PhD THESIS

Composition, authenticity and oxidative stability of some essential oils as free and microencapsulated formulae

(SUMMARY)

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INTRODUCTION

Essential oils, also known as volatile oils, mainly contain components that evaporate easily, hydrocarbons such as mono- and diterpenes, terpenoid derivatives (alcohols, aldehydes and ketones, esters and ethers derived from terpenes) representing 90- 98%, but also less volatile components (FISHER et al, 2007; GYAWALI et al, 2014; RIVERA et al., 2015; YU et al., 2019). Dozens of articles have been reported in recent years on the chemical composition of volatiles from aromatic plants, either from wild flora or cultivated in continental, tropical and subtropical areas. Their composition is greatly influenced by genotype (species, cultivar, ecotype), ecological (geographic area, climate, soil composition) and technological factors (cultivation, raw material storage and processing techniques) (JOHNSON, 2017). Essential oils are widely used as natural food flavors, being responsible for their attractive smell and taste. Among the indigenous sources of essential oils used as culinary spices, one can mention aromatic plants from the *Lamiaceae* family (thyme, sage, oregano, mint, marjoram and basil), from conifers (juniper, pine), and spice flavorings (cloves and cinnamon).

Since the Middle Ages, interest in essential oils has been due to their antioxidant, bactericidal, virucidal, fungicidal, antiparasitic, insecticidal effects, and this interest is amplified nowadays, due to their applications in pharmacology, medicine, food industry, under different pharmaceutical formulas and cosmetics (BAKKALI, 2008; JOHNSON et al, 2017; KHANEGHAH, 2017; DERBASSIN et al, 2022; BAPTISTA-SILVA et al, 2020).

For the protection of food, especially fatty ones, the addition of herbs and spices, but also their extracts in the form of essential oils, are preferred natural options because they can prevent and control rancidity, delay the formation of toxic oxidation products, maintain nutritional quality and prolong the shelf life of food products (FISHER et al, 2007; DERBASSIN et al, 2022). At the same time, synthetic versions of compounds with "identically natural" aromas can be found on the market, some with codes marked E, others being undeclared, which falsify OE. Their authentication and traceability is an important area of investigation (TUREK et al, 2013).

Essential oils from aromatic plants are highly valued by consumers nowadays, due to their antioxidant and free radical scavenging effects (BAKKALI et al, 2008; DO et al, 2015). Moreover, as demonstrated in the last decades, through various in vitro or in vivo experiments, essential oils act as antimicrobials, protecting against respiratory, infectious and cardiovascular diseases (RIVERA et al, 2015; BAPTISTA-SILVA et al, 2020).

Many components of essential oils have been identified as effective antibiotics, e.g. carvacrol, thymol, eugenol, cinnamic aldehyde and cinnamic acid. Essential oils are important in the food industry, as their antimicrobial effect has been proven, during processing or for the preservation of different food matrices, being used including in packaging (KHANEGHAH, 2017; FIGUEROA et al, 2019; FERNANDEZ LOPEZ, 2018; JU, 2023). Recently, the characterization of the volatile fractions of essential oils from aromatic plants from the *Lamiaceae* family was published in relation to their antimicrobial and antioxidant activities (BOZIN et al., 2006; FALEIRO, 2011; TUREK, 2013).

Food flavors include different types of compounds classified into three categories: natural, synthetic and released during processing. Some of them are volatile and are known as "flavors", as is the case with volatile oils, of natural or synthetic origin. The different types of flavor compounds are presented in Fig.1.

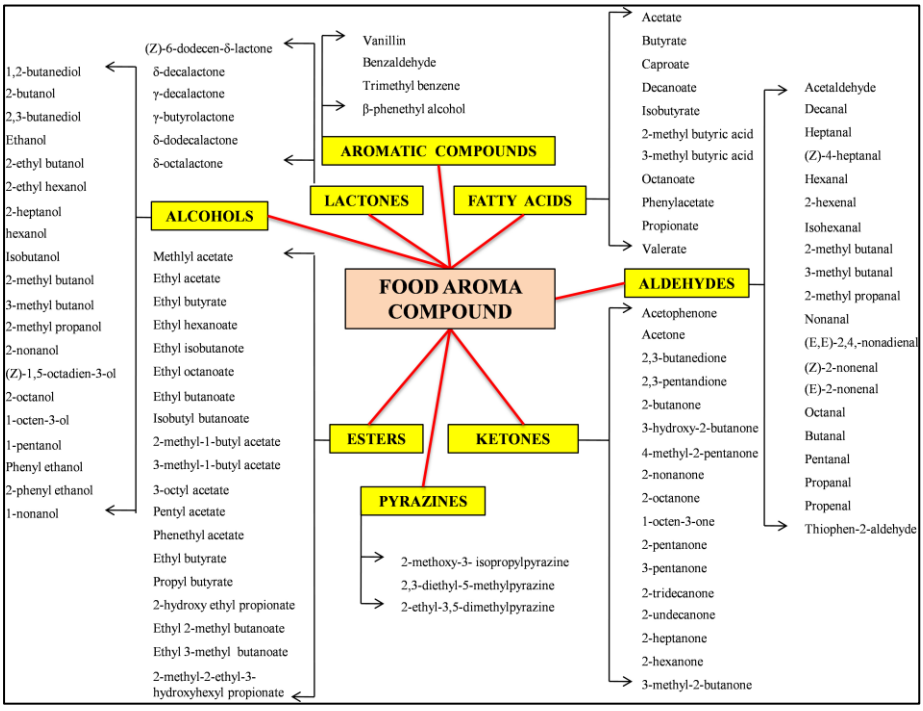


Fig.1. Classification of plant and food aroma compounds (SHARMA et al., 2020)

The role and benefits of aromatic plants in food systems, the associated active ingredients and their antioxidant, antimicrobial and general protective action is briefly presented in Fig.2.

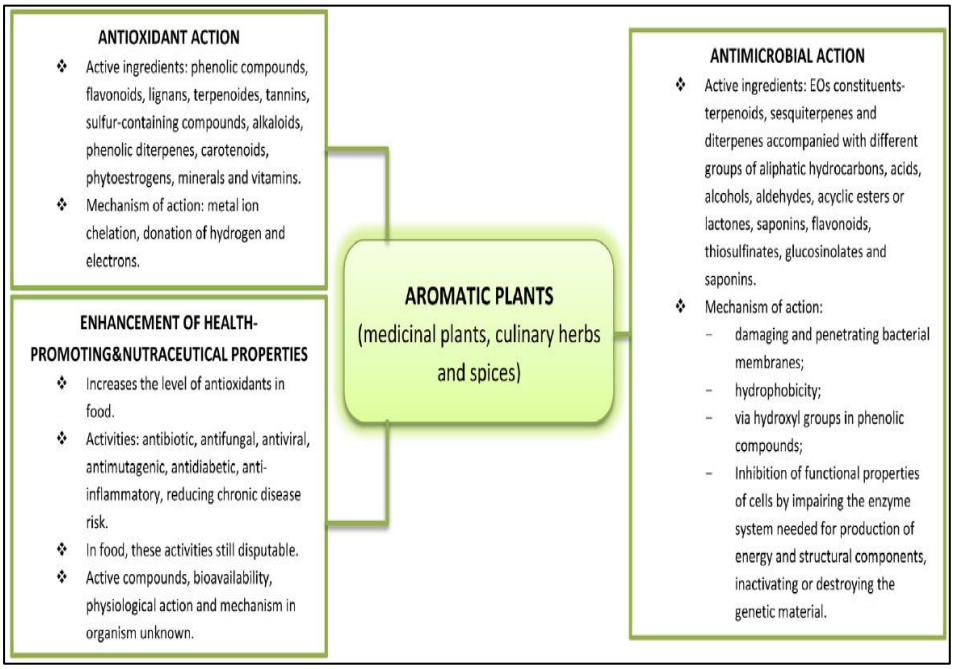


Fig. 2. The benefits of aromatic plants (Source: FILIPCEV, 2020)

Essential oils used as natural flavors contain a variety of compounds from those mentioned above, are extracted from aromatic and medicinal plants, or seeds, spices, vegetables or fruits and are added to food in the form of powders, or aqueous extracts or oily, these being described in the EMA monographs (https://www.ema.europa.eu/en/medicines/herbal) and in the EUROPEAN PHARMACOPEIA (2017, 2020).

Essential oils usually include complex mixtures of several volatile or non-volatile molecules, obtained by hydro distillation or by extractions with supercritical fluids (carbon dioxide). An impressive progress in the research of aromatic plants has been observed in the last decades, as consumers are concerned about the quality of food and prefer spices and flavors derived from plants (ISTUDOR, 2001; FERNANDEZ LOPEZ, 2018; JU, 2023).

Recent systematic reviews have been devoted to culinary herbs and their essential oils with antibacterial activities and potential applications in food (SVOBODA et al, 2004; BAKKALI, 2008; CHASSAGNE et al., 2021). Also, verifying the authenticity of essential oils, along with other secondary plant metabolites, is of high scientific interest and is important for gaining customer trust.

The quality, authenticity and safety of essential oils are of great scientific and applied interest, they involve high-resolution analytical procedures, especially gas-chromatography coupled with mass spectrometry, vibrational spectrometry (Infrared or Raman), nuclear magnetic resonance (SOCACIU et al, 2009; DO et al, 2015; FRANCA et al, 2017; LI et al, 2019; TAYLAN et al, 2021; CEBI et al, 2021).

AIMS AND OBJECTIVES OF THE PHD THESIS

Six types of essential oils (Thyme, Oregano, Juniper, Tea-tree, Clove and Cinnamon) as well as the products Biomicin, Biomicin forte and Biomicin Urinar (oily formulae), were used to obtain microencapsulated formulae on solid supports of fructose or maltodextrin.

The research carried out in the framework of this doctoral thesis had as its general aim the study of the composition, authenticity and oxidative stability of these six essential oils in free form and in oily and microencapsulated formulas on solid supports.

The specific objectives targeted four research directions:

1. Obtaining and characterizing original formulas that include the six types of essential oils: oily formulas and solid formulas obtained by microencapsulation on fructose or maltodextrin solid supports.
2. Determination of the quality and authenticity of free essential oils by gas chromatography with mass spectrometry detection(GC-MS) coupled with multivariate statistical analysis.
3. Evaluation of the quality and authenticity of essential oils by infrared spectroscopy (FTIR-ATR), before and after the induction of thermal oxidation.
4. Comparative study of oxidative stability, peroxidation indices and *cis-trans* isomerizations for essential oils in oily and microencapsulated formulas.

Modern methodologies were applied (GC-MS, FTIR-ATR) associated with statistical analysis specific to modern metabolomics' approaches, which establish non-targeted and targeted metabolic biomarkers of authenticity, stability and quality of essential oils and derived formulas. These studies are original and represent a modern model of approach in Food Science and Technology, in pharmacology and biomedical research.

Figure 3 summarizes the objectives and methods used in these studies.

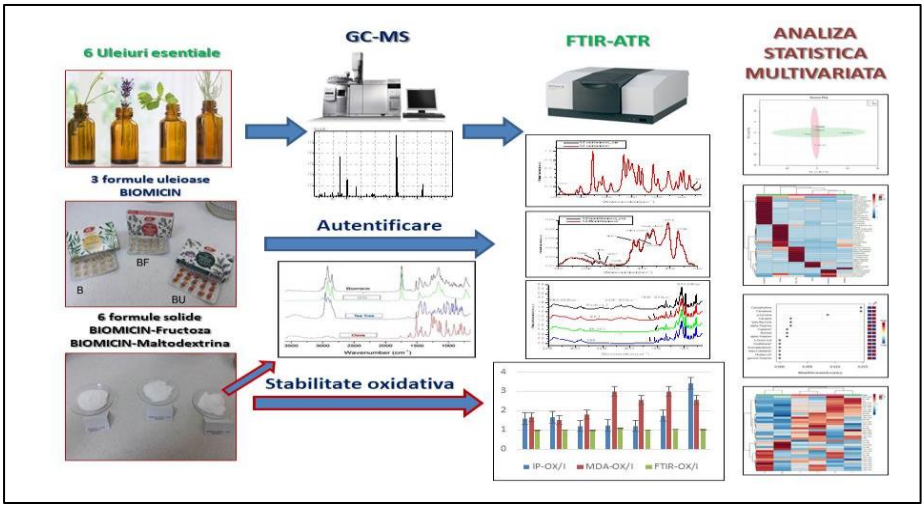


Fig.3. The products and methods used in this PhD thesis.

THESIS STRUCTURE

The first part represents a literature review, included in chapters 1-3 and deals with general theoretical aspects related to essential oils, microencapsulation matrices and techniques, as well as modern methods of assessing the quality and authenticity of essential oils by GC-MS and FTIR-ATR and statistical analysis. The personal contribution is presented in chapters 4-7 and includes the experimental results obtained by the essential oils, of their oily and microencapsulated formulae, targeting the composition and oxidative stability.

ORIGINAL CONTRIBUTION: RESULTS

The research undertaken in the doctoral thesis was carried out in the Biochemistry laboratories from the USAMV Cluj-Napoca campus and the LICSA laboratory, both belonging to the Faculty of Food Science and Technology. The essential oils and oily formulas of the Biomicin range were obtained from the company SC Fares SA Orăștie, a national producer of teas and food supplements based on plants and essential oils.

Chapter 4 included the materials and methods used to obtain and characterize formulas that include essential oils, from oily formulas (Biomycin, Biomicin forte, Biomicin urinary), to those obtained by microencapsulation on solid supports of fructose and maltodextrin. To evaluate the quality of oily formulas,

standardized techniques were used (relative density, refractive index, peroxide index, rotational power, determination of lipid peroxidation by the malondialdehyde test).

There were presented techniques considered currently the most advanced for the separation, identification, and authentication of marker molecules from products containing essential oils, namely Gas Chromatography with detection by Mass Spectrometry (GC-MS) and Fourier transform Infrared Spectroscopy (FTIR-ATR).

The analytical studies were completed by following the oxidative stability of these formulas, either by thermally induced oxidation on the oily formulas from the Biomicin products, or by solar oxidation induced on the solid formulas microencapsulated on maltodextrin and fructose matrices.

Chapter 5 included the results of GC-MS analyzes performed to determine the quality and authenticity of essential oils. The spectra obtained and a statistical analysis of the results are presented, using an approach based on metabolomic analysis, a combination of the separation and precise identification of molecules from different essential oils combined with a modern and updated statistical tool provided by the Metaboanalyst 5.0 software.

The specific molecules of each of the six free-form essential oils (Thyme, Juniper, Oregano, Tea-tree, Clove and Cinnamon) were identified. Multivariate analysis reflected the predictability of about 15 potential biomarkers such as Thymol and p-Cymene for Thyme, α-Pinen, β-Myrcene and Sabinene for Juniper, Carvacrol for Oregano, Terpinene derivatives for Tea Tree, Eugenol , Eugenol Acetate for Clove, Cinnamaldehyde for Cinnamon.

Table 5.1 shows the comparative composition (%) of the volatile fraction, calculated from the GC-MS spectra of the six essential oils, with the identification of the components.

Table 5.1.

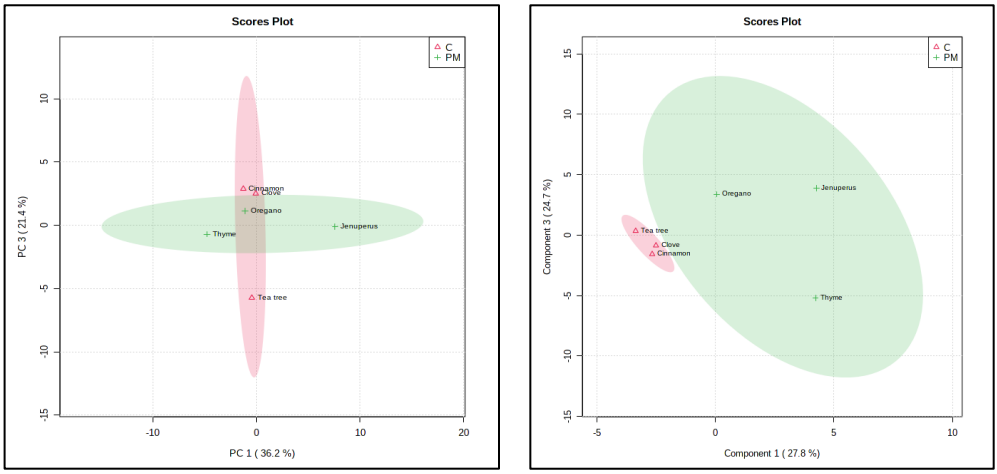
Comparative composition (%) of the volatile fraction of six essential oils, calculated from the GC-MS spectra, with the identification of components.

Molecule	Cimbru	Ienupăr	Oregano	Arbore ceai	Cuişoare	Scorţişoară
α-Terpinene	1.70	0.48	0.51	9.96	0	0
γ-Terpinene	8.91	2.17	4.27	19.79	0	0
Himbaccol*	0.12	0	0	0	0	0
β-Cubebene*	0	1.80	0	0	0	0
Aromadendrene	0	0.19	0	1.44	0	0
Viridiflorene*	0	0	0	1.30	0	0

1-Octen-3-ol	0.72	0	0	0	0	0
3-Carene *	0.12	0	0	0	0	0
1-Terpinen-4-ol	1.46	3.46	0.58	44.45	0	0
α-Terpineol	0.16	0.06	0.72	4.12	0	1.41
α-Caryophyllene	0	1.12	0.3	0.02	0.57	0
α-Cubebene	0.10	0.73	0	0	0	0
α-Phellandrene*	0.20	0	0	0.35	0	0
α-Pinene	1.16	36.41	0.21	2.63	0	0.89
Isothymol methyl ether	0.81	0	0	0	0	0
Thymol methyl ether	0.64	0	0	0	0	0
β-Myrcene	1.76	15.38	0.23	0.41	0	0
β-Pinene	0.24	3	0.44	0.56	0	0.18
β-Linalool	5.03	0	2.53	0	0	4.63
α-Thujene	0.85	1.40	0.23	0.56	0	0
*trans*Sabinene hydrate*	0.35	0	0	0.17	0	0
Sabinene	0	10.31	0	0.22	0	0
Borneol	1.82	0	0.95	0	0	0
Camphene	1.67	0.3	0.14	0	0	0
Camphor	1.32	0	0.83	0	0	0
Carvacrol	2.34	0	75.82	0	0	0
Caryophyllene	7.50	5.41	2.33	0	0	0
Copaene*	0.14	0.53	0	0.18	7.59	3.3
β-Elemene	0	0.67	0	0.07	0.16	0
Pseudolimonene	0.06	0.37	0	0	0	0
α-Terpinolene	0.15	1.27	0	3.25	0	0
δ-Cadinene*	0.14	1.57	0	1.59	0	0
D-Limonene	0.80	8.17	0.67	1.38	0	1.81
Eucalyptol	1.76	0	1.52	1.69	0	3.74

Eugenol	0	0	0	0	79.67	2.03
Eugenol acetate	0	0	0	0	11.69	0
p-Cymene	19.94	2.90	5.98	2.67	0	2.05
Thymol	38.00	0	1.59	0	0	0
γ-Muurolene	0	0.4	0	0	0	0
β-*cis*-Ocimene	0		0	0.14	0	0
Germacrene B*	0	0.84	0	0	0	0
Cinnamyl acetate	0	0	0	0	0	2.76
Cinnamaldehyde	0	0	0	0	0	76.92

The multivariate statistical analysis through the algorithms used (PCA, PLSDA, Random Forest, Heatmap) reflected the discrimination between sample groups (C and PM) and individual essential oils, represented by specific biomarker molecules. Figure 5.2a includes the PCA score plot highlighting the sample differentiation and similarities of group C (spices) and PM (herbs) based on their compositional variability. The co-variance for the first 3 components reached the value of 57.6%. Figure 5.2b represents the PLSDA score graph, the co-variance for the first 3 components being 52.5%.



(a) **(b)**

Fig. 5.2 (a) PCA score plot (PC1 *vs* PC3) showing differentiation between groups C and PM (b) PLSDA scores plot (PC1 vs PC3) showing significant discrimination between groups C and PM. PM- medicinal plants (Thyme, Oregano, Juniperus); C- condiments (Cinnamon, Clove, Tea tree).

Both graphs confirm a significant differentiation between the two groups. It should be noted that Juniper oil is clearly different in its composition and also that of Thyme. Figure 5.3. represents the Heatmap using a Euclidean distance and the Ward algorithm. Cluster analysis dendrograms are shown (top) with correlations and similarities between samples in group C (red). A significant differentiation of the Oregano sample is observed, which does not cluster with the other oils in the PM group (green). The heatmap also shows which are the molecules that explain the differentiation, respectively the similarity between these oil samples.

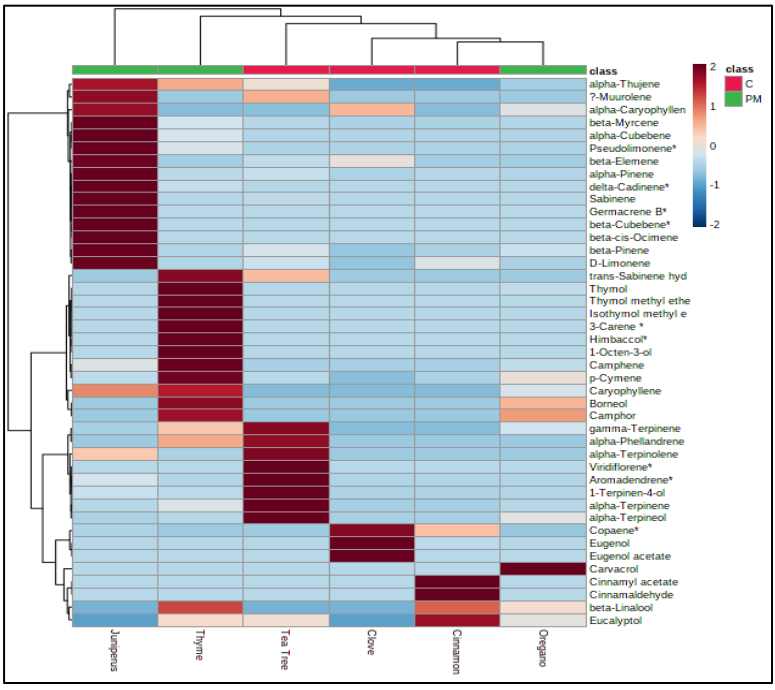


Fig. 5.3. Heatmap: correlations between samples (top) based on the cluster analysis dendrogram and between samples and molecules (bottom and right), showing specific signatures of each sample.

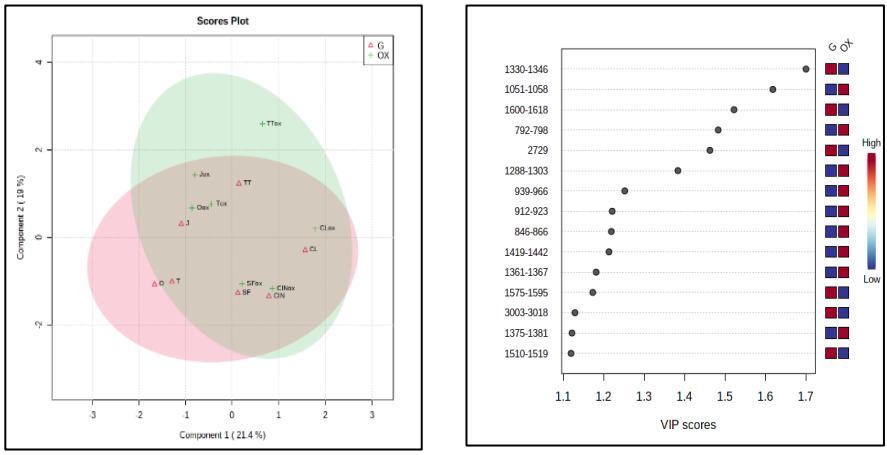
This study provides a precise evaluation and interpretation of the authenticity of these oils based on the identification of biomarkers, being possible to identify adulterations or oxidative degradations, including the identification of volatile additives called "identical natural" oils that are actually synthetic or degraded specimens.

Chapter 6 includes the results of the evaluation of the quality and authenticity of the six essential oils by FTIR-ATR spectrometry, before and after controlled thermal

oxidation. An assessment of the oxidative stability was made in parallel by classical methods (peroxide index and malondialdehyde test) and by the FTIR-ATR method. The

results of the FTIR spectra were interpreted by multivariate statistical analysis to make specific "identity cards" for each oil, based on specific IR absorption wavenumbers and signal intensities in certain spectral domains, to be considered as authenticity markers. By thermally induced oxidation it was shown that almost all essential oils tested showed good oxidative stability. Positive and significant correlations were found between the values of the Peroxide Index and malodialdehyde, obtained by routine chemical methods, and the intensities of the FTIR signals at specific wave numbers (966/925 cm^{-1}), as markers of the oxidative stability of essential oils. The FTIR spectral analysis provided additional and complementary information, highlighting the absence of *cis-trans* isomerizations.

The PLSDA and Random Forest multivariate statistical analysis and intuitive Heatmap maps were useful to select and identify authenticity, quality biomarkers and also to highlight spectral fingerprint differences after induced thermal oxidation. Based on spectral fingerprints and matrices representing wavenumbers vs. intensity, for each essential oil sample, before and after induced thermal oxidation, PLSDA and Random Forest analysis showed the differences between the initial (G) and after oxidation (OX) time. Figure 6.4A and B represent the plot of the PLSDA score and the VIP scores that identified the IR areas (wavenumbers) that discriminate between the G and OX groups.



A. **B.**

Fig. 6.4. The PLSDA Score plot for the discrimination between samples at initial (G) and final stage (Ox). B. VIP scores corresponding IR zones which discriminate between groups G and Ox. T-Thyme; O-Oregano; TT-Tea tree; J-Juniper; CIN-Cinnamon; CL-Clove.

The FTIR-ATR spectroscopy has once again proven to be a reliable, rapid and non-destructive, easy-to-use method for controlling the quality, authenticity and safety of essential oils in oily formulations. Figure 6.8. integrate the data obtained by the three methods, considering the average values (determined in triplicate) of the IP-OX/I, MDA-OX/I and FTIR-OX/I ratios determined for each of the seven oils.

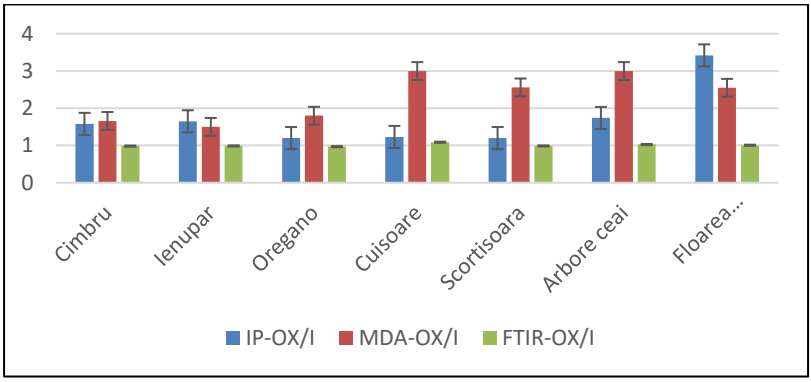
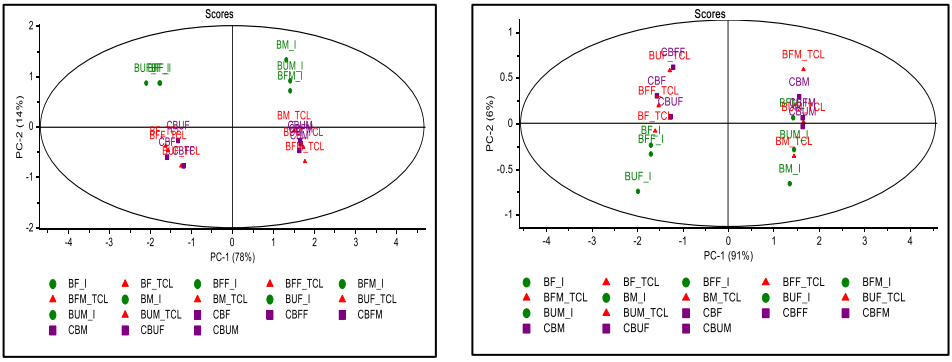


Fig. 6.8. Mean Values (x ± SD) of the ratios IP-OX/I, MDA-OX/I şi FTIR-OX/I for the seven oils mentioned in the legend.

In **Chapter 7** the results reflect the comparative oxidative stability of oily and microencapsulated formulations on solid supports. The FTIR-ATR spectrometry was used to determine the specific fingerprint of these products and evaluate their stability after solar irradiation. The investigations and results presented demonstrated that the FTIR-ATR-MIR technique can be applied successively, successfully in the oily or microencapsulated formulas on solid support. The FTIR-ATR-MIR absorption spectra of the essential oils show characteristic C-H stretching vibrations (~2900 cm^{-1}), C=O stretching (~1700 cm^{-1}), O-H stretching (~3400 cm^{-1}) and C-O (~1100 cm^{-1}) specific to terpenoid components, and are dominated by vibrational modes of monoterpenes at 886, 1436 and 1644 cm^{-1}. The shape of the spectra and the intensities of the signals reflected a good discrimination between the three oily formulas as well as the microencapsulated ones. The statistical analysis was particularly useful in this case as well, for identifying and discriminating the differences between the profiles of the three formulas on fructose or
maltodextrin supports. The PLSDA plots showed that the maltodextrin solid formulations were able to better discriminate the different essential oil ingredients.

Using the Unscrambler 10.x software and algorithms, the principal component score graphs (PCA) corresponding to the IR region 3600-650 cm^{-1}

(Fig.7.5.a) and 1800-650 cm^{-1} (Fig.7.5.b) were obtained , highlighting the differences between the fingerprints of the microencapsulated products Biomicin (B), Biomicin Forte (BF) and urinary Biomicin (BU) on fructose (F) or maltodextrin (M) supports, in the initial stage (I), after solar irradiation (TCL) and after storage in capsules (C).



(a) **(b)**

Fig. 7.5. Principal component (PCA) score plots corresponding to the IR region 3600-650 cm^{-1}(a) and 1800-650 cm^{-1} (b) showing the comparative fingerprints of microencapsulated products Biomicin (B), Biomicin Forte (BF) and Biomicin urinary (BU) on Fructose (F) and maltodextrin (M) as matrices, at initial stage (I), aftersolar irradiation (TCL) and after storage in capsules (C).

A general picture of the comparisons between the samples and the significant wave numbers was obtained through the Heatmap graphs and the Random Forest Analysis showed which are the most relevant wavenumbers capable of discriminating the different essential oils adsorbed on these matrices.

Specific absorption wavenumbers and signal intensities before and after exposure to light also indicated good stability of these solid formulations, as well as differences between fructose and maltodextrin. Maltodextrin is recommended for encapsulation having the advantage of lower hygroscopicity (low moisture retention), better stability and better availability to be included in food matrices (for example candies, gums) or in food supplements. Compared to oily formulations, more exposed to oxidation and more difficult to introduce into food matrices, microencapsulated solid formulations are recommended, especially on maltodextrin. The contribution of each ingredient in the essential oils category of Thyme, Oregano, Juniper, Tea Tree, Clove, Cinnamon in oily formulas and in microencapsulated solid formulas were easily identified and evaluated semi-quantitatively by specific FTIR absorption wavenumbers and signal intensities .

These data complemented studies based on GC-MS analysis that could highlight and identify individual molecules in the six essential oils, finding their major

biomarkers of authenticity, e.g. thymol and p-cymene for Thyme, α-pinene, β-myrcene and sabinene for Juniper, carvacrol for Oregano, terpinene derivatives for Tea Tree, eugenol, eugenol acetate for Clove, cinnamaldehyde for Cinnamon. Similar studies have been carried out for the free essential oils of Thyme (SATYAL et al., 2016), Oregano (GONG et al., 2016; KULA et al., 2007), Juniper (FALCÃOA et al, 2018), Tea Tree [RAYMOND et al., 2017; GALLART-MATEUA et al., 2018), Clove (HAMEED et al., 2021) and Cinnamon (BRODOWSKA et al., 2016). Some studies were validated by GC-MS, being focused on the taxonomy and purity of essential oils, and statistically interpreted (SU et al., 2019). They demonstrated that FTIR spectrometry is a reliable method for evaluating the specific biomarkers of these oils and plant species.

None of these studies analyzed essential oils microencapsulated on solid matrices. That is why the research presented in this PhD thesis completes the existing data, demonstrated the advantages of FTIR spectroscopy for the quality, authentication, and safety of essential oils in any formula.

GENERAL CONCLUSION

The objectives of this study were achieved experimentally, so that:

1. Six types of essential oils (Thyme, Juniper, Oregano, Tea Tree, Clove and Cinnamon) were obtained and characterized, as free or included in different formulas (oily or solid on different bioavailable supports) using microencapsulation techniques by adsorption on fructose or maltodextrin
2. The quality and authenticity of the six free essential oils were evaluated by gas chromatography coupled with mass spectrometry (GC-MS), highlighting the authentication marker molecules of each type of essential oil.
3. Spectral fingerprints of the free essential oils and the oily formulas of the Biomicin products (Biomycin, Biomicin Forte and Biomicin Urinar) were made by Infrared spectroscopy (FTIR-ATR), before and after the induction of thermal oxidation.
4. A comparative study of oxidative stability, peroxidation indices and cis-trans isomerizations was carried out by complementary methods (chemical and FTIR-ATR spectroscopic) for these oily formulas of essential oils and microencapsulated formulas on solid supports of fructose and maltodextrin.
5. All experimental data were statistically processed by multivariate and univariate analysis (One way ANOVA) using specialized software, such as Unscrambler 10.x and Metaboanalyst 5.0,, a software specialized in metabolomic analysis.

The data obtained highlighted significant aspects regarding the usefulness of these techniques for the separation and identification of biomarkers of authenticity and quality of essential oils in different formulas.

ORIGINALITY AND INNOVATIVE CONTRIBUTIONS

The results obtained in the presented studies included different aspects of originality and innovative contributions, from a technical point of view and statistical processing. Thus, we mention:

• The use of original protocols for obtaining oily formulas that contain mixtures of essential oils with targeted functionality (Biomicin, Biomicin forte and Biomicin Urinar) that contain mixtures of bioactive molecules from essential oils with antibacterial action from different plants.

• Application of original protocols for obtaining microencapsulated solid formulas on bioavailable supports of fructose and maltodextrin, using optimal ratios between essential oil concentrations and solid matrices. Fructose has not been reported so far to be used as an encapsulation matrix, despite its natural origin and high nutritional quality.

• Application of complementary methods and techniques (GC-MS, FTIR-ATR) associated with statistical analysis specific to modern metabolomic approaches, which establish in a non-targeted and targeted manner the metabolic biomarkers of authenticity, stability and quality of essential oils and derived formulas.

• Application of a simplified protocol for short-time FTIR-ATR-MIR analysis of a large number of samples, identifying the specific phenotype of each type of ingredient (essential oil) in complex mixtures, from oily or solid formulas. Such a procedure allows a quick and precise evaluation of essential oils' authenticity, quality, stability and traceability in different matrices, during storage or along the production chain.

These original studies that include innovative components represent an updated model of approach in Food Science and Technology, in pharmacology and biomedical research. In this context, the results presented in these studies offer beneficial application perspectives in the field of Food Science and Technology. The studies carried out were published in international journals with impact factors:

1. POPA R.M., SOCACI S., FARCAS A., SOCACIU C., 2021, Comparative Authenticity Signatures of Six Essential Oils Used as Food Flavors: a Gas chromatography - Mass Spectrometry Approach, *Bull USAMV Food Sci Technol, 78, 88-100.*
2. POPA R.M., FETEA F., SOCACIU C., 2021, ATR-FTIR-MIR Spectrometry and pattern recognition of bioactive volatiles in oily vs microencapsulated food supplements: authenticity, quality and stability, *Molecules, 26, 4837.*
3. POPA R.M., FETEA F., SOCACIU C., 2021, Attenuated total reflectance - fourier transform infrared spectroscopy applied for the evaluation of essential oils' pattern recognition and thermooxidative stability: a comparative study, *Studia UBB Chemia, LXVI, 4, 33-50.*

FUTURE RESEARCH AND PERSPECTIVES

This study can provide an example of a systematic approach, through complementary methods and techniques (GC-MS and FTIR-ATR) combined with statistical analysis, to be applied in the future to different formulas containing plant essential oils, and will make possible to evaluate their quality, authenticity and traceability in various agri-food or cosmetic products.

Since essential oils are frequently used, free or in different formulas, as ingredients in food and food supplements or phytocometics, it is possible to identify fakes (synthetic volatile samples or essential oils degraded by oxidation).

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