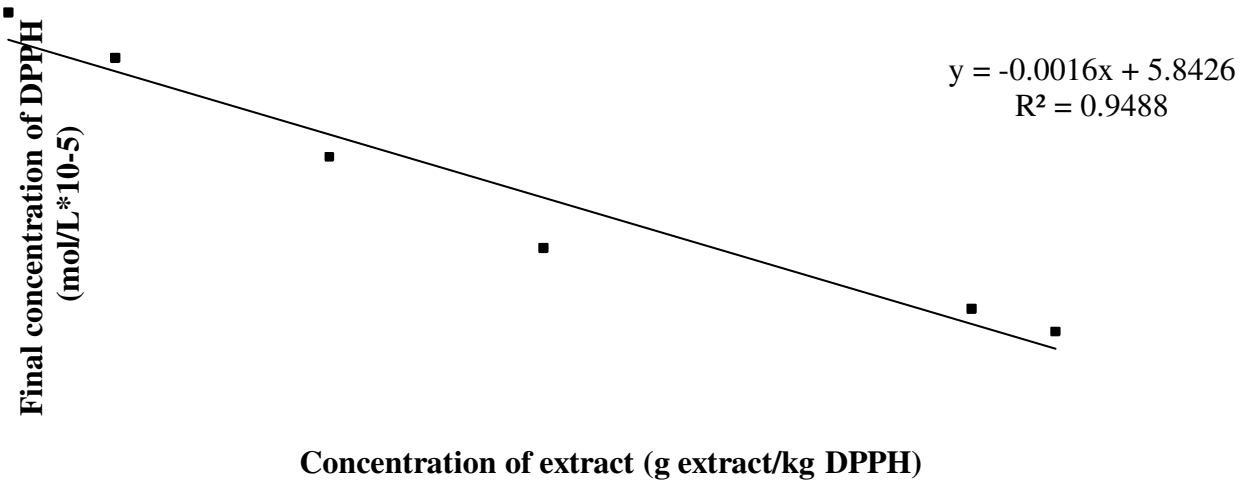


Figure 21b: Calculation of EC_{50}

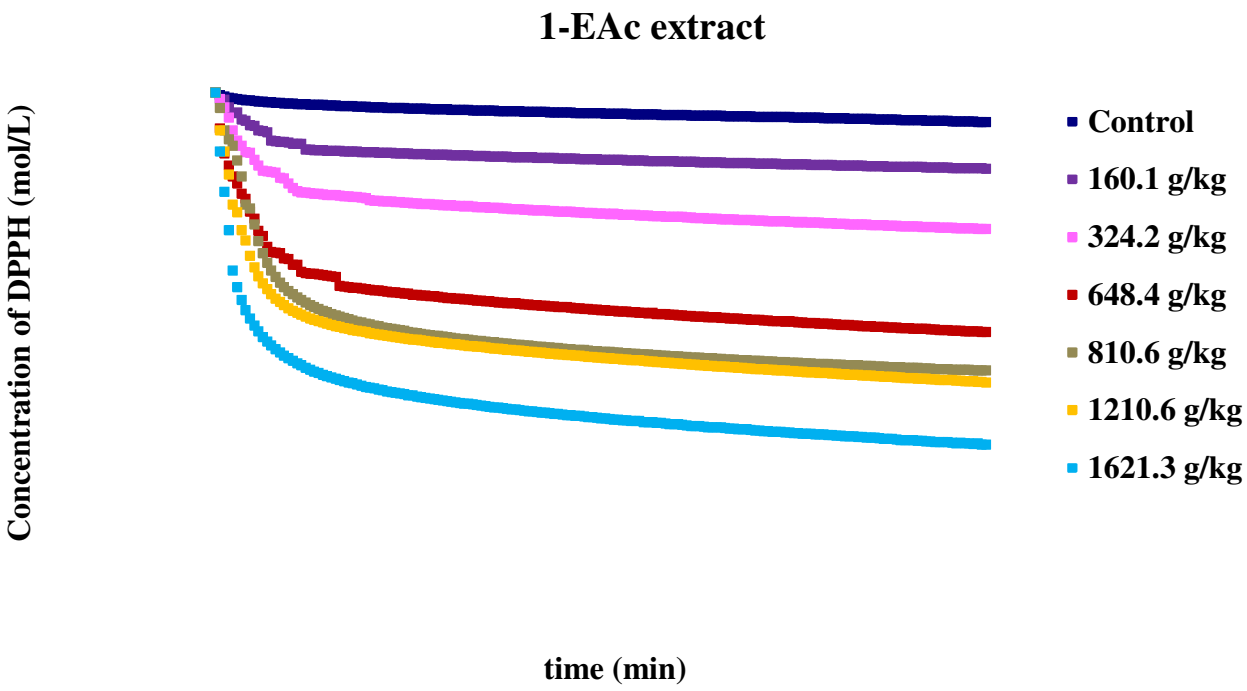


Figure 22a **Depletion of DPPH in the one-step extraction with ethyl acetate**

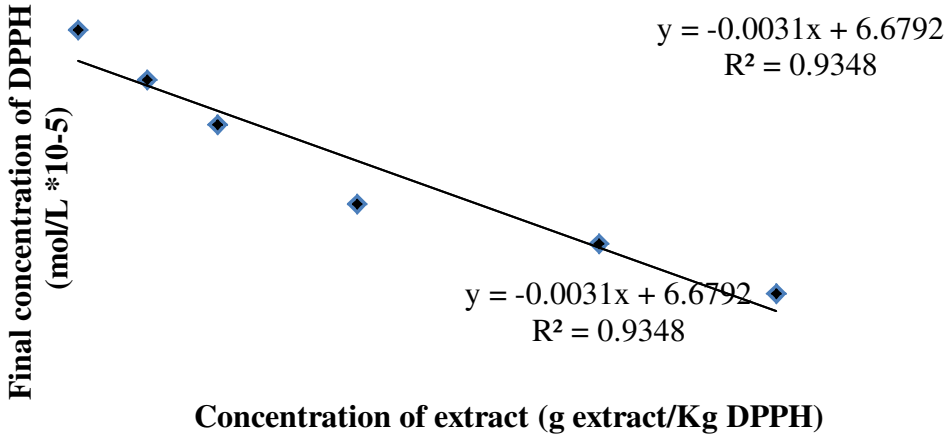
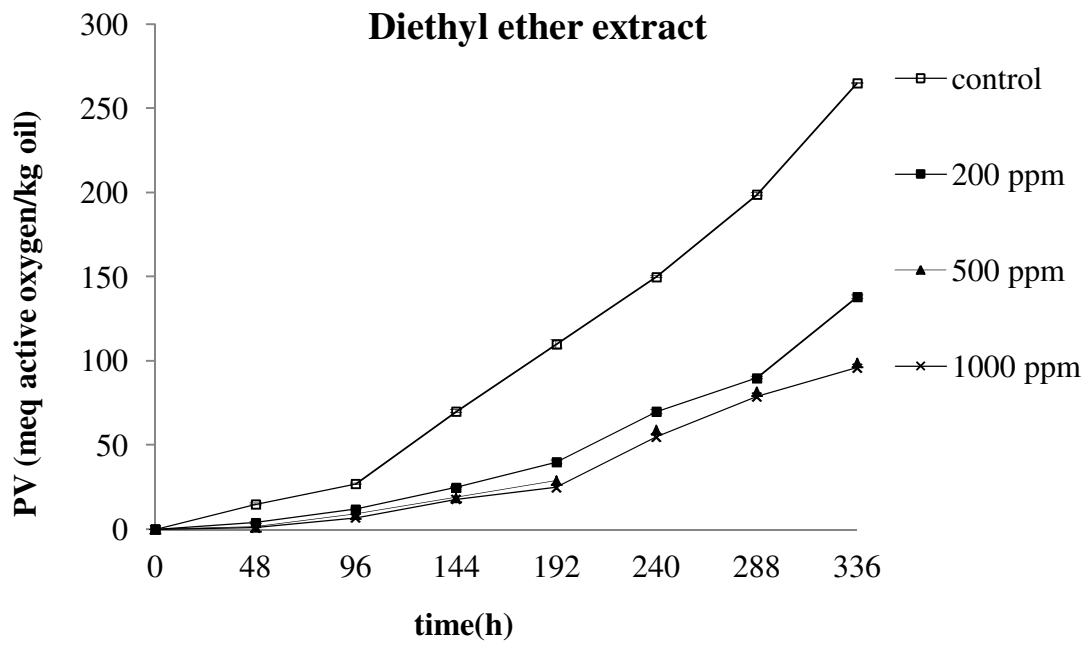
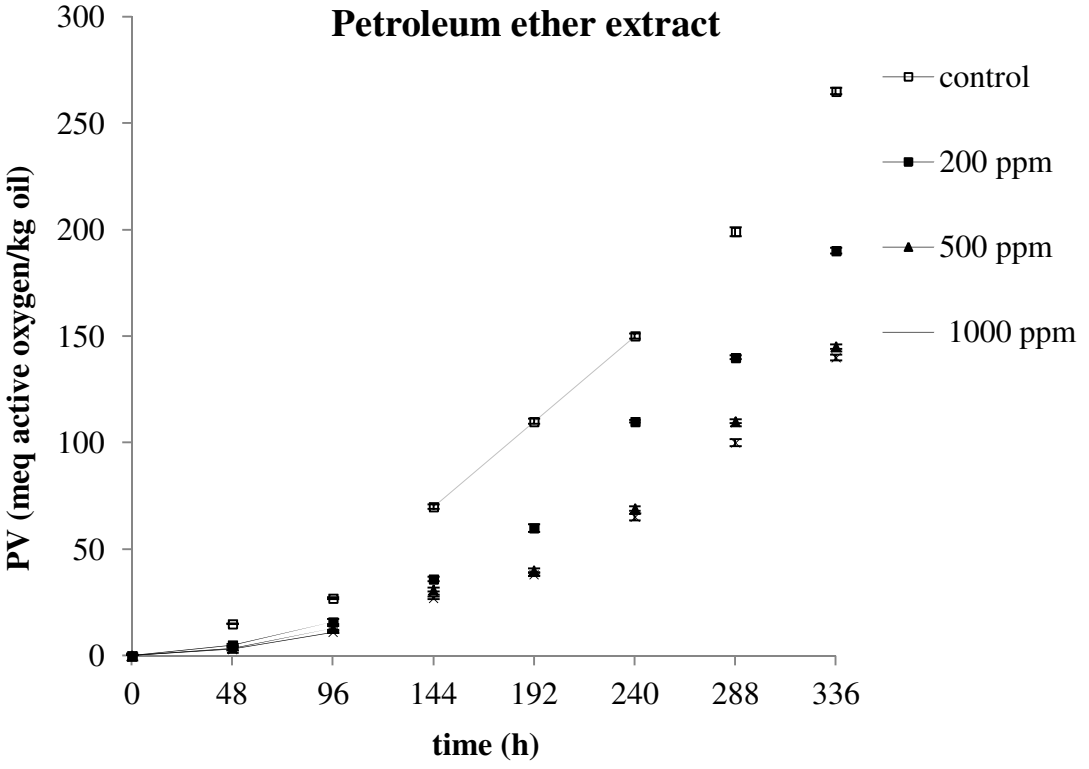


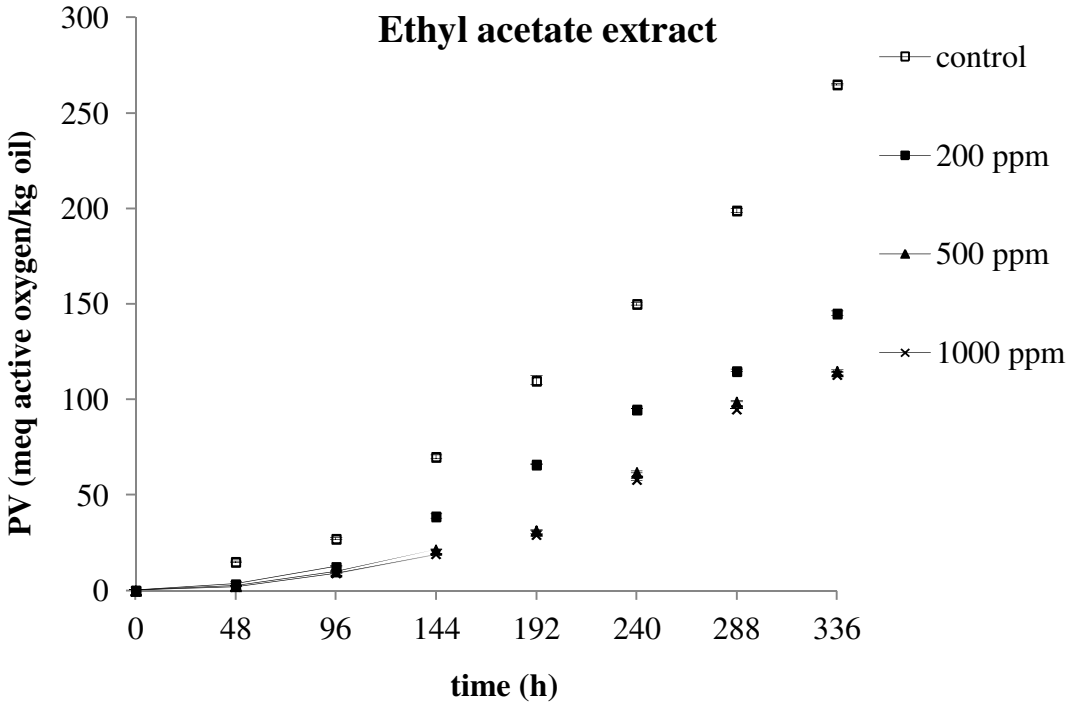
Figure 22ab **Calculation of EC_{50}**



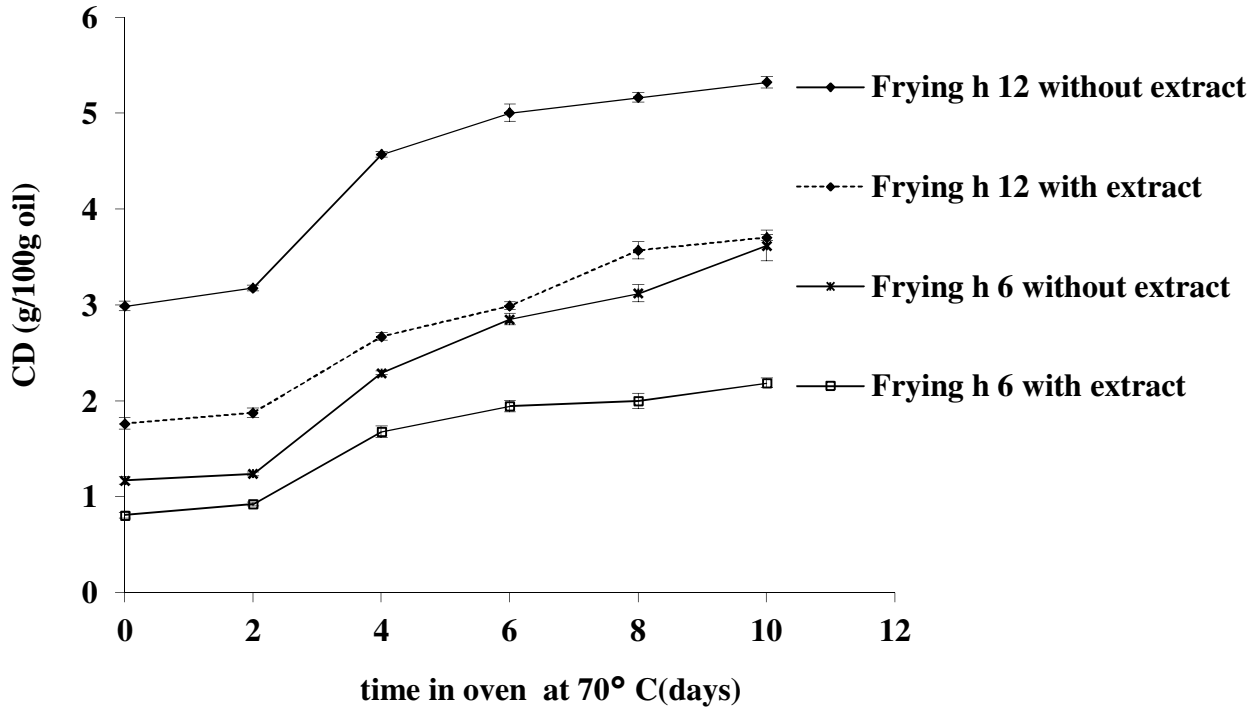
Figures 23a Effect of increase of concentration of diethyl ether extract(D on PV of corn oil stored under acceleration oxidation at 70 °C.



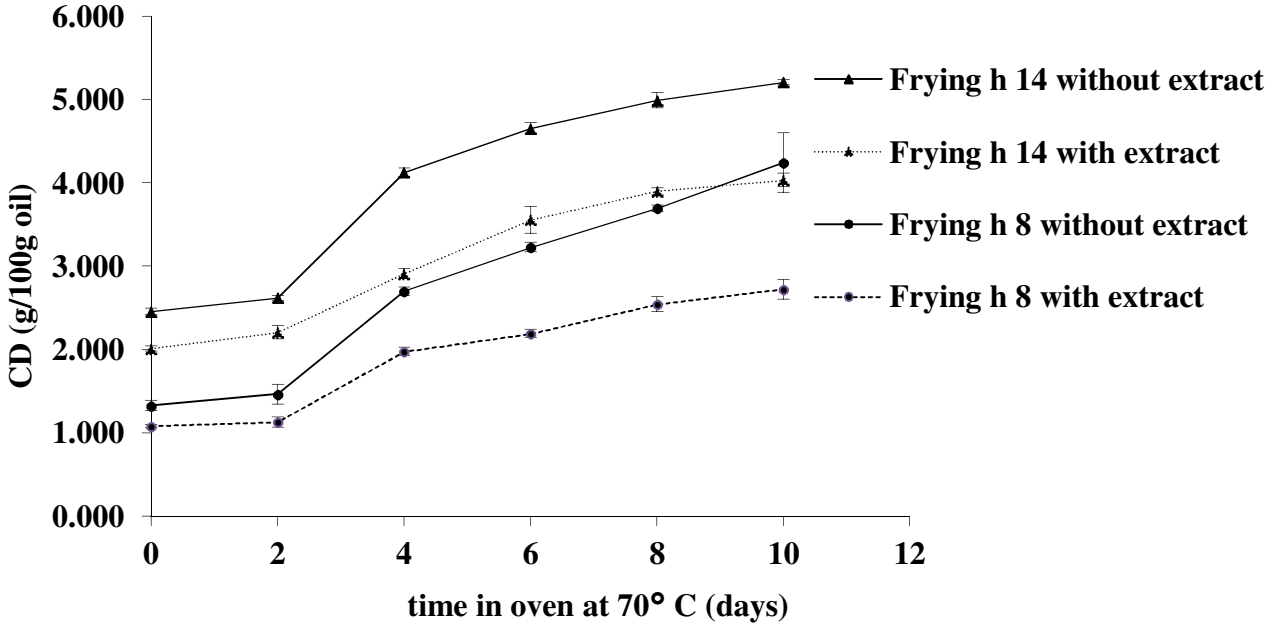
Figures 23b Effect of increase of concentration of pretrolium ether extract (P) on PV of corn oil stored under acceleration oxidation at 70 °C.



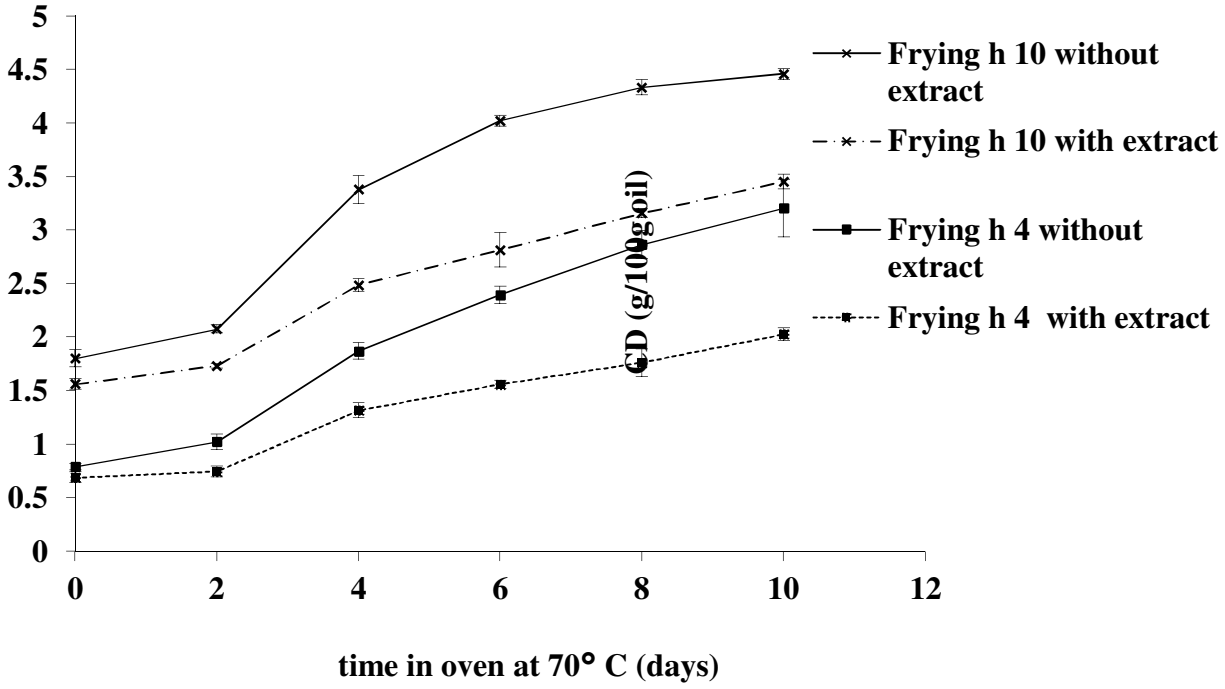
Figures 23c Effect of increase of concentration of ethyl acetate extract (EAc) on PV of corn oil stored under acceleration oxidation at 70 °C.



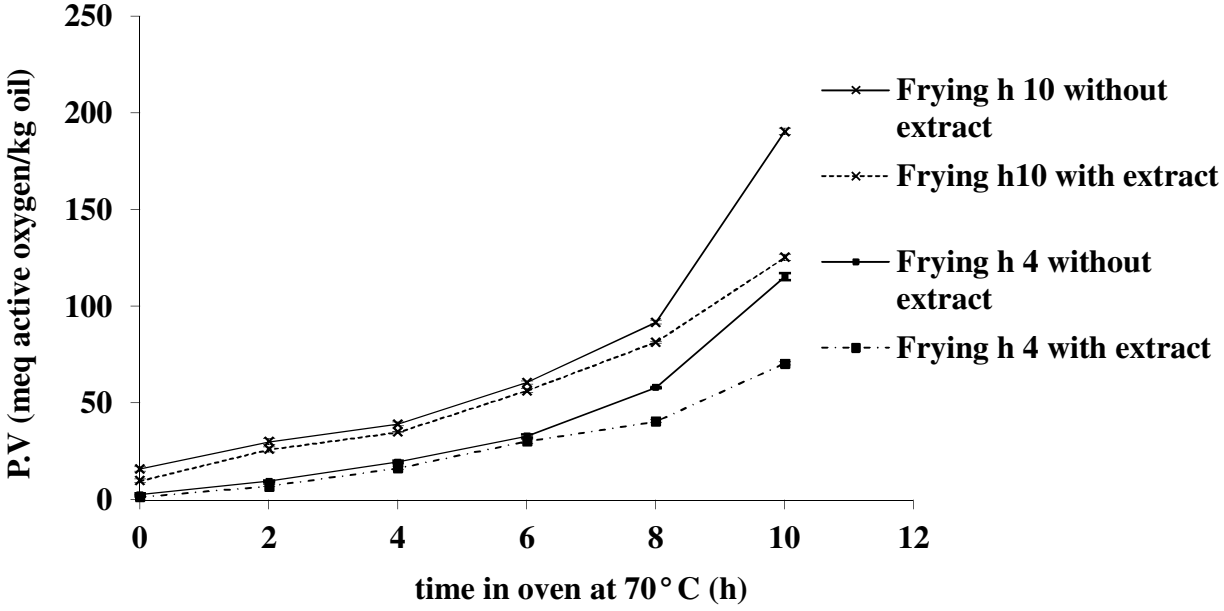
Figures 24a Effect of adding *M.syriaca* in frying oil of increase of CD of the oil extracted from potato chips of frying hours 12 and 6, stored under accelerated oxidation at 70°C.



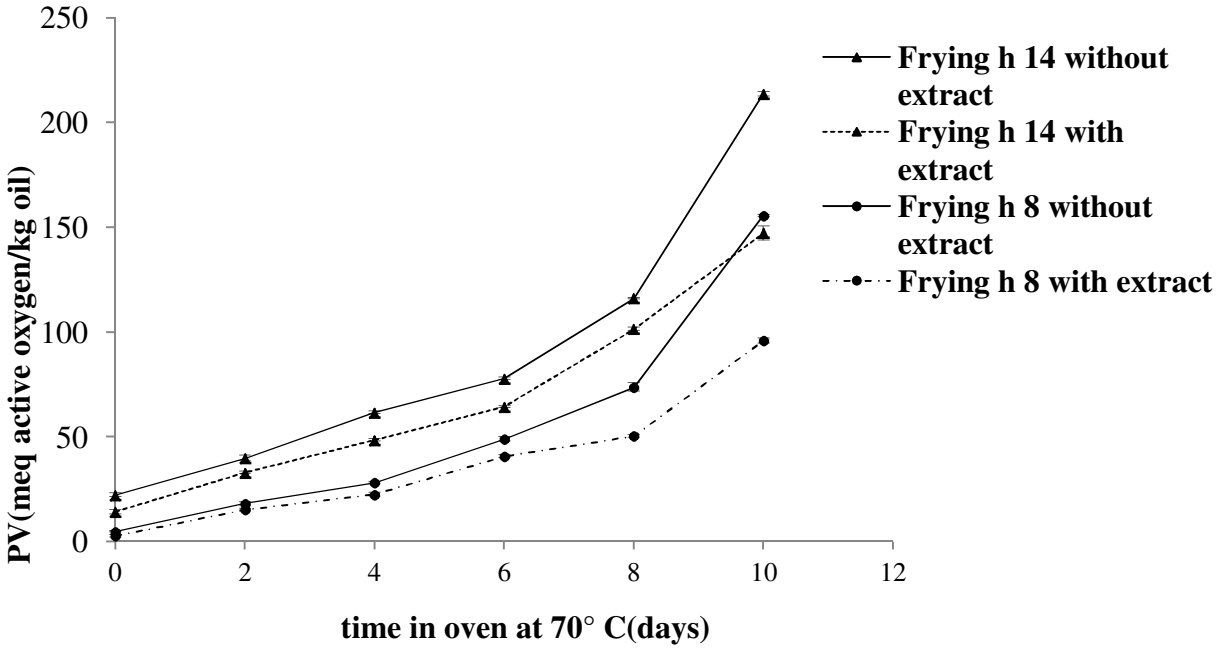
Figures 24b Effect of adding *M.syriaca* in frying oil of increase of CD of the oil extracted from potato chips of frying hours 14 and 8 stored under accelerated oxidation at 70°C.



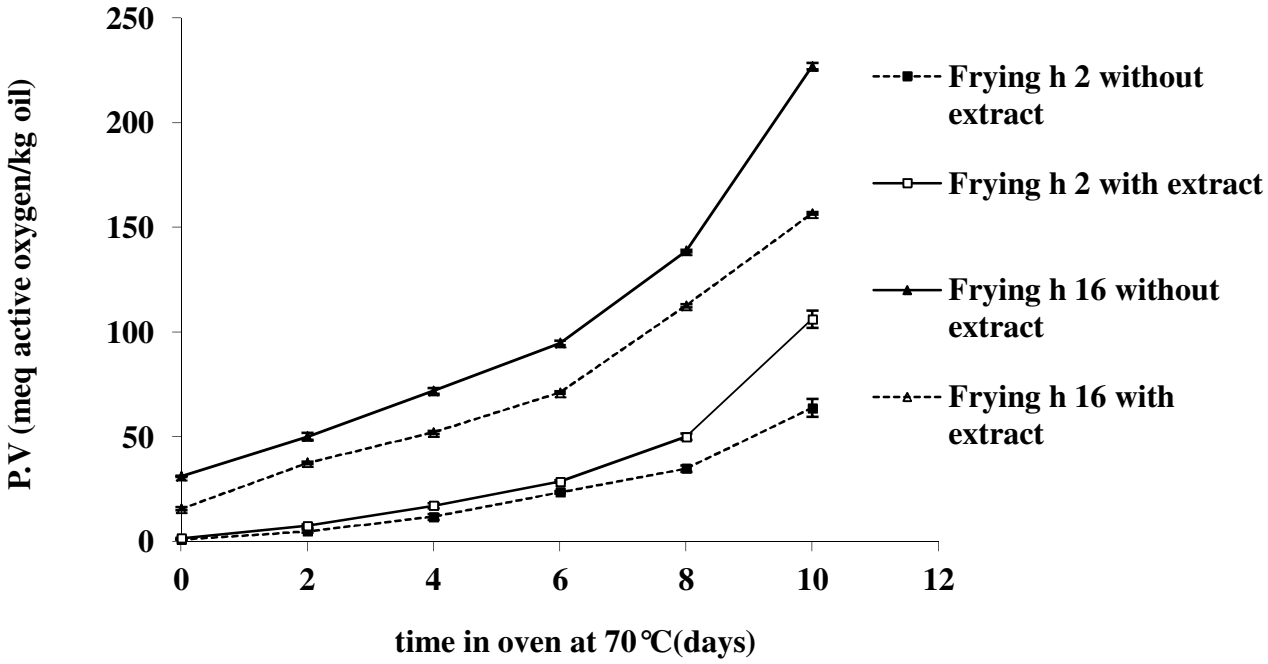
Figures 24c Effect of adding *M.syriaca* in frying oil of increase of CD of the oil extracted from potato chips of frying hours 10 and 4 stored under accelerated oxidation at 70°C.



Figures 25a Effect of adding *M.syriaca* in frying oil of increase of PV of the oil extracted from potato chips of frying hours 10 and 4 stored under accelerated oxidation at 70°C.



Figures 25b Effect of adding *M.syriaca* in frying oil of increase of CD of the oil extracted from potato chips of frying hours 8 and 14 stored under accelerated oxidation at 70°C.



Figures 25c Effect of adding *M.syriaca* in frying oil of increase of CD of the oil extracted from potato chips of frying hours 2 and 16 stored under accelerated oxidation at 70°C.

List of publications

1. **Antioxidant properties and composition of *Majorana syriaca* extracts. (European Journal of Lipid Science)**

2. **Antimicrobial and Antioxidant activity of *Majorana syriaca* in Yellowfin Tuna.**
   (International Journal of Food Science and Technology)

3. **Antioxidant effect of *Majorana syriaca* extract in bulk corn oil and o/w emulsion after applying high hydrostatic pressure.**
   (Journal of Food Chemistry)

4. **Inhibition of Lipid Oxidation in fried chips and cookies by *Majorana syriaca.***
   (International Journal of Food Science and Technology)