MICROWAVE EXTRACTION OF ACTIVE PRINCIPLES FROM MEDICINAL PLANTS

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Scopul acestui studiu a fost de a mări viteza de extracţie şi de a îmbunătăţi randamentul şi calitatea produselor, asociind câmpul de microunde cu extracţia compuşilor activi din plante de flavonoide şi acizi fenolici din Cynara scolymus frunze.

În comparaţie cu metodele clasice de extracţie la temperatură înaltă, extracţia cu microunde asigură purităţi mai mari în compuşi activi, evitând în acelaşi timp supra-expunerea locală, un lucru foarte important pentru industrie.

Metoda de cuantificare spectrofotometrică a fost utilizată în separarea şi determinarea cantitativă flavonoide şi acizi fenolici, componente din extracte vegetale de Cynara scolymus.

The aim of this study was to increase extraction rate, yield and quality of products, associating microwave field with extraction of plant's active compounds such as flavonoids and phenolic acids from Cynara scolymus (CS) leaves.

Compared to classical hot extraction methods (CE), microwave extraction (MAE) provides higher purities in active compounds while avoiding local over-exposure, very important assets for industry.

Spectrophotometry was used to separate and quantify flavonoids (FV) and phenolic acids (FA) in herbal extracts of Cynara scolymus.

Keywords: microwave-assisted extraction (MAE), flavonoids, phenolic acids

1. Introduction

Over the past 15 years, researchers and food manufacturers have become increasingly interested in phenolic acids, mainly due to the current growing interest for natural antioxidants. The phenolic compounds are among the phytochemical constituents that are responsible for the beneficial effects of the herb Artichoke (*Cynara scolymus* L.). They posses good antioxidant properties, due primarily to flavonoids and phenolic acids, particularly chlorogenic acid (5-caffeoylquinic acid, cynarin), dicaffeoylquinic acids, caffeic acid and flavonoids (luteolin-7-O- and apigenin glycosides). Their antioxidant activity is mainly due

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to their redox properties, which allow them to act as reducing agents, hydrogen donors, free radical scavengers, singlet oxygen quenchers and metal chelators [1].

Cynara scolymus (CS) leaves extracts have long been used for their choleretic and hepatoprotective activities and ability to lower cholesterol in humans, which are often related to the cynarin content [2].

The active compounds studied contain many hydroxyl groups thus conferring them a high solubility in water or in mixture of water and alcohols. The toxicity restriction imposes as safe solvents only water and lower alcohols (methanol or ethanol) [3].

The classical process of effective constituents extraction from a plant material by means of a solvent is via their diffusion through the cell walls and generally involves two main stages: first, dissolution of the soluble constituents on or near surfaces of solid plant particles (so called, washing), and second, mass transfer of soluble constituents from the plant material into the solution by diffusion and osmotic process (so called slow extraction). Usually, the latter stage is slower than the former one and is responsible for limiting the rate of extraction process which drives a number of difficulties. Effective constituent's recovery and purity are both limited by the inherent diffusion characteristics: slow extraction rates require large capital equipment for a given level of throughput; lengthy exposure to heated solvents cause product degradation; and the need to re-extract biomass with fresh solvent in multi-stage extractions can result in high solvent and energy costs [3].

Process intensification has become a very interesting approach, transforming current practices in biochemical engineering and bringing forth new developments in equipment, processing techniques and operational methods. This development aims at more compact, safe, energy efficient and environmentally friendly process. Several unconventional processing techniques rely on alternative forms of external energy, used to increase the internal kinetic energy of the molecules and, sometimes, to break the cells' wall.

In recent years, there is steady progress in extraction technology with the development of new and simpler sample preparation methods. In order to increase the productivity, several intensification techniques like supercritical fluids extraction (SFE), microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), pressurized liquid extraction (PLE) and pressurized hot water extraction (PHWE) were associated with extraction of plant's compounds to improve the yield and quality of extracted products [3].

From these, ultrasound assisted and microwave extractions emerged as two promising techniques from an economical point of view, being inexpensive, simple and efficient. During the last few decades, ultrasound and microwave irradiation has received a lot of attention and widespread research is going on in

these areas. Significant enhancement of selectivity's, rates and yields in medicinal plant extraction has been achieved by means of US and MW irradiation [3-9].

The use of microwave energy for the extraction of active substances from plant materials results in faster heating, since the each molecule exposed to the microwave field is directly affected, reduced thermal gradients, since heat is volume generated, reduced equipment size, because of the higher process rates, and increased productivity, through a better usage of the same equipment process volume [4]. In case of microwave irradiation on biological material, electromagnetic waves are indeed absorbed selectively by media possessing a high dielectric constant resulting in more effective heating. During absorption, the microwaves' energy is converted into kinetic energy, thus enabling the selective heating of the microwave-absorbent parts of the plant material. The volume increased in this way makes cells explode, releasing their content into the liquid phase. When the liquid phase absorbs the microwaves, the kinetic energy of its molecules increases, and consequently, the diffusion rate increases too, resulting in faster mass transfer [8].

MAE is a feasible green solvent extraction method as it utilizes water or alcohols at elevated temperature and controlled pressure conditions. Water is a highly polar solvent with a high dielectric constant (ε) at room temperature and atmospheric pressure due to the presence of extensive hydrogen-bonded structures. Hence, traditionally water is not considered as a suitable extraction fluid for non-polar or organic compounds at room temperature. Various reports have shown that at certain temperature and applied pressure, the polarity of water can be varied close to those of alcohols. When the temperature of water is raised, there is a steady decrease in its permittivity, viscosity and surface tension but an increase in its diffusivity characteristics. With enough pressure to maintain water in the liquid phase at elevated temperature, the initial value of the dielectric constant of 80 at 25^{o}C decreases to 27 at 250^{o}C and 50 bar, which falls between those of methanol (ε = 33) and ethanol (ε = 24) at 25^{o}C. Under these conditions, water behaves like certain organic solvents which can dissolve a wide range of medium and low polarity analytes [6, 7].

The extraction mechanism in MAE is supposed to involve four sequential steps. The first step is the desorption of solutes from the various active sites in the sample matrix under the pressurized and elevated temperature conditions. The second step may involve the diffusion of extraction fluid into the matrix. Next, depending on the sample matrix, the solutes may partition themselves from the sample matrix into the extraction fluid. The enhancement on the extraction efficiency of MAE can be attributed to: (1) an improvement in the solubility and mass transfer effects and (2) an increased disruption of surface equilibrium [3]. There are reduced viscosity and improved diffusivity of water which allow better penetration through the matrix particles. Both the high temperatures and pressures

could disrupt the surface equilibrium. The increased temperature can overcome the solute–matrix interaction caused by *van der Waals* forces, hydrogen bonding, dipole attraction of the solutes molecules and active sites in the matrix. Thus, the supplied thermal energy can disrupt cohesive (solute–solute) and adhesive (solute–matrix) interactions, providing the necessary activation energy required for desorption process. The transfer of the analytes from matrix to solvent is achieved by the diffusion and convection processes [9]. However, thermally labile compounds are degraded at elevated temperatures. Sufficient pressure is required to be exerted on water when temperatures above its boiling point are used. The presence of pressure could facilitate extraction from samples where analytes are trapped in the matrix pores. This pressure forces the water into areas of the matrices which are not normally covered if water at atmospheric pressure is used [3].

Microwave intensification needs special equipment to be functional, which means higher investments, and electricity to produce waves, which means higher operating costs than classical techniques [9].

MAE has applications in natural compounds extraction such glycosides, alkaloids, carotenoids, terpenes, polyphenols, essenti oils [7-9].

This paper describes the extraction of steviol glycosides, flavonoids and polyphenols from herbs by MAE and makes a comparative study with the traditional extraction technologies. In recent years, there are new techniques reports for steviol glycosides, flavonoids and polyphenols in some related literatures but use of the microwave-assistant extraction was infrequent for the herbs studied [9-10].

The objective of the present experimental work is to investigate the influence of some parameters: microwave temperature/power and extraction time on extraction yields.

2. Materials and methods

2.1. Reagents and plant material

Gallic acid and Rutoside (Sigma) were used as standard chemicals for spectrophotometric analysis.

Folin-Ciocalteu reactive, sodium carbonate 10%, sodium acetate 100 g/L and aluminum trichloride 25g/L were used as reagents in spectrophotometric analysis.

The plant material consists of dried leaves of Cynara scolymus (artichoke) harvested in Romania. They were stored in dark hermetic tight bags to protect them from humidity and light and, before each bunch of experiments, they were cut into pieces of the appropriate equivalent diameter.

2.3. Classic extraction

To illustrate the benefits and, thus, the necessity of MAE, a classical thermal extraction method was used as control, under the same conditions.

The thermal extraction was done in Erlenmeyer flasks: 10 g plant material with optimum particles size of 0.315 mm equivalent diameter was mixed with 50% solution water/ethanol using an optimum ratio of 1/8 (w/v) sample weight to solvent volume. The optimum values were chosen based on a preliminary study which followed the optimum temperature due to the higher extraction yields at room temperature.

In this case, samples were taken at 1, 2, 3, 5 and 8 minutes at temperatures of 40^0C to 90 ^{0}C for all active compounds.

All final extracts were filtrated through a 0.45 μm filter prior to spectrophotometer and HPLC analyses.

2.4. Microwave assisted extraction

A closed vessel system *(Model Initiator 2.0, Biotage)* (under controlled temperature range between 40 and 250°C, power range of 0-400 W and pressure range of 0-20 bar) was employed.

For a closed vessel MAE system, the parameters that could affect the extraction efficiency were sample size, nature of solvent and its volume, pressure, temperature, microwave power and extraction time.

The power setting (microwave power) and pressure are important experimental variables for closed vessel MAE, but they directly dependent on the temperature. Thus, temperature was the preferred controlled variable to avoid degradation of the target compounds and to achieve the maximum efficiency.

Using the same proportions, i.e. 0,4 g plant material with 0.315 mm equivalent diameter was mixed with solvent, placed into the closed microwave assisted extractor and irradiated for 1, 2, 3 and 5 minutes at 70^0C, 90^0C and 100^0C, with stirring.

After the samples were submitted to MAE, the extract was filtered through a 0.45 μm filter before HPLC and spectrophotometer analyses.

2.5. Extractive values

According to the Romanian Pharmacopoeia (10th edition) approximately 2g (2 ml) of extract was placed into a flat-bottomed glass dish (36 mm diameter and 28 mm height) covered to prevent evaporation of solvent before weighting. After weighting, the extract was dried in oven at 103^0C for 3h. The content of extractive substances in the plant material was calculated from the mass of dry extract and the initial mass of plant subject to experiment. The concentration of

extractive substances in the liquid extract was calculated from the mass of dry extract and the volume of liquid extract. The extractive value of the soluble compounds from the extract was calculated as a mass percentage of dry residue (g/100g extract).

2.6 Determination of flavonoids and phenolic acids percentages using spectrophotometer

Determination of the phenolic acids and flavonoids content was performed according with the method presented in Romanian Pharmacopeia (10th edition), implemented using a Spekol 1000 Series spectrophotometer with VIS detector.

All the calculations concerning the quantitative analysis were performed with external standardization by measurement of the absorbance of a specific wavelength of light.

Stock solutions of gallic acid (0.1g/L) and rutoside (0.1 g/L) (Sigma) were prepared in distilled water (v/v). Standard series in the concentration range of 25-200 μg/mL for flavonoids and 4-16 μg/mL for phenolic acid were obtained from the stock solution.

Volumes of 2 mL solution prepared from each sample were placed into quartz cuvettes into the spectrophotometer and analyzed.

The total content of phenolic acids was determined with Folin-Ciocalteu reactive and Na_2CO_3 10% (Sigma), considering the maximum of absorption of the obtained coloration, with respect to the calibration curve. The phenolic acids are expressed in mg/g gallic acid correspondents at a final set concentration of 16 μg/mL.

The total content in flavonoids was measured with ethanol solution of aluminum trichloride 25 g/L and sodium acetate 100 g/L (Sigma), throughout the evaluation of the maximum of absorption of obtained coloration, with respect to the calibration curve. The total content of flavonoids is expressed in mg/g rutoside correspondents related to a final set concentration of 200 μg/mL.

The blank solutions were prepared in the same conditions as the samples and were used for all spectrophotometric experimental studies.

Quantification was realized by measuring the absorbance at the wavelength 430 nm for flavonoids and 765 nm for phenol acids to determine the concentration of a known solute in a given solution by the application of the Beer-Lambert law, which states that the concentration of a solute is proportional to the absorbance. Optical density, or OD, is the absorbance per unit length. The results were obtained as a mean value of two separate samples.

The concentrations of flavones and total phenolic acids were calculated as that of rutoside and gallic acid respectively. The results could be described by mass fraction (%) = [C x V x D] / [m x 100] x 100, where C (mg/mL) is the

flavone or phenolic acid content of solution calculated by standard curve; V is the volume of solution; D is the total dilution value and m(g) is mass of the sample

3. Results and Comparative Discussions

The results of microwave extraction employed are presented, *per se* or compared to the classic thermal extraction, in Figs. 1 to 4.

3.1. The Effect of Extraction Temperature on the Extraction Yield and Purity of the Active Constituents

Theoretically, the extraction temperature can increase the extraction rate, the extraction yield and the degradation of unstable compounds.

Fig. 1 (a and b) presents these effects of microwave extraction temperature on the extraction yields. The initial increase of extraction temperature had a positive effect on the extraction yields while extraction temperature higher than 70^0C in both case of FA and FV had a negative effect on the extraction yield, which was attributed to the thermal oxidation of the hydroxyl of glycoside and phenolic compounds. So 70^0C was the maximum allowable extraction temperature in the present experiment and were considered as the optimum for achieving a high recovery yield of active constituents.

As shown in Fig. 1 (a and b) the extraction yields of flavonoids and total phenolic acids increased with temperature until reaching their maximum values at optimum temperature, and subsequently decreased at higher temperatures.

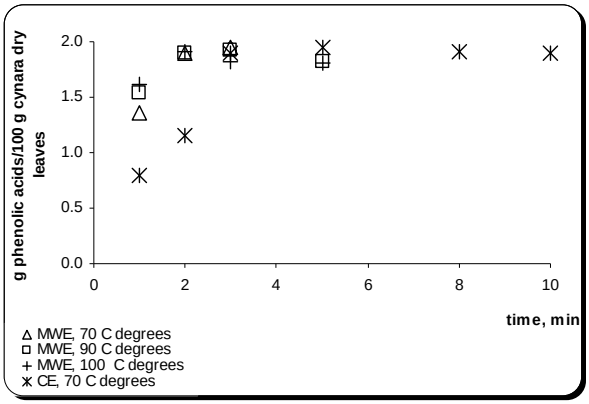

Fig. 1. The effect of the microwave temperature extraction on the recoveries in time: a. flavonoids

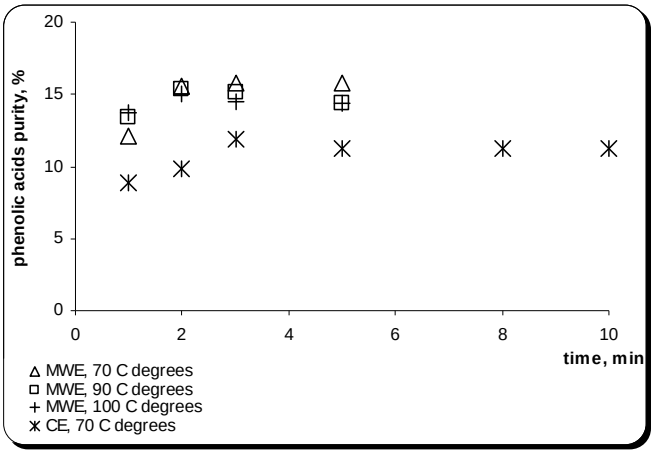

Fig. 1. The effect of the microwave temperature extraction on the recoveries in time: b. phenolic acids

Fig. 2 (a and b) depicts the effective substances purity at three increasing temperature values of the microwave field. The main drawback is the danger of overexposure, when the valuable species ends up being destroyed, the decrease in the concentration of effective constituents for long times.

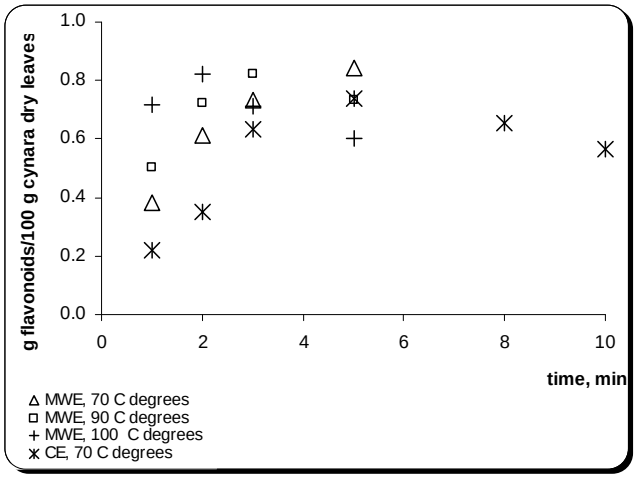

Fig. 2. The effect of the microwave temperature extraction on the purity of active constituents in time: a. flavonoids

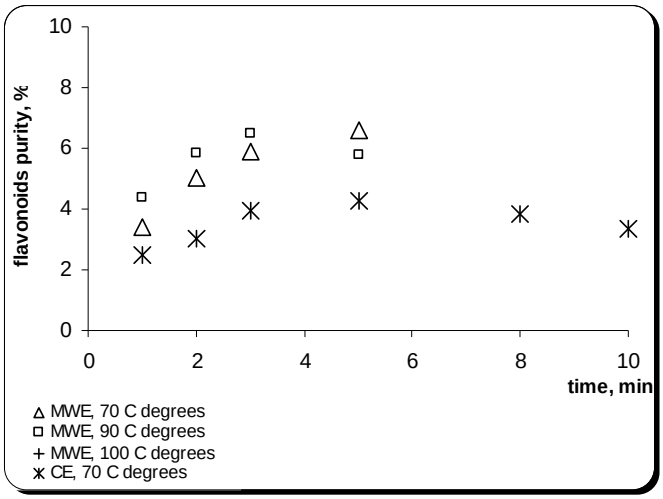

Fig. 2. The effect of the microwave temperature extraction on the purity of active constituents in time: b. phenolic acids

3.2. The effect of Extraction Time on the Extraction Yield and Purity of the Active Constituents

At 70^0C, the extraction time is in direct proportion to the extraction yield. Fig. 1 (a-d) illustrates that the extraction yield augmented with the increase of the extraction time up to 5 minutes in both cases. When the extraction time was longer than 3 min, the extraction yield decreased. The decrease of extraction yield derives from the thermal oxidation caused by the excessive exposure. As can be seen, 3-5 minutes was enough for the process to complete.

3.3. Classic thermal extraction (CTE) results

In order to consider classic thermal extraction as the control method for the microwave assisted extraction, we tried to follow the same extraction temperature profile for CTE. Fig. 3 shows that the extraction temperature profile for CTE was found to be similar to that of MAE for the four effective constituents.

From Fig. 1 (a and b), it can be observed the main downside of classic thermal extraction - the thermal degradation of the valuable compound which starts manifesting with increasing the temperature at high values or using long extraction time. Both FV and PF were stable up to 70^0C. Thus, we considered the optimal temperature of CTE at 70^0C, after that the yield started to decrease at 100^0C.

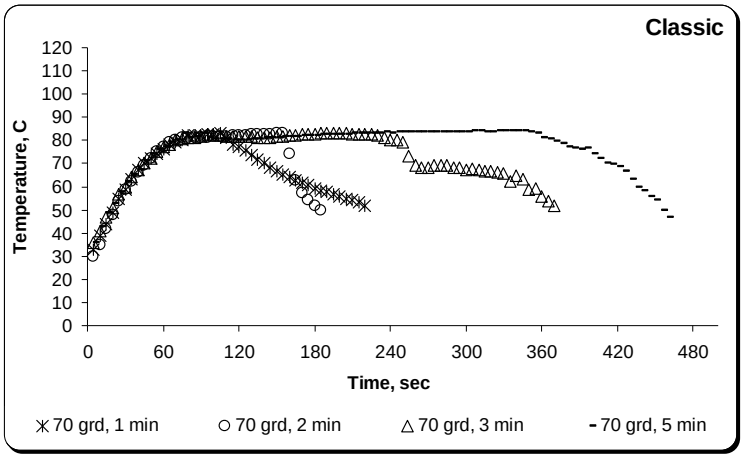

Fig. 3. The classic vs. microwave temperature profiles in time: a. classic

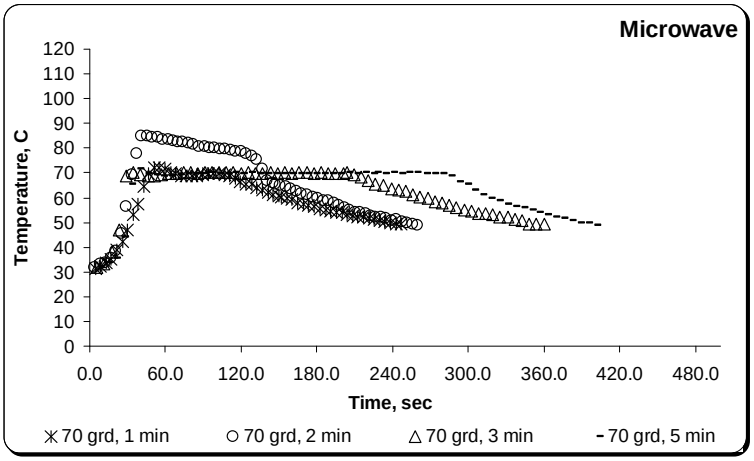

Fig. 3. The classic vs. microwave temperature profiles in time: b. microwave

3.4. Comparison of the Microwave Extraction with the Classic Thermal Method

The main factors impacting the effects of microwave extraction were the temperature and extraction time. A normal correlation was found to exist between the extraction time and the extraction temperature (Fig. 1 a and b). Although not unexpected, a linear correlation between the concentration and the extraction temperature in MAE was also observed (R^2 = 0.983 for FV-CS and 0.999 for FA-

CS, see Fig. 4a). The results from Figs. 1 and 2 show a remarkable dependency of the concentration in effective constituents upon the temperature used to intensify the mass transfer. The supplemental kinetic energy introduced from outside into the liquid phase through microwave energies and stirring, modifies the extraction process rate, without altering the thermodynamic equilibrium.

Compared to conventional CTE, MAE increased the efficiency in yields while the time of execution is reduced.

The conventional extraction of active substances from medicinal plants, is an asymptotic process, governed by thermodynamic equilibrium. The equilibrium is reached when the driving force becomes null.

Using MAE as intensification technique, a supplemental kinetic energy is introduced from outside into the liquid phase through heating. Thus the kinetic energy of the contacting fluid increased, improving both the extraction rate due to the effect of the effective heating with the disruption of weak hydrogen bounds generated by the dipole rotation of the molecules, and also increased the mass transfer by diffusion inside the plant matrix, obtaining a higher driving force than CTE with a significant improvement in productivity, as can be observed for all experiments.

The optimum extraction conditions were confirmed as follows: 0.315 mm equivalent diameter plant material mixed with 50% solution water/ethanol using a ratio of 1/8 (w/v) sample weight to solvent volume; extraction temperature was considered to be 70 ^{0}C for FV and FA and the extraction time was 3-5 minutes in both cases. The extraction rate of active constituents under this condition was 2.01g FA/100g CS dry leaves and 0.84g FV/100g CS dry leaves respectively. Compared to thermal extraction, when the extract is subject to thermal degradation through overheating, the microwave technique ensure higher purity values in active substances 31.37% in FA and 6.6% from CS (see Fig. 2 a and b) together with a much lower possibility of overexposure, i.e. the waves' field could be cut off rapidly, contrary to the thermal field.

The extraction was performed under the optimal conditions, varying the time to find the efficiency of the method. The aforementioned experimental parameters might synergistically affect the extraction efficiency. As demonstrated in Fig. 4, the extraction efficiency increased markedly when extending the extraction time from 1 to 2 minutes, a further increase in extraction time resulting in a slight decrease in the efficiency; this is in concordance with the mechanism of microwave extraction. Raising the extraction temperature usually decreases the interaction between matrix and analytes, which results in better extraction efficiency. The prevention of degradation of thermally labile analytes dictates the upper temperature limit.

The effect of microwave extraction temperature at the efficiency of active compounds, instead of classic temperature had different behaviors and is shown in Fig. 4.

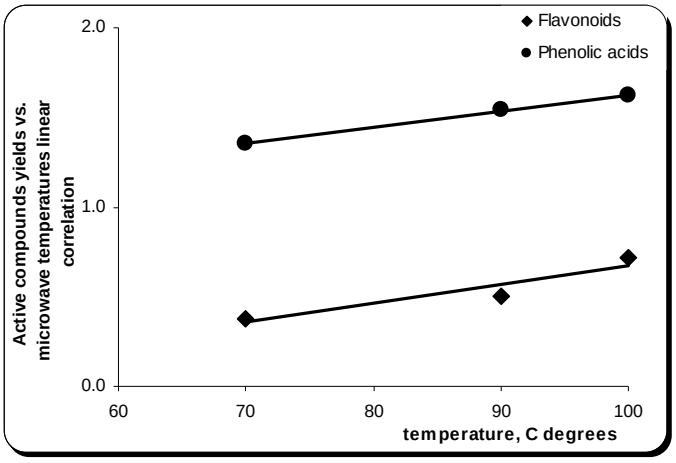

Fig. 4. The effect of the microwave temperature extraction in time: a) on the active compounds yields

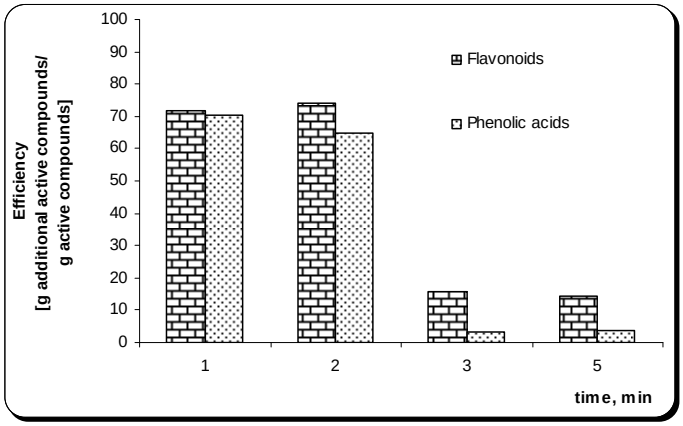

Fig. 4. The effect of the microwave temperature extraction in time: b) on the efficiency of extraction

Under the same optimized conditions, the MAE produced higher efficiency than that of CE (70.56% FA-CS and 74.14%-FV-CS enhanced). It was noted that MAE could achieve higher extraction efficiency within a relatively short time (1-2 minutes).

From Fig. 4, MAE, which involved desorption, diffusion and dissolution of the sample cell, demonstrated to be efficient in the extraction of FV and FA. The initial desorption process seems to determine the overall extraction rate. From

Figs. 1 and 2, it was noted that exhaustive extraction was observed using MAE. Our data showed that the CE did not imply a complete desorption process; the heated solvent was not able to penetrate into the sample core to release more target compounds and thus resulted in lower extraction efficiency as compared to MAE. With microwave extraction, even without a continuous supply of extraction fluid, the combination of microwave energy with solvent was able to penetrate deep into the plant matrix core. The direct heating mechanism for MAE allowed maximum transfer of energy to heat up the aqueous solvent in the closed vessels. The heating enhanced the solvent's permittivity and diffusivity which in turn disrupted the in-depth solute-matrix interactions to release more target compounds which were embedded inside the sample core. These compounds would then diffuse out of the core and dissolve into the extraction fluid.

4. Conclusions

In order to increase the process rate, yield and quality of products, microwave field was associated with extraction of plant's active compounds such as flavonoids and phenolic acids from *Cynara scolymus*.

The purpose of the experiment was to compare this intensification technique with the classical extraction method like thermal extraction, studying the influence of the temperature and time to determine the optimum domain in a reliable extraction protocol.

Over other extraction methods, MAE has excellent advantages, such as higher yields, purity and efficiency. In comparison with classic thermal extraction, the MAE proved to be a simpler but more effective procedure to obtain active compounds from medicinal plants. With respect to thermal extraction, the microwave extraction technique emerged as secure and worthy method to improve either a rather time consuming or an energy intensive (far from optimal conditions) process.

Applying MAE method to extract active compounds from medicinal plants is feasible and reliable. Our experimental results are fairly comparable and even with a smaller raise in active substances yields than the published findings from literature.

It is very probable that MAE will become an important tool in industrial applications for enhancement of extraction processes in the near future. Despite the certain limitations discussed as compared to certain classical method of extraction, MAE is a feasible green alternative method to be exploited in the future technologies for more analytes to be used on a bigger scale. This simple technique utilizes cheap and non-toxic solvents. Under optimized conditions, MAE could be a suitable technique for scale up to handle larger sample sizes for industrial applications.

Like CTE, MAE intensification needs special equipment to be functional, which means higher investments, and electricity to produce the ultrasonic waves, which means higher operating costs than maceration. So, taking into account the efficiency of the intensification methods, a soundly economic analysis should be done, in order to consider suitable the microwave extraction procedure.

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