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Hydrodiffusion assistée par micro-ondes. Nouvelle technique d'eco-extraction d'antioxydants

par

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Scientific Publications

1. Clean recovery of antioxidant flavonoids from onions: Optimising solvent free microwave extraction method

Zill-e-HUMA, Maryline ABERT-VIAN, Jean Francois MAINGONNAT, Farid CHEMAT

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2. A remarkable influence of microwave extraction: Enhancement of antioxidant activity of extracted onion Varieties

Zill-e-HUMA, Maryline ABERT-VIAN, Anne-Sylvie FABIANO-TIXIER, Mohamed ELMAATAOUI, Olivier DANGLES, Farid CHEMAT

(Submitted to ***Food Chemistry****)*

3. Vacuum microwave hydrodiffusion and gravity technique: an idea towards improvement with application of vacuum system

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(Submitted to ***Journal of Food Engineering****)*

4. Solvent free microwave assisted extraction of antioxidants from sea buckthorn (Hippophae rhamnoides) food byproducts

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Microwave assisted separations: Green chemistry in action

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Poster: Clean recovery of antioxidant flavonoids from onions: Optimising solvent free microwave extraction method

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Oral presentation: Eco-friendly efficient extraction of onion antioxidants by using microwave energy and earth gravity

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5. 25th International Conference on Ploy phenols (ICP), 24-27 Aug 2010, Montpellier, France

Poster: Recovery of antioxidants with clean noval extarction techniques

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Synopsis

Il semblerait que ce soit les pharaons, dont l'histoire remonte à plus de 4 000, ans qui furent les premiers à maîtriser la technique d'extraction et à tirer parti du règne végétal dans un souci nutritionnel, esthétique et spirituel. Des sesquiterpènes spécifiques aux extraits d'encens ont été découverts dans les bandages de momies. Les civilisations grecques et romaines ont aussi apporté de grandes découvertes dans ce domaine, ils ont mis en place la base de la distillation comme l'invention de l'ambix utilisé aussi bien pour extraire que pour distiller. Plus tard, la civilisation arabo-musulmane développa le commerce des épices et des aromates, et donna une grande impulsion à l'art de la distillation et de l'extraction. C'est Geber (721-815), qui inventa l'alambic à des fins d'alchimie, mais le nom de ce procédé resta incontestablement associée à Avicenne (930-1037) qui inventa le réfrigérant et distilla pour la première fois l'éthanol dont le nom est tiré de l'arabe « elkohol ». Ce dernier a autant révolutionné la médecine, l'art culinaire que l'extraction des molécules bioactives comme les colorants, les antioxydants et les arômes.

De nos jours, il est devenu difficile de trouver une analyse en recherche ou une ligne de production en industrie qui, directement ou indirectement, n'utilise pas l'extraction. La recherche dans ce domaine ne cesse de croître pour trouver ou inventer de nouveaux procédés plus efficaces en terme de réduction de temps, de rendement et de sélectivité, mais aussi pour œuvrer vers une chimie « durable » utilisant moins de solvant, moins d'énergie et diminuant les rejets aussi bien de CO_2 que liquides et solides.

L'hydrodiffusion générée par micro-ondes est l'une des plus récentes. Inspirée d'un ancien procédé de distillation dite descendante, appelée aussi hydrodiffusion, utilisé par les alchimistes pour la distillation, elle consiste en une distillation à l'aide d'un alambic utilisant un feu de bois comme source de chaleur, ou plus récemment des énergies fossiles. L'hydrodiffusion générée par micro-ondes a été développée en remplaçant ces énergies dites fossiles par un chauffage micro-ondes plus performant, plus spécifique et beaucoup moins polluant qu'un chauffage conventionnel.

Ce mémoire de thèse composé de quatre chapitres a pour but de présenter le travail effectué pour la conception, la mise au point, le développement, la validation et la valorisation de cette nouvelle technique d'extraction sans solvant assistée par micro-ondes.

Une premier chapitre de ce mémoire est consacrée aux fondements et considérations théoriques de l'énergie électromagnétique, de la façon dont les micro-ondes sont produites et de leur cheminement jusqu'à la zone de traitement de l'échantillon. Quelques applications originales permettront d'illustrer le chauffage par micro-ondes, ses caractéristiques, sa sélectivité, son efficacité et son originalité. Les procédés et découvertes les plus significatives concernant l'extraction par micro-ondes seront ensuite répertoriés afin de rappeler l'évolution de la technique d'extraction par micro-ondes et de décrire en dernier lieu les réalisations les plus remarquables dans le domaine de l'extraction des produits naturels par micro-ondes.

Le deuxième chapitre de ce manuscrit relève des résultats, de leur discussion, et des techniques employées afin de proposer une procédure d'extraction innovante en vue de l'extraction des antioxydants contenus dans les matrices alimentaires solides. Ce chapitre s'articule selon trois sections distinctes : dans la première partie, la mise au point, l'optimisation et l'application du nouveau dispositif de laboratoire, permettant l'extraction de polyphenols à partir d'oignons seront discutées. L'originalité de l'appareillage réside en une extraction rapide, non destructrice et généralisable à une multitude de matrices végétales qui sera discutée dans les deux autres parties. Enfin, l'approche écologique apportée par le procédé sera aussi discutée.

Le troisième chapitre montre les potentialités d'évolution de ce procédé. Nous avons combiné l'hydrodiffusion générée par micro-ondes avec le procédé d'aspiration ou de création du vide. Cela a permis d'obtenir de meilleurs rendements et une sélectivité accrue, tout en diminuant les temps d'extraction.

Le dernier chapitre propose une comparaison de ce système avec d'autres procédés d'extraction innovants comme les ultrasons. Cela permet d'avoir une vue plus globale et d'appréhender les avantages et inconvénients de notre procédé vis-à-vis des autres procédés existants pour une potentielle application industrielle.

GENERAL INTRODUCTION

The importance of extraction step in the analytical procedure and especially in food, pharmaceutical and neutraceutical industries, whose products have direct interaction with human health and consists diverse range of plant extracted components, has gained renewed attention in the last few decades. Existing conventional extraction methods are known for their economic impact due to high energy consumption (extraction step often used more than 70% of total process energy), large amount of toxic solvent and time utilization in completion of extraction step and environmental impact by rejecting CO_2 and untreatable wastes. Recently evolve concept of green extraction demands for the development and utilization of techniques with highly efficient approach for reducing energy consumption and generation, no or atleast less utilization of solvents with reduce generation of hazardous wastes. Extraction of components by extensively used operations like solvent extraction or leaching is usually enhanced by assistance of different processes like mechanical fragmentation, pressing, or heating which along with generation of large amount of heat and wastes also resulted with degradation of sensitive components. These shortcomings have led the extraction chemists and industrialists to the consideration of use of new "green" techniques in separation. Microwave energy has been developed recently for the extraction of organic compounds from environmental matrices after its use in inorganic chemistry for trace metal analysis. The development in microwave techniques has occurred because of a need for a rapid, safe and cheap method.

Microwave energy as a non contact alternative heat source is being utilized efficiently in the field of extraction. Several classes of compounds such as essential oils, aromas, pigments, antioxidants have been extracted successively by consuming only fraction of energy in comparison to conventional extraction methods. Microwave extraction techniques by giving high purity of final product have also secured the intensive energy expenditure in the purification and separation step. Various microwave assisted extraction methods have been developed to counteract the limits of conventional extraction techniques. Recently, we have developed a new extraction technique microwave hydrodiffusion and gravity (MHG) and applied it for antioxidants extraction from vegetal materials along with exhibiting its unique character of fast and differential heating.

Chapter 1 presents a complete picture of current knowledge on microwave-assisted separations of food and natural products. It provides the necessary theoretical background and some details about extraction by microwaves, the technique, the mechanism, some applications, and environmental impacts. Microwave-assisted separation is a research topic which affects several fields of modern chemistry. All the reported applications have shown that microwave-assisted separation is an alternative to conventional techniques for food and natural products. The main benefits are decreases in extraction times, the amount of energy and solvents used, and CO_2 emissions.

Chapter 2 consists of two distinct parts. Initially, the design and optimization of this clean and environmental benign extraction technique (MHG) has done for flavonoids extraction along with detailed prediction of temperature distributions in different parts of matrix during microwave heating. In the second part the competency evaluation of this technique is done by analysing the antioxidant activity of the extracts. The purpose behind this generalization step was, to offer ultimately a green (solvent free), simple (one step), fast and effective antioxidant extraction method.

In chapter 3 the effect of vacuum is described after designing and optimizing Vacuum microwave hydrodiffusion and gravity (VMHG), which is the refitted form of solvent free microwave hydrodiffusion and gravity (MHG) system. VMHG is developed with the theme to minimize the limitations observed in MHG extraction system. Here, we have used this apparatus first time for extraction of onion by-products.

In the last chapter of this study, we have tried to make comparison, by extracting onion flavonols with another new extraction method, ultrasound assisted extraction (UAE). The present study reports on the extraction of polyphenols especially flavonols from onion (*Allium cepa* L.) by-products by using water having pH 7 as solvent. Furthermore, the product quality is assessed by quantification of compounds and its antioxidant activity.

Chapter I

Microwave assisted – separations: Green chemistry in action

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I.1. INTRODUCTION

With the increasing energy prices and the drive to reduce CO_2 emissions, chemical and food industries are challenged to find new technologies in order to reduce energy consumption, to meet legal requirements on emissions, product/process safety and control, and for cost reduction and increased quality as well as functionality. Separation technology (such as extraction, distillation, and crystallization) is one of the promising innovation themes that could contribute to sustainable growth of chemical and food industries. For example, existing extraction technologies have considerable technological and scientific bottlenecks to overcome: often requiring up to 50% of investments in a new plant and more than 70% of total process energy used in food, fine chemicals and pharmaceutical industries. These shortcomings have led to the consideration of the use of new "green" techniques in separation, which typically use less solvent and energy, such as microwave extraction, supercritical fluid extraction, ultrasound extraction, ultrafiltration, flash distillation, controlled pressure drop process, and subcritical water extraction. Separation under extreme or non-classical conditions is currently a dynamically developing area in applied research and industry.

Using microwaves, extraction and distillation can now be completed in minutes instead of hours with high reproducibility, reducing the consumption of solvent, simplifying manipulation and work-up, giving higher purity of the final product, eliminating post-treatment of waste water and consuming only a fraction of the energy normally needed for a conventional separation method such as distillation or solvent extraction. Several classes of compounds such as essential oils, aromas, pigments, anti-oxidants, and other organic compounds have been extracted efficiently from a variety of matrices (mainly animal tissues, food, and plant materials). The advantages of using microwave energy, which is a non contact heat source, includes: more effective heating, faster energy transfer, reduced thermal gradients, selective heating, reduced equipment size, faster response to process heating control, faster start-up, increased production, and elimination of process steps [1-2].

This chapter presents a complete picture of current knowledge on microwave-assisted separations of food and natural products. It provides the necessary theoretical background and some details about extraction by microwaves, the technique, the mechanism, some applications, and environmental impacts.

I.2. BASIC PRINCIPLES

I.2.1. Importance of the separation step

Food and natural products are invaluable resources, useful in daily life as food additives, flavours, fragrances, pharmaceuticals, colours or directly in medicine. This use of plants has a long history all over the world, and over the centuries, humanity has developed better methods for the separation of these components from such materials. These primary and secondary metabolites are generally present at low concentrations. Before such substances could be used, they have to be extracted from the plant matrix. Different methods are used for this purpose, e.g. hydro-distillation, steam distillation, cold pressing, simultaneous distillation-extraction, solvent extraction, ultra-filtration, crystallisation... Nevertheless, these molecules are well known to be thermally sensitive and vulnerable to chemical changes.

In general, a separation procedure for food components from vegetables, fruits, spices or other complex food matrices comprises two steps: preparation (grinding, cutting, crushing, milling...) and extraction (e.g. single-step solvent extraction, Soxhlet extraction, steam distillation and simultaneous distillation-extraction). While the preparation step is complete after only 15 to 30 minutes, extraction takes at least several hours. It is frequently carried out by prolonged heating and stirring in boiling solvent. Thus, the principal limiting step of a separation operation is the extraction of the analyte from the matrix, which consists in transferring the desired compounds into solvent. The conventional solvent extraction procedure represents 90% of the total processing time. It is thus important to shorten this limiting step. The choice of the technique is the result of a compromise between efficiency and reproducibility of extraction, ease of procedure, together with considerations of cost, time, degree of automation and safety.

I.2.2. Microwave heat transfer

Microwaves (MWs) are electromagnetic waves with a frequency range from 100 MHz to 3 GHz. MWs comprise electric and magnetic field components and thus constitute propagating electromagnetic energy. This energy acts as a non-ionizing radiation that causes molecular motions of ions and rotation of the dipoles, but does not affect molecular structure.

When dielectric materials containing either permanent or induced dipoles are place in a microwave field, the rotation of the dipoles in the alternating field produces heat. More precisely, the applied microwave field causes the molecules, on average, to spend slightly more time orienting themselves in the direction of the electric field rather than in other directions. When the electric field is removed, thermal agitation returns the molecules to a disordered state in the relaxation time and thermal energy is released. Thus microwave heating results from the dissipation of the electromagnetic waves in the irradiated medium. The dissipated power in the medium depends on the complex permittivity of the material and the local time-averaged electric field strength.

In conventional heating, heat is transferred from the heating medium to the interior of the sample, while in microwave heating; heat is dissipated volumetrically inside the irradiated medium. MWs are volumetrically distributed heating, and heat transfers occur from the sample to the colder environment. This causes an important difference between conventional and microwave heating. In conventional heating, heat transfer depends on thermal conductivity, on the temperature difference across the sample, and, for fluids, on convection currents. As a result, the temperature increase is often rather slow. By contrast, in microwave heating, due to the volumetric heating effect, much faster temperature increases can be obtained, depending on the microwave power and the dielectric loss factor of the material being irradiated.

Although microwaves heat volumetrically, it is well known that the electromagnetic field distribution is not even in the irradiated material, thus the energy is not homogeneously dissipated. The electric field distribution depends on the geometry of the heated object and the dielectric properties. For media which readily absorb microwaves, i.e. for which the loss factor is > 5, the penetration depth Dp at which the dissipated power is reduced to 1/e of surface value might be a limiting factor:

$$D_p \approx \frac{\lambda_0}{2\pi} \frac{\sqrt{\varepsilon'}}{\varepsilon''} \qquad (1)$$

where λ_0: wave length ; ε' : dielectric constant, ε'' : loss factor. If this dimension is much smaller than the dimension of the object being heated, localized surface heating will occur. For more transparent media, e.g. with a loss factor < 0.01, penetration depth will not be problematical but dissipating enough power will. In addition, the occurrence of standing wave

patterns will result in “hot spots” if the power dissipation is faster than the heat transfer to surrounding colder areas. As a rule of thumb; a standing wave pattern can occur if the characteristic dimension of the object is several half wave lengths larger than the wavelength of radiation in the material. Hot spots have been observed in poorly conductive materials (solids or highly viscous media) and at the surface of boiling liquids by IR measurements [3].

Microwave ovens can have monomode or multimode cavity (Figure 1). The monomode cavity can generate a frequency, which excites only one mode of resonance. The sample is placed on the maximum of the electrical field, as the distribution of the field is known. The multimode cavity is large and the incident wave is able to affect several modes of resonance.

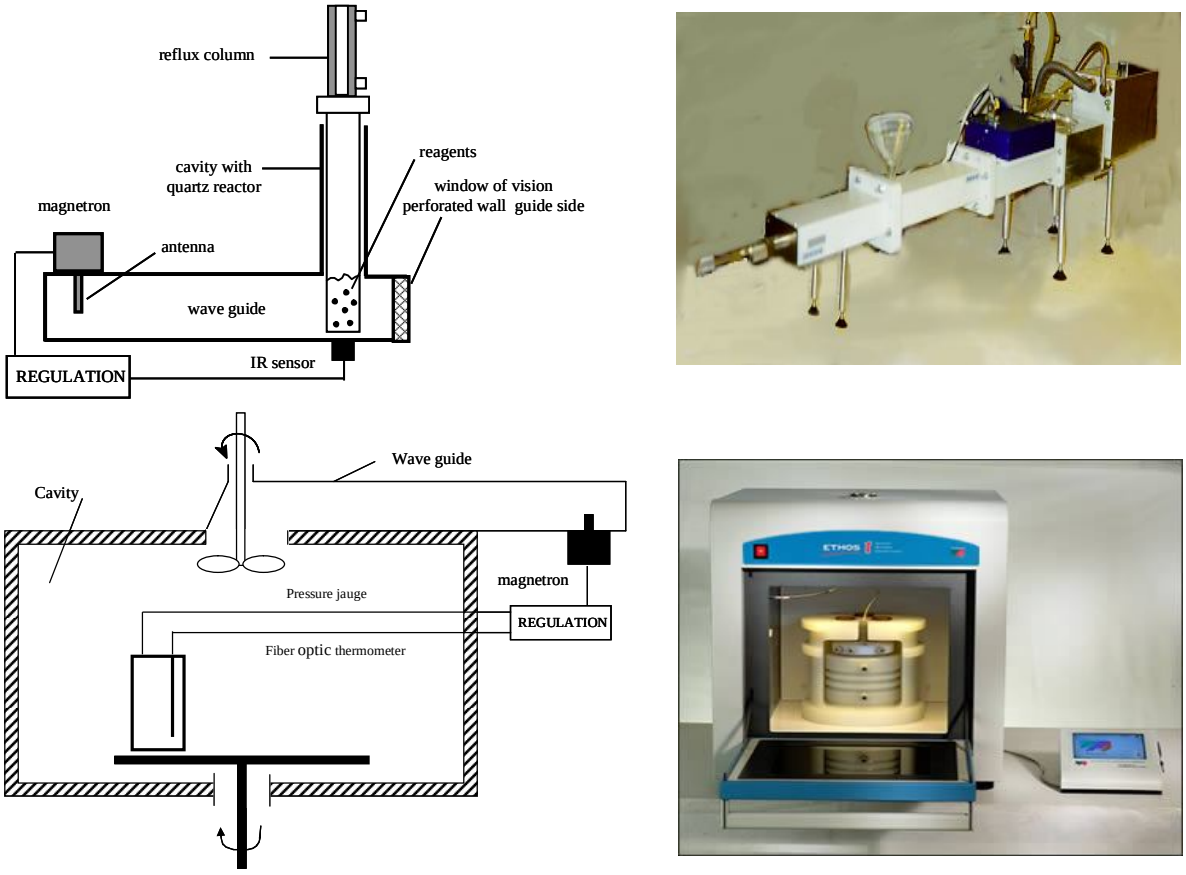

Figure 1: Monomode and multimode microwave ovens

The influence of microwave energy on chemical or biochemical reactions is strictly thermal. The microwave energy quantum is given by the usual equation $W = h \nu$. Within the frequency domain of microwaves and hyper-frequencies (300 MHz - 300 GHz), the corresponding energies are respectively $1.24\ 10^{-6}$ eV - $1.24\ 10^{-3}$ eV. These energies are much

lower than the usual ionisation energies of biological compounds (13.6 eV), of covalent bond energies like OH (5 eV), hydrogen bonds (2 eV), Van der Waals intermolecular interactions (lower than 2 eV) and even lower than the energy associated to Brownian motion at 37°C (2.7 10^{-3}eV). From this scientific point of view, direct molecular activation of microwaves should be excluded. Some kind of step by step accumulation of the energy, giving rise to a high-activated state should be totally excluded due to fast relaxation. The question and the debate of the non thermal effect of microwave give a lot of damage for the reputation of this technology and its application in industry.

I.3. MICROWAVE SEPARATION TECHNIQUES

Use of microwave energy was described for the first time in 1986 simultaneously by Gedye [4] and Giguere [5] in organic synthesis and by Ganzler [6] and Lane [7] for extraction of biological samples for analysis of organic compounds. Since then, numerous laboratories have studied the synthetic and analytical possibilities of microwaves as a nonclassical source of energy. Over 2000 and 500 articles have been published on the subject of microwave synthesis and extraction, respectively [8, 9].

In the last decade there has been an increasing demand for new extraction techniques, amenable to automation, with shortened extraction times and reduced organic solvent consumption, to prevent pollution and reduce the cost of sample preparation. Driven by these goals, advances in microwave extraction have given rise to three classes of techniques such as microwave-assisted solvent extraction (MASE), microwave-assisted distillation (MAD) and more recently microwave hydrodiffusion and gravity (MHG). Over the years, procedures based on microwave extraction have replaced some of the conventional processes and other thermal extraction techniques that have been used for decades in chemical laboratories.

I.3.1. Microwave-Assisted Solvent Extraction (MASE)

MASE consists of treating an organic solvent (extractant) in contact with the sample, dry or wet, with microwave energy. The partitioning of the analytes from the sample matrix to the extractant depends on the temperature and the nature (polarity) of the solvent.

According to the dielectric characteristics of the solvent and the sample matrix, two cases should be considered:

- the solvent absorbs all the microwave energy ; the polar solvent (ethanol, methanol, water, ..) heats up until it reaches the boiling point, diffuses into the sample matrix and solubilizes the analytes. The heat transfer in the solid matrix is made by conduction from solvent. In this case, the mechanism of extraction assisted by microwaves is not fundamentally different from that of the classical solid-liquid extraction. However, microwaves present an instantly controllable energy source and adjustable with precision.

-the second scenario consists of direct heating of a wet matrix which directly absorbs microwaves; target compounds migrate out from the matrix through the transparent non-polar, solvent (hexane, toluene...). This process has been introduced by Paré who has developed and patented a family of technologies called Microwave-Assisted Process (MAP) [10-13] for extraction of various chemical categories such as essential oils from plant material, coloring agents for the food and cosmetic industries, oil from oil-seeds, etc…. This approach is considered to support sustainable development as it requires less energy and solvent than conventional processes, while generating fewer wastes. Liquid-phase MAP extraction process is based upon the ability of a matrix to absorb microwave energy. The absorption efficiency is largely related to the moisture contents of the material; the water molecules convert the microwave energy into heat and the result is a sudden rise in temperature inside the material. According to Paré [14], when the plant cells are subjected to severe thermal stress and localized high pressures, the pressure build-up within the cells exceeds their capacity for expansion, and causes their dislocation more rapidly than in conventional extraction and leads the release of their contents in the middle of extraction.

The application of microwaves energy to the samples may be performed using two technologies: closed extract vessels under controlled pressure and temperature, and open vessels under atmospheric pressure.

Closed vessel systems are generally advised for extractions under drastic conditions such as high extraction temperature. Most available closed-vessel systems are based on multi-mode microwaves; however, the advantages of high pressure vessels combined with focused heating have led to the development of systems that combine both approaches and operating at a very high pressure and temperature. The solvent can be heated above its boiling point at atmospheric pressure, thus accelerating the mass transfer of target compounds from the sample matrix [15]. This system is schematized in Figure 2.

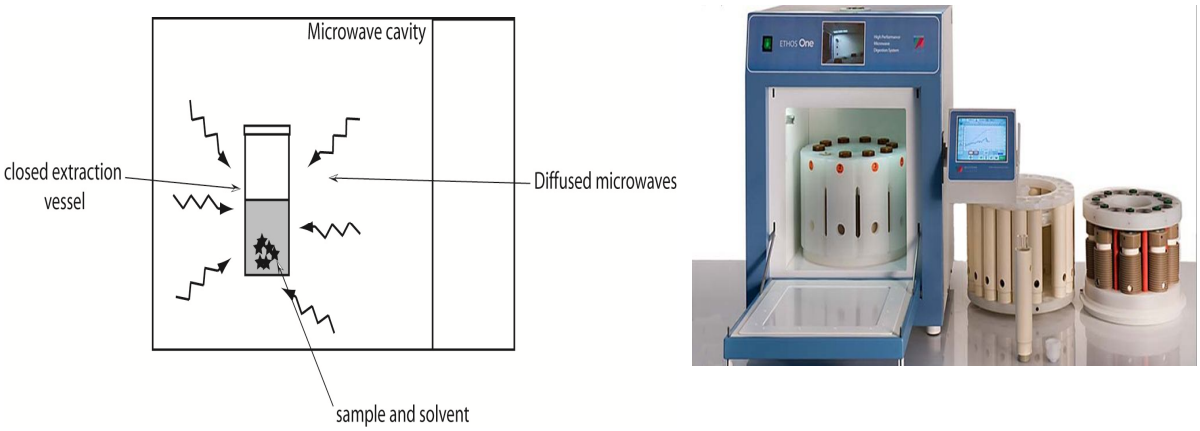

Figure 2: Microwave assisted solvent extraction

In the so-called open systems, extractions proceed under atmospheric pressure. As a consequence, the maximum possible temperature is determined by the boiling point of the solvent at that pressure. A number of applications have reported the use of open vessel systems with multi-mode and mono-mode microwave oven. The solvent is heated and refluxed through the sample, and in this case the microwaves are focused on the sample placed into the vessel allowing homogeneous and very efficient heating. This technique is called Focused Microwave-Assisted Solvent Extraction (FMASE) [16] (Figure 3). Compared to closed vessel extractions, open vessels offer increased safety in sample handling and, furthermore, they allow larger samples to be extracted.

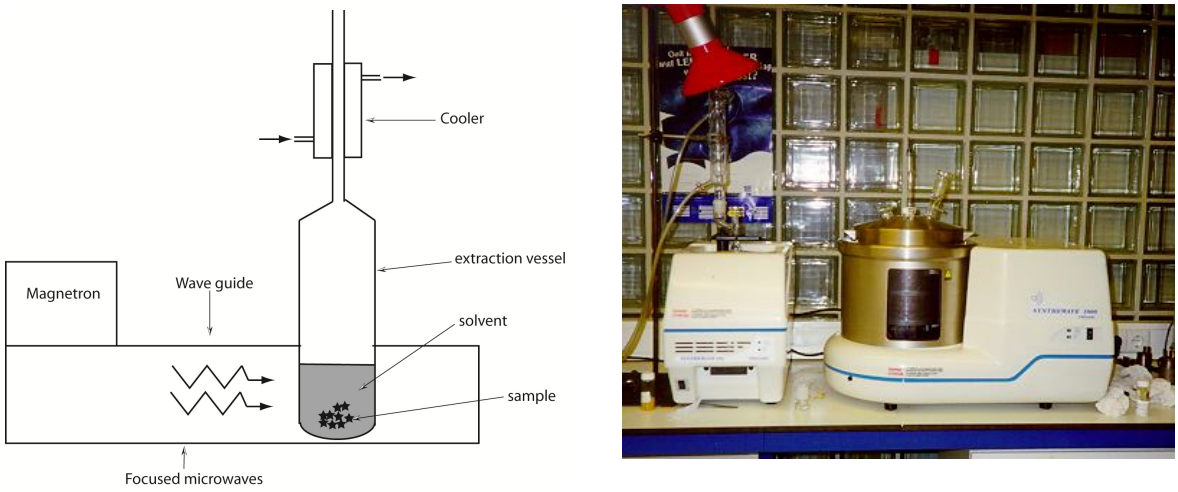

Figure 3: Focussed microwave assisted solvent extraction

MASE has been considered as a potential alternative to traditional solid-liquid extraction for the extraction of substances from natural matrices. It has been used for several reasons: (1) reduced extraction time, (2) reduced solvent usage and (3) improved extraction yield. After observing the potential of microwaves, scientists are continuously busy in

inventing new techniques with assistance of microwaves. Along with the invention of new techniques, they have also derived different extraction techniques from MASE such as: microwave-integrated Soxhlet extraction (MIS), Ultrasound and microwave assisted extraction (UMAE), dynamic microwave assisted extraction (DMAE), on-line dynamic microwave assisted solvent extraction (On-line DMASE), U-column microwave assisted solvent extraction (U-column MASE).

I.3.2. Microwave-Assisted Distillation (MAD)

MAD techniques (Figure 4) were developed for the isolation of essential oils from plant material. The drawbacks linked to the common extraction methods of essential oils have led to the development for new alternative extraction processes using microwave energy. The extraction of essential oils is currently obtained by introducing the plant material in a multi-mode microwave cavity.

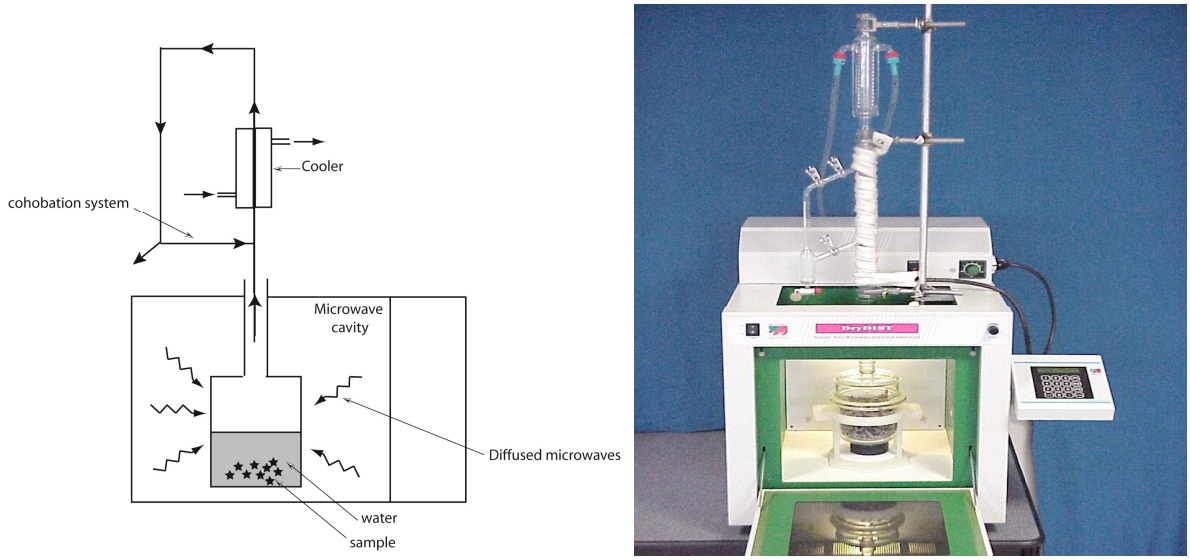

Figure 4: Microwave assisted distillation

One of the first methods using microwave-assisted extraction of essential oil was presented in 1989 [17]. The essential oil of *Lippia sidoides* was extracted using microwave energy and compressed air only. Inspired by classical steam distillation, the Compressed Air microwave distillation (CAMD) technique used compressed air instead of vapour to extract the volatile oil. Typically, plant material is placed in a reactor inside the microwave cavity and then heated. At the same time, a compressor located outside the cavity forces compressed air into the reactor. Volatile oil and vapour are then driven to the recovery flask outside the

cavity. In 5 min CAMD provides an essential oil which is qualitatively and quantitatively identical with that produced by the conventional hydrodistillation method.

Vacuum Microwave Hydrodistillation (VMHD) was elaborated and patented by Archimex [18]. This technique is based on selective heating by microwaves combined with sequential application of a vacuum. The plant material is placed in a microwave cavity with water to refresh the dry material. The plant material is then exposed to microwave radiation to release the natural extract. Working the pressures between 100 and 200 mbar enables the evaporation of the azeotropic water–volatile oil mixture from the biological matrix. The procedure is repeated in a stepwise fashion to extract all the volatile oil from the plant. Upto 30 $kg.h^{-1}$ material can be treated [18, 19].

According to the patents, VMHD provides yields comparable to those obtained by traditional hydrodistillation but with extraction times only one tenth of those required with hydrodistillation. The thermally sensitive crude notes seem to be preserved with VMHD, in contrast to conventional hydrodistillation. VMHD is suggested as an economical and efficient technique to extract high-quality natural products on a large scale [20-22].

Microwave-assisted desorption coupled to in situ headspace solid-phase microextraction (HS–SPME) was first proposed as a possible alternative pretreatment of samples collected from workplace monitoring. Therefore, pretreatment that takes a short time and uses little or no organic solvents has led to the recent development of a new extraction technique. Solid-phase micro-extraction (SPME) coupled with GC analysis has been used successfully to analyze pollutants in environmental matrices. Microwave Headspace (MHS) has been developed to achieve one-step, in situ headspace sampling of semivolatile organic compounds in aqueous samples, vegetables, and soil [23-27].

Stashenko [28, 29] used the classical technique of hydrodistillation in association with microwave energy. It has been called Microwave Hydrodistillation (MWHD). Part of the conventional equipment, in which the plant material is usually immersed in water, is placed inside the microwave cavity, whereas the cooler and the recovery system for the essential oil are situated outside the microwave cavity. Essential oils are obtained more rapidly but with yields and quality comparable with those obtained by hydrodistillation.

Solvent-free Microwave Hydrodistillation (SFME) is a recent method of extraction, patented in 2004, with the specific objective of obtaining essential oil from plant material [30, 31]. Based on a relatively simple principle, SFME involves placing the vegetable material in a microwave reactor without addition of solvent or water. SFME is a combination of microwave heating and distillation, and is performed at atmospheric pressure. In terms of quality and quantity, SFME seems to be more competitive and economic than classical methods such as hydro or steam distillation [32, 33].

I.3.3. Microwave Hydrodiffusion and Gravity (MHG)

Microwave Hydrodiffusion and Gravity (MHG) (Figure 5) [34] is a new and green technique for the extraction of essential oils. This green extraction technique is an original "upside down" microwave alembic combining microwave heating and earth gravity at atmospheric pressure. MHG was conceived for laboratory and industrial scale applications for the extraction of essential oils from different kind of aromatic plants. Based on a relatively simple principle, this method involves placing plant material in a microwave reactor, without adding any solvent or water. The internal heating of the *in situ* water within the plant material distends the plant cells and leads to the rupture of glands and oleiferous receptacles. The heating action of microwaves thus frees essential oil and *in situ* water which are transferred from the inside to the outside of the plant material.

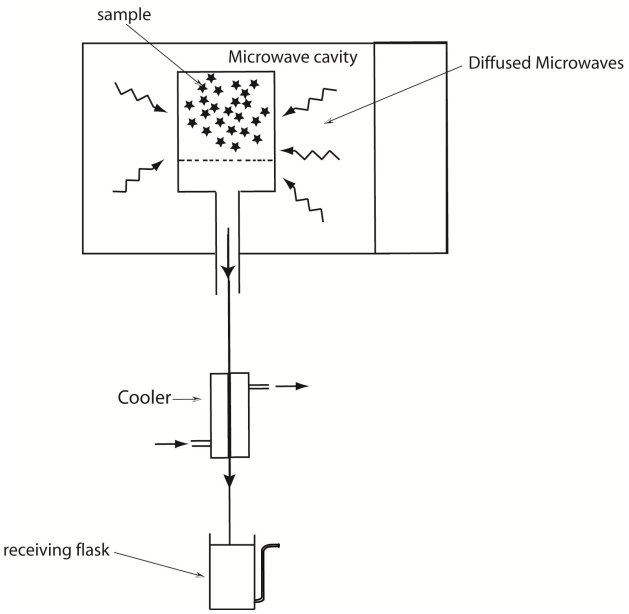

Figure 5: Microwave hydrodiffusion and gravity

This physical phenomenon, known as hydrodiffusion, allows the extract (water and essential oil), diffused outside the plant material, to drop by earth gravity out of the microwave reactor and fall through the perforated Pyrex disc. A cooling system outside the microwave oven cooled the extract continuously. Water and essential oil are collected and separated in a vessel traditionally called the “Florentine flask». The essential oil, being lighter than water, floats at the top while water goes to the bottom and can be easily separated. It is important to note that this green method allows to extract essential oils without distillation and evaporation which are the most energy consuming processes between the unit operations. MHG is neither a modified microwave-assisted extraction (MAE) which uses organic solvents nor a SFME which evaporates the essential oil with the *in situ* water or a modified hydrodistillation which uses a large quantity of water in energy consumption.

I.4. MICROWAVE-ASSISTED SEPARATION: MAIN APPLICATIONS IN GREEN CHEMISTRY

I.4.1. Flavours and fragrances

The history of flavour and fragrances is intimately interwined with that of the human race. At each stage of human development the use of flavour and fragrances has reflected society. In our early history we used aromas to protect against insects. As society became more complex so did fragrances and flavour, they also became much more spiritual. Over the course of time, and with the discovery of many thousands of species of plants, countless numbers of such flavour and fragrances have found their way through essential oils into everyday life. Essential oils produced from individual aromatic plants are never used directly. They are further formulated to make flavour and fragrances for a wide range of end uses: in foods and drinks and confectionery items; in products for personal use such as perfumes, deodorants, shampoos, bath lotions, toilet soaps, toothpastes and mouth washes; in pharmaceutical preparations where flavours are added to make the product more appealing or to mask the taste of less agreeable ones; in items used about the house or office or in industry such as air fresheners, laundry soaps, detergents, cleaning agents; the list is endless.

The chemicals responsible for the flavour or aroma are organoleptic compounds i.e., the compounds that affect the sense organs. They are present in their sources at various concentration levels. Normally, these compounds have molecular weight below 300 and are relatively volatile [35]. Natural flavour and fragrances or aromatic compounds are stored in

certain parts of plants such as in leaves: basil, bay leaf, cinnamon, common sage, eucalyptus, lemon grass, melaleuca, oregano peppermint, pine, rosemary, tea tree, thyme, winter green; berries: allspice, juniper; seeds: almond, anise, celery, cumin, nutmeg oil; bark: cassia, cinnamon, sassafras; wood: camphor, cedar, rosewood, sandalwood; rhizome: ginger; resin: frankincense, myrrh; flowers: chamomile, clary sage, clove, geranium, hyssop, jasmine, lavender, marjoram, orange, rose; root: vetyver, valerian; peel: bergamot, grapefruit, lemon, lime, orange.

Recovery of flavour and fragrances from their sources is crucial, because of their short life span. Along with having volatile nature their yield and quality also depend on the environmental conditions, genetic variability and maturation stage of plants. Conventional methods which are in continuous use for extraction of flavour and fragrances are not very useful, because of discrimination and transformation processes due to high temperatures and acidic conditions. Along this, the extraction of active constituents with these techniques is time and solvent consuming, thermally unsafe and the analysis of numerous constituents is limited [36].

With growing flavour and fragrance industry and increasing demand of more natural products, the need of novel methods in extraction became more intense. The use of microwave energy in sample treatment has attracted growing interest in the past few years, which have resulted the development of several techniques such as microwave-assisted solvent extraction (MASE), vacuum microwave hydrodistillation (VMHD), microwave hydrodistillation (MWHD), compressed air microwave distillation (CAMD), microwave headspace (MHS) and solvent free microwave hydrodistillation (SFME) [1]. Microwave assisted innovative techniques proves to be very useful in rapid yield extraction and obtaining high quality aromatic compounds from garlic, lavender flowers, orange peel and rosemary leaves as summarized in Table 1.

Lucchesi [32] have extracted essential oils by SFME from three aromatic herbs: basil, garden mint and thyme. With this technique, isolation and concentration of volatile compounds were performed in a single step, without adding any solvent or water. Essential oils extracted were richest in terms of amount of oxygenated compounds; eugenol (43.2%) in basil, carvone (64.9%) in mint and thymol (51%) in thyme as compared to conventional method. Actually higher abundance of oxygenated compounds in essential oil is related to the rapid heating of polar substances by microwaves and to the smaller amount of water used,

which prevented the decomposition of principal oxygenated constituents by thermal and hydrolytic reactions. Additionally, the SFME method also offers a reduced environmental burden as it rejects less CO_2 in atmosphere (200g CO_2 per gram of essential oil compared to traditional method which was rejecting 3600g CO_2 per gram of essential oil).

Table 1: Flavour and Fragrances

Matrix	Analyte	Technique	Operating Conditions and Remarks	References
Garlic cloves	Aroma compounds	MWHD	P.atm, 700 W, 100 mL H_2O with 3 different solvents diethyl ether, hexane and ethyl acetate 50 mL,30 min. MWHD results drastic increase in yield of vinyl dithiin isomers	[38]
Magnolia bark	Volatile compounds	MAE-HS-SPME	P.atm, 640 W, saturated NaCl 10 mL, 70°C with stirring at 1000 rpm for 30 min. 32 VOCs were detected with higher % of β-Eudesmol, best tech for more long chain alkanes	[39]
Lippia alba (Mill.) Verbenae family	Volatile secondary metabolites	MWHD	P.atm, 800 W, 1L H_2O, 30 min. Yield (0.69%), Carvone (57.2%), Limonene (29.58%). Same number of components extracted with conventional method; similar yield in less time	[29]
Lavender flowers	Essential oil	SFME	P.atm, 500 W, 200 mL H_2O, Energy required 0.13 kWh, 10 min. Yield (8.86%), Linalool (47.8%). Similar yield in less time	[40]
Orange peel	Essential oil	SFME	P.atm, 200 W, 100°C, Energy required 0.25 kWh, 30 min. Yield (0.42%) more oxygenated fraction (11.7%) versus traditional method	[37]
Aerial parts of Origanum plant	Essential oil & extract	SFME	P.atm, 850 W, 20 min. SFME oil contained higher amount of oxygenated compounds (87.4%) i.e. thymol (81.1%)	[41]
Fruits of Xylopia aromatica Lam. Plant	Volatile secondary metabolites	MWHD	P.atm, 800 W, 2L H_2O, 30 min. "Microwave Oil" richer in β-phellandrene (65%)	[28]
Basil, Garden mint, Thyme	Essential oil	SFME	P.atm, 500 W, 100°C, Energy required 0.25 kWh, 30 min. "Microwave essential oil" richer in oxygenated compounds	[32]
Spearmint and pennyroyal	Essential oil	MHG	P.atm, 500 W, 15 min. Similar chemical profile obtained in less time	[34]

P.atm (Pressure atmospheric)
MWHD (microwave hydrodistillation)
MAE-HS-SPME (microwave assisted extraction-head space-solid phase microextraction)
SFME (solvent free microwave extraction)
MHG (microwave hydrodiffusion and gravity)

Along with microwave offering advantages like; less extraction time, less solvent consumption, high efficiency, high yield and reproducibility, organoleptic properties of essential oil were also improved. Essential oil obtained by SFME from fresh orange peels were colourless and having fresh, light and sweet citrusy odour as compared to the pale yellow oil having pungent smell extracted with traditional methods [37]. These results clearly explained efficiency of microwaves in extraction step and their importance in green chemistry.

I.4.2. Antioxidants

The term antioxidant originally was referred to a chemical that prevented the consumption of oxygen. In the late 19th and early 20th century, chemists have extensively studied the antioxidants for their uses in industrial processes, such as the prevention of metal corrosion, the vulcanization of rubber, and the polymerization of fuels in the fouling of internal combustion engines. Their uses ranged from food storage to the vulcanization of rubber, but it was the identification of vitamins A, C, and E as antioxidants that led to the realization of the importance of antioxidants in biochemistry of living organisms. Later, the explanations for the effects of antioxidants on cancer susceptibility and overall health expanded rapidly with research into mechanisms, molecular targets, and molecular interactions [42].

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent, thus produce free radicals which start chain reactions. If free radicals reach high levels, oxidative stress in human body would be created, which leads to a variety of biochemical and physiological lesions and often results in metabolic impairment and cell death. On the other hand, high levels of active oxygen and free radicals could also cause lipid oxidation which led to a highly deteriorative process and unacceptable properties of foods as well as a loss in nutritional value [43]. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. Most commonly known antioxidants are: Vitamin A and Carotenoids (Carrots, squash, broccoli, sweet potatoes, tomatoes, kale, collards, cantaloupe, peaches and apricots); Vitamin C (Citrus fruits, green peppers, broccoli, green leafy vegetables, strawberries and tomatoes); Vitamin E (Nuts and seeds, whole grains, green leafy vegetables, vegetable oil and liver oil); Selenium (Fish and shellfish, red meat, grains, eggs, chicken and garlic);

Polyphenols (soy, red wine, grapes, pomegranate, cranberries, tea); Body enzymes (superoxide dismutase, catalase, glutathione peroxidase).

The presence of antioxidants is essential not only for the better human health but also for the quality, retention and safety of foods. In current years, intensive research on natural antioxidants has attained considerable attention because synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have been proved to cause one or the other health effects because of possessing certain toxicity and being responsible for carcinogenesis and liver damage.

A number of articles have publicized that plants possess potent antioxidants which act as inhibitors of lipid peroxidation and scavengers of free radicals in the form of phenolic compounds, vitamins and flavonoids [44]. Therefore, the development and isolation of natural antioxidants from natural plant have become the focus of the research on antioxidant. It is always difficult to isolate the antioxidants from plant matrix due to the co-extraction of various undesirable by-products, such as oils, waxes, pigments, etc. Therefore, cleaning and multi-step fortification of extracts usually cause the technical hitches, such as time-consumption and remarkable increase in the price of the final products. Effective extraction should be economical, safe, require less time and recover maximum possible number and amount of the antioxidants present in the plant. Different extraction techniques, such as soxhlet, supercritical fluid extraction, percolation and dispersed-solids have been employed to isolate antioxidants from the plants but none of them can be succeeded in achieving this purpose.

Recently microwave-assisted extraction (MAE) has been used as an alternative laboratory scale extraction method, which proved to be significantly faster. MAE also requires less solvent and offer higher recoveries in comparison to conventional extraction methods [45] as explained in Table 2.

Hemwimon [46] have made a comparison between the microwave-assisted extraction (MAE) of antioxidative anthraquinones from the roots of Morinda citrifolia and extraction with other conventional techniques (maceration and Soxhlet) and ultrasound-assisted extraction (UAE). The efficiency of extraction using MAE (720W, 15min, 60°C, 10ml EtOH:H_2O (80:20)) was found higher than maceration (3 days, 25°C, 10ml EtOH), UAE (15.7W, 60min, 60°C, 10ml EtOH) and was also comparable with that of Soxhlet (4h, boiling

point, 200ml EtOH). The main reason behind the higher anthraquinones recovery with MAE was the dipole rotation of the polar solvent in the microwave field.

Table 2: Antioxidant Extracts

Matrix	Analyte	Technique	Operating Conditions and Remarks	References
Sweet grass leaves	Polyphenols	MAE	P.atm, 200 W, 30 mL acetone, 10:1*, 80°C, 15 min. Best recoveries of antioxidants; 5,8-dihydroxycoumarin (0.42%) and 5-hydroxy-8-*o*-β -D-glucopyranosyl-benzopyranone (0.11%) obtained during one-step extraction	[45]
Green tea leaves	Polyphenols	MAE	P.atm, 700 W, 100 mL ethanol (50%), 20:1*, 4 min. Yield 29.59% polyphenols in very short time	[47]
Noni plant roots	Anthraquinones	MAE	P.atm, 720 W, 60°C, 10 mL ethanol (80%), 100:1*, 15 min. Higher yield (95.91%) obtained with higher antioxidant activity	[46]
Sea buckthorn (seeds, leaves, pulp and fruits)	Phenolic constituents	MAE	P.atm, 150 W, 60°C, 50 mL ethanol, 10:1*, 20 min. Total phenolic content varies from 9.3 to 23.5 mg of GAE/g, with highest amount of Rutin compound (365µg/g)	[44]
Longan peel	Phenolic compounds	MAE	P.atm, 500 W, 80°C, 50 mL ethanol (95%), 10:1*, 30 min. Microwave extract possess abundant phenolic content (96.78 mg/g); excellent scavenging ability comparing to synthetic antioxidant BHT.	[43]
Platycladus orientalis (Book-leaf Pine)	Flavonoids	DMAE	P.atm, 80 W, 5 mL methanol (80%), 500:1*, 5 min. Yield of flavonoids was found 1.72% by DMAE coupled with on-line derivatization and UV-vis detection in closed and automated system, very short time and little solvent quantity required	[48]
Plants of Labiatae, Verbenaceae and Styracaceae	Total Phenolic compounds	MAE	P.atm, 750 W, 20 mL acetone (60%), 20:1*, 4 min. Higher yield (23.8mg of gallic acid/g) of TPC found in *Rosmarinus officinalis*	[49]
Dried roots of *Rhodiola* L. family	Salidroside and tyrosol	MAE	P.atm, 400 W, 5 mL methanol (50%), 5:1*, 5 min.Good recoveries (94.4 to 123%) of salidroside and tyrosol obtained from *Rhodiola* L. samples from five different growing areas	[50]
Grape skin and seeds	Phenolic compounds	MAE	P.(1-10 atm), 500 W, 65-140°C, 20 mL methanol (100%), 20 min. Flavanols was mostly found in skin but absent in grape seeds; catechin was abundant in seeds	[51]
Olive leaves	Oleuropein and related biophenols	MAE	P.atm, 200 W, 8 mL ethanol (80%), 8:1*, 8 min. The main compounds ranged from 631 (verbacoside) to 23200mg/kg (oleuropein)	[52]

*Solvent to material ratio (mL/g); DMAE (dynamic microwave assisted extraction)

The extract from MAE had higher antioxidant activity than those of UAE and maceration. The exposure of the maceration extract to unfavourable conditions like light and oxygen had caused its lower activity due to long extraction time. On the other hand, UAE did not require long extraction time but due to formation of free radicals by ultrasonication has caused the oxidation and degradation of anthraquinones. Similarly, the best recoveries of antioxidants in short time and with less polar solvent consumption were also observed from sweet grass by Grigonis [45]. Along with obtaining higher yield, Pan [43] has extracted phenolic compounds with admirable scavenging ability comparing to synthetic antioxidants from longan peel with MAE. These publications clearly reveal the potential of microwaves in the field of extraction of different vital compounds from green plants.

I.4.3. Fats and oils

Fats and oils are an important part of any well designed dietary plan. They directly impact on our health in profound ways, as much or perhaps more than any other single dietary component. The distinction between a fat and an oil is purely an accidental one depending upon the environment in which the substance happens to be placed. If the substance is solid at ordinary temperatures, it is termed as fat; if fluid, an oil. This is merely a distinction of convenience, since all oils are solidified at lower temperatures and all fats melted at higher temperatures. Plant lipids comprise a complex mixture of monoglycerides, diglycerides, triglycerides and free fatty acids associated with some minor constituents, such as squalane, tocopherols, sterols, phosphatides, alkaloids, flavonoids, waxy materials, pigments and volatiles that provide the taste and odour of the oils. Naturally occurring fats and oils are essentially triglycerides. Triglycerides consist of three fatty acids bound to a glycerol backbone. The length of the fatty acid chain as well as its configuration and relative degree of saturation determine how the fatty acid will act within our body. All fats and oils have three types of fatty acids:

1. Saturated: Mostly found in animal sources such as milk, cream, butter, cheese, meat, poultry and also in coconut and palm oil. The amount of saturated fat taken is directly related to cholesterol levels in blood and appears to raise low density lipoprotein (LDL) or bad cholesterol.
2. Monounsaturated: These are considered as best type of fat, found in plant sources such as olives, avocadoes, canola and peanut oils. Most margarines and

hydrogenated vegetable oils are highly monounsaturated and also seem to lower LDL level and raise high density lipoprotein (HDL) levels.

3. Polyunsaturated: They are found in vegetable oils and fish, appear to aid lower cholesterol level [53].

The function of fats and oils in the diet is mainly to furnish energy to operate the animal machine. It also stored in the body as a reserve, in adipose tissues under skin against the risk of a future period of food shortage. Fats have a number of important functions in the body. As well as being a concentrated source of energy, fats act as carriers for fat-soluble vitamins A, D, E and K. Fats are also essential for maintaining the integrity and fluidity of cell membranes and are also precursors of many hormones. In addition to edible oils, the plant and animal lipids find a number of important applications in foods, pharmaceuticals and cosmetics. The requirement of pharmaceutical industry in case of lipids is polyunsaturated fatty acids with high grade of purity, while the food industry requires triglycerides with certain degree of unsaturation. In recent years there has been a growing interest in oils enriched with different omega-3 polyunsaturated fatty acids such as alpha-linolenic acid, docosaheanoic acid (DHA; 22:6, n-3) and eicosapentanoic (EPA; 20:5, n-3)) acid for human nutrition and long term health benefits. Because they are beneficial: in the treatment of many inflammatory diseases like asthma, cancer, arthritis and atherosclerosis, in prevention of excessive blood clotting and in reducing the risk of becoming obese. The recovery of Omega three fatty acids is highly beneficial from its natural sources like salmon, flax seeds, walnuts, marine fish, algae and seaweeds [54].

The interest in dietary fat is a growing trend of today, causes increase in consumer demand to reduce total fat contents in food in order to improve human health. This is the main cause which increases the use of more precise methods for fat extraction. Vegetable and cereal fats and oils are mostly found in greatest abundance in fruits and seeds. While in roots, stalks, branches and leaves they are rarely present in quantities large enough for commercial purposes. Seeds are very hard and complex matrix due to its characteristics such as particle size and less moisture content and these characteristics offer hazards in extraction of lipid content. Although most of the lipids they contain are readily extractable with some solvents, but the remaining lipid material strongly bound to the matrix and exhaustive treatments are required to segregate the lasting small parts [55]. For example, Elkhori [56] has described microwave-assisted extraction (MAE) of cocoa powder with hexane/isopropanol, resulted

rapid determination of fat contents with recoveries similar to or better than conventional method with added advantages of low consumption of solvent, short extraction time, low energy consumption and excellent reproducibility.

Table 3: Fat and Oil Extraction

Matrix	Analyte	Technique	Operating Conditions and Remarks	References
Olive seeds	Fat content	FMASE	P.atm, 80 W, 100 mL n-hexane, 7 cycles, 20-25 min. No significant difference in yield ; Substantial shortening of extraction time	[55]
Olive seeds	Oil	MIS	P.atm, 720 W, 300 mL n-hexane, Energy required 0.5 kwh, 32 min. Microwaves used as only heating source; prevents pollution through 90% solvent recovery	[59]
Oleaginous seeds	Oil	FMASE	P.atm, 150 W, 100 mL n-hexane, 65cycles, 180 min.Very similar amounts of saturated and unsaturated fatty acids obtained; with less solvent consumption in lesser time	[60]
Cocoa powder (CP) and nibs (CN)	Fat content	MAP	P.atm, CP: 250 W, 1 mL H_20, 30 mL n-hexane and then 10 mL isopropanol, 7.6 min. CN: 250 W, 10 mL HCl (50%) and then 90 mL petroleum ether, 13 min. Complete fat extraction obtained in few minutes	[56]
Soybean germ and seaweed	Oil	UMAE	P.atm, US (21 kHz, 50 W)/ MW (100 W), 40 mL n-hexane, 45°C, 60 min. Yield: soybean germ (14.1%) and soybeanweed (24%); extraction time reduced up to 10-fold with 50-500% increase in yield	[61]
Bakery products	Fat content	FMASE	P.atm, 100 W, 125 mL n-hexane, 12 cycles, 60 min for cookies and 35 min for snacks. Comparable extraction efficiencies and precision; drastic reduction in time	[62]
Holm Oak acorns	Oil	FMASE	P.atm, 300 W, 100 mL n-hexane, 15 cycles, 30 min. *trans* fatty acid-free oil obtained due to less exposure to drastic conditions	[57]
Rose hip seeds	Oil	MASE	P.atm, 300 W, 35 mL n-hexane, 40°C, 30 min. Favourable fatty acid composition found for palmic, oleic and linolenic acids; higher concentration of most elements obtained	[63]
Oleaginous seeds	Oil	MASE	P.atm, 90 W, 50 mL tert-butyl methyl ether, 60 min. Highest amount of free fatty acids obtained with triglycerids as major class	[64]
Olive seeds	Oil	MIS	P.atm, 720 W, 300 mL d-limonene, 32 min. No significant difference in yield; Nice bio-solvent alternative to petroleum solvents obtained	[58]

UMAE (ultrasound microwave-assisted extraction)
MASE (microwave-assisted solvent extraction)
MIS (microwave-integrated soxhlet extraction)

Another growing trend in the development of new extraction process is to combine traditional and novel techniques as described in Table 3. Focused microwave-assisted Soxhlet extraction was used by Pérez-Serradilla [57] for acorn oil determination, which resulted *trans* fatty acid-free oil, probably because of less exposure of drastic conditions in 30 min, which is much less than the time required by Soxhlet (8 h) and stirring (56 h) reference methods. One of the common drawbacks of all these techniques is addition of environmental pollution due to the use of traditional petroleum solvent n-hexane. But this problem also has been solved with the development of a new technique microwave-integrated Soxhlet (MIS) extraction using d-limonene a bio-solvent obtained from lime peel, a new alternative of petroleum solvents. This technique along with reduced environmental burden also gave best yield, in less time, with more solvent recovery [58].

I.4.4. Natural food colours

Man has always been interested in colours, the art of dyeing has a long past. Primitive men used plant dyestuff for colouring himself and also the skin of his animals at the moment of different occasions like religious festivals as well as during wars. They believed that the colour would protect them, give them magical powers, and help them to achieve victory in war. And now, the use of colours has become so much a part of mans customs that it is difficult to imagine a modern world without colours. The art of dyeing spread widely as civilization advanced. Plants are dominated as sources of natural dyes, mainly all natural colorants and dyes came from plant. Almost all parts of the plants like root, bark, leaf, fruit, wood, seed, flower, etc. produce colours. Green in most leaves is surely the most ever-present plant colour. The green pigment chlorophyll in leaves, basically use for conversion of the energy from the sun into a usable chemical energy. Different colours in flowers help the plants in attracting insects and other animals which in turn play key role in plants pollination and finally reproduction. Along with performing these servings for plants, these natural colours also used to impart colour to an infinite variety of materials like textiles, leather, paper, wood varnishes, ink, fur, foodstuff, cosmetics, medicine, toothpaste, etc.

These natural colours are principally due to the occurrence of the one or more of the groups of colour compounds, such as carotenoids (impart yellow, orange and red colours in annatto, carrots, oranges, prawns, red peppers, saffron, tomatoes, palm fruit), anthocyanins (impart red, purple and blue colours in black grapes, cherries, red cabbage, strawberries), betanin (impart pink colour in beetroot), chlorophyll (impart green colour in alfalfa grass,

parsley, spinach), curcumin (impart yellow colour in turmeric), and other flavonoids which imparts yellow, red, blue and purple colours [65]. As far as the chemistry of colouring pigments is concerned, a pigment molecule has two principal chemical groups, chromophores and auxochromes. The chromophore is usually an aromatic ring having unsaturated bonds and is associated with the colouring property. The number of unsaturated bonds decides the intensity of the colour. The second group is auxochrome which helps the pigment molecule to combine with the substrate, thus imparting colour [66].

Colour additives have long been used as a means of enhancing the aesthetic value of foods. Commonly the food colorants are used in beverages, dairy products powders, jellies, confections, condiments, icings, syrups, baked goods, snack foods, puddings, sherbets, gelatines, custards, dessert powders etc. Due to the large usage area and increasing market demand, most colourings used today are artificial, that is made mostly from petrochemical coal-tar dyes. The introduction of synthetic dyes caused rapid decline in the use of natural dyes, which were completely replaced by the artificial dyes within a century. However, artificial colours are suspected to release harmful chemicals that are allergic, carcinogenic and harmful to human health. While, natural dyes and colorants along with giving harmonizing, soft, gentle, calm and restful effect are non-poisonous, non-carcinogenic, less toxic, less polluting, less health hazardous. Above all they are environmentally friendly and can be recycled after use. Although natural colours have several advantages but they are not commercially succeed like synthetic dyes, because of some problems like difficulty in the collection of plants, lack of consistency, lack of availability of precise practical knowledge of extraction.

Microwave energy has become one of the modern technologies to overcome the limitation of natural colours as explained in Table 4 with different examples. Lycopene has been used as natural food colorant for many years but the conventional methods which were in regular use for its extraction, mostly lead to accelerate the oxidative decomposition of lycopene. Lianfu and Zelong [67] have described the extraction of lycopene from tomatoes with combined innovative techniques ultrasound and microwave assisted extraction (UMAE) in comparison to ultrasound assisted extraction (UAE). The results obtained with UAE did not increase the lycopene yield (89.4%) along with enhancement of extraction efficiency in shorter time. The reason behind this was production of hydroxyl radicals by acoustic cavitation of ultrasound in extracts, due to the presence of small amount of water in extract,

resulted in decomposition of lycopene. But the yield (97.4%) was increased when extraction was performed with UMAE, with the assistances of acoustic cavitation and fast heating of microwaves, in shorter time and with less solvent consumption. Similarly, higher yield of anthocyanins in red raspberries was also observed by Sun [68] with optimal conditions of

Table 4: Natural Food Colours Extraction

Matrix	Analyte	Technique	Operating Conditions and Remarks	References
Tomato paste	Lycopene	UMAE	P.atm, US (40 kHz, 50 W)/ MW (98 W), 21.2 mL ethyl acetate, 10.6:1*, 6.1 min. 97.4% yield of lycopene proved it to be a more efficient & attractive extraction method	[67]
Dried rhizomes of *Curcuma longa* L., Turmeric	Curcumin	MASE	P.atm, 140 W, 8 mL of methanol (as modifier) followed by 40 mL acetone, 20:1*, 4 min. Dual heating phenomenon of solvent & matrix resulted 27% more efficient extraction	[70]
Safflower *Flos carthami*	Safflower yellow	On-line DMASE	P.atm, 60 W, 4 mL methanol (60%), 1.33 mL/mg*, solvent flow rate 1.0 mL/min, 4 min. 11.35% yield of safflower yellow obtained; extraction can easily be monitored	[69]
Red Raspberries	Anthocyanins	MASE	P.atm, 366 W, 240 mL 1.5 M HCl – 95% ethanol (15:85), 4:1*, 12 min. 98.33% yield was obtained; 12 kinds of Anthocyanins extracted without any destruction of its chemical structure	[68]
Paprika	Carotenoids	MASE	P.atm, 50 W, 60°C, 30 solvent mixtures; acetone, dioxane, ethanol, methanol, tetra-hydrofuran (15%, 30%, 45%, 60%, 75% and 90%), 2 min. Extraction efficiency increases with organic solvents	[71]
Cape Jasmine	Yellow pigment	U-coloumn MASE	60°C, 700 W, 70-95°C, 30.7 mL H_2O, 1.6 min. 50% higher yield obtained in comparison to conventional method	[72]
Rabdosia serra ‘Maxim.) Hara	Yellow pigment	MASE	P.atm, 464 W, 120 mL alcohol (95%), 60:1*, 5.8 min. Extraction rate increases upto 90.6% in lesser time	[73]
Rubiaceae plants	Alizarin and purpurin	MASE	P.atm, 600 W, 120°C, 20 mL methanol (30%), 15:1*, 20 min. Relative recoveries was higher than 140%; 14 samples extracted simultaneously	[74]

microwave assisted extraction. MAE proved to be more rapid and efficient as it extracts different kinds of anthocyanins without any destruction of chemical structure in very short time, due to intensive disruption of tissue structure under microwave irradiation. Along with resolving problems related to higher yield and extraction in minimum time microwave

assisted extraction has also solved the problem of automation as described by Chen [69]. These authors have extracted Safflower yellow from *Flos Carthami* by using dynamic microwave assisted extraction coupled with on-line detection by spectrophotometery. This method along with providing rapid measurements also offers convenience for obtaining continuous measurements.

I.4.5. Miscellaneous

Nutrient sufficiency is the basis of good health and nutrient availability to people is primarily determined by the output of foods produced by different green plants. Nature provides us with all the essential nutrients for human growth, development and good health. Along with providing these essential nutrients, green plants also become cure for many diseases, because of having a number of active ingredients which possess anti-oxidants, anti-malarial, anti-fungal, anti-microbial, anti-diarrhoea, anti-diabetic, anti-cancer properties. These active ingredients play a key role in human medicine. Plants contain a number of broad categories of significant plant constituents. These includes alkaloids, amino acids, peptides and proteins, glycosides, acids, terpens, phenols, essential oils, carotenoids, vitamins, acids, selenium, indoles, coumarins, flavonoids, isoflavones, isothiocyanates and thiocyanates, plant sterols, saponins, allium compounds and limonene [75]. Plants typically produce these compounds in low quantities and some of these nutrients are depleted or sometime completely lost during the processes of harvesting, storage and ingestion, then it becomes absolutely essential to supplement our foods with these nutrients. Consequently, it is required that these nutrients should be close to their original form, for this it is preferable that they must be derived from natural sources, in the form of natural extracts.

Extraction of these compounds from their sources is the most critical step, because of the strong interactions normally occurring between the vegetal matrices and analytes. Depending on the extraction of desired end products a number of different extraction processes are available, such as maceration, leaching, percolation, magnetic stirring. These processes vary from simple Soxhlet extraction to complicated ultrasound-assisted extraction, extraction by means of supercritical fluids, extraction by pressurized liquids and enzymatic extraction. The main motive behind the development of these novel techniques was the increasing demand for methods that permit the extraction process to be automated, less exposure of operative personnel, consumption of solvents to be reduced and extraction time to be shortened [76].

In addition to these techniques, microwave-assisted extraction is also emerging as an attractive alternative to conventional extraction methods. Initially, it was only employed for digestion of different matrices as a heating source, now it is widely accepted in analytical laboratories and in industries for extraction at large scale. Table 5 clearly shows importance of microwaves, as extraction of different useful metabolites carried out from several vegetal matrices. Pectin is a structurally complex polysaccharide and its transfer in the extraction processes is difficult from raw material. Conventionally, pectin is extracted in acidic solution at high temperature which is normally a time consuming process and the pH, temperature and time also have effects on the yield and quality of pectin. This problem has been solved by the use of microwaves in assistance to acid leaching by Fishman [77]. They extracted pectin from flavedo, albedo and pulp of lime by microwave heating under pressure indicated that molar mass, size and intrinsic viscosity was increased as compared with pectin extracted by conventional heating techniques.

Table 5: Miscellaneous

Matrix	Analyte	Technique	Operating Conditions and Remarks	References
Rhizoma coptidis	Alkaloids	MASE	P.atm, 40 mL Oligoethylene glycol monoalkyl ether (Acidified Genapol X-080) 5%, 20:1*, 100°C, 10 min. Efficient recovery of alkaloids obtained (93.6-94.7%) due to combine extraction of microwaves and cloud-point preconcentration	[79]
Peppers	Capsaicinoids	MASE	P.atm, 500 W, 25 mL ethanol (100%), 50:1*, 125°C, 5 min. Higher amount of capsaicin (451.6 μmol/kg) and dihydrocapsaicin (265.4 μmol/kg) obtained; no degradation of compounds	[80]
Chickpea seeds	Saponins	MASE	P.atm, 300 W, 1000 mL ethanol (70%), 250:1*, 80°C, 20 min. 25 mg saponin found in each g of chickpea seed; DDMP-conjugated saponin identified as major saponin with antifungal properties	[81]
Ginseng	Saponins	MASE	P.atm, 300 W, 50 mL methanol (80%), 10:1*, 72.2°C, 2 min. More yield of saponins obtained in few seconds without any degradation	[82]
Ganoderma atrum	Triterpenoid saponins	MASE	P.atm, 800 W, 75 mL ethanol (95%), 25:1*, 90°C, 5 min. Highest yield (0.968%) obtained in lesser time; with less intensive labour; large scale efficient extraction possible	[83]
Lime flavedo, albedo and pulp	Pectin	MASE	P. 3.40atm, 630 W, 25 mL of pH 2 HCl, 25:1*, 140°C, 3 min. Pectin molecules exist in networks, microwave irradiations resulted feasible extraction	[77]
Apple pomace	Pectin	MASE	P.atm, 499.4 W, 29 mL of pH 1.01 HCl, 14.5:1*, 20.8 min. 0.315 g pectin content obtained in far less time than that of conventional method	[78]

Similarly, optimization of microwave-assisted extraction of pectin from apple pomace by response surface methodology was reported by Wang [78]. Like pectin alkaloids are also active ingredients which reveal a great variety of biological and pharmacological activities. However, the separation of these alkaloids from the herb becomes a problem due to the extensive use of organic solvents in traditional extraction techniques. To overcome this problem Sun [79] offers a novel technique which is fast, simple and free of organic solvents. They employed non-ionic surfactant oilgoethylene glycol monoalkyl ether (Genapol X-080) as an alternative solvent for microwave-assisted extraction of alkaloids from Rhizoma Coptidis. The use of acidified Genapol X-080 enhanced the recovery of alkaloids upto 92.8% in one step. Similarly, Barbero [80] have also reported the highest reproducibility (6%) of capsaicinoids in peppers with microwave-assisted extraction, under optimised extraction conditions in order to avoid possible thermal degradation.

I.5. MICROWAVE-ASSISTED SEPARATIONS: IMPORTANT PARAMETERS AND MECHANISM

In conventional separations (Figure 6), mass transfer occurs from the inside to the outside while heat transfer occurs from the outside to the inside. For microwave separations, the two transport phenomena are in the same direction from the inside of the extracted material to the bulk solvent. The acceleration of extraction rates under microwaves could be due to a synergy combination of the two transfer phenomena mass and heat acting in the same direction. In Microwave assisted-separations, heat is dissipated volumetrically inside the irradiated medium, while in conventional separations; heat is transferred from the heating medium to the interior of the sample. Microwaves are volumetrically distributed, and heat transfers occur from the sample to the colder environment. This causes an important difference between conventional and microwave heating. In conventional heating, heat transfer depends on thermal conductivity, on the temperature difference across the sample, and for fluids, on convection currents. As a result, the temperature increase is often rather slow. By contrast, in microwave heating, due to the volumetric heating effect, much faster temperature increases can be obtained, depending on the microwave power and the dielectric loss factor of the material being irradiated.

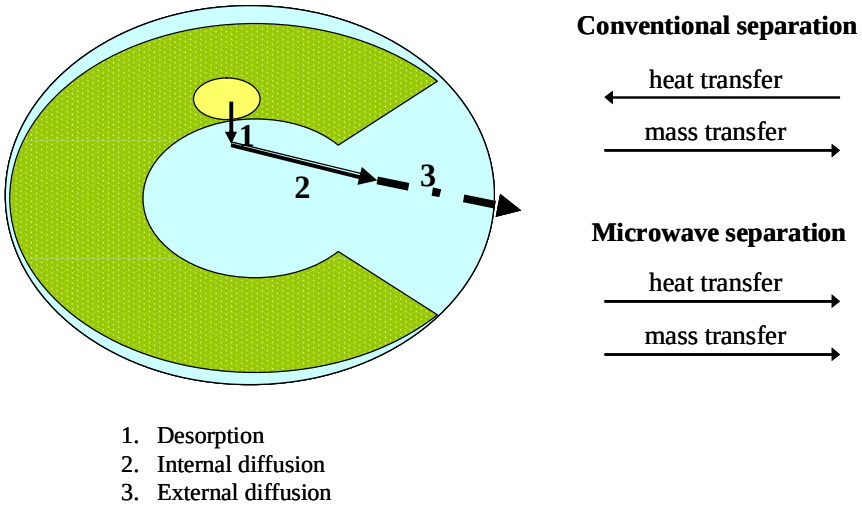

Figure 6: Mechanism of microwave assisted separation of natural products

Moisture content of the plant material, microwave power and extraction time affects not only the yield of secondary metabolites but also its composition. The sample moisture content under a microwave treatment is critical, since water is an excellent absorber of microwave energy. This strong absorption provides the increase of the temperature inside the sample leading to the rupture of the cells by the *in situ* water. The required microwave input power is directly related to the sample size and weight. The power must be sufficient to reach the boiling point of the water (distillation) or solvent (extraction) which fixes the separation temperature. However, the power should not be too high otherwise loss of secondary metabolites compounds would result. Extraction time is the major factor directly affecting the yield of using microwaves. As time increases, the yield increases almost linearly. Finally, the extraction time must be optimized to maximize the yield of the extraction without affecting the extracts quality. Moreover, the separation time with microwaves must be much lower than conventional separations to be economically and environmentally viable.

I.6. MICROWAVE-ASSISTED SEPARATIONS: SAFETY, ENERGY AND ENVIRONMENTAL IMPACT

Microwave solvent extraction process is simple and can be readily understood in terms of the operating steps to be performed. However, the application of microwave energy can pose serious hazards in inexperienced hands. A high level of safety and attention to details when planning and performing experiments must be used by people using microwave ovens. They have to ensure that they seek proper information from knowledgeable sources and that

they do not attempt to implement this type of energy unless proper guidance is provided. Only approved equipment and scientifically sound procedures should be used.

Microwaves are proposed as a “green” separation method suitable for secondary and primary metabolites contained in food and natural products. The reduced cost of separation is clearly advantageous for the microwave method in terms of energy, solvent used and time. Conventional procedure required an extraction time of 8 h. The microwave method generally required heating for 30 min only. The energy required to perform the two extraction methods are respectively 8 kWh for conventional Soxhlet (electrical energy for heating and evaporating) and 0.5 kWh for MIS (electrical energy for microwave supply). The power consumption has been determined with a Wattmeter at the microwave generator entrance and the electrical heater power supply. Regarding environmental impact, the calculated quantity of carbon dioxide emissions to atmosphere is higher in the case of conventional extraction (600 g CO_2/ g product) than for microwave extraction (40 g CO_2/ g of product). These calculations have been made according to the literature: to obtain 1 kWh from coal or fuel, 800 g of CO_2 will be emitted to atmosphere during combustion of fossil fuel. Further experiments were made with the aim of measuring the ability of each technique to allow solvent recovery. As microwave technology allowed a recovery of almost 90% of solvent used, more than 50% of solvent were lost during conventional investigation, namely, Soxhlet extraction followed by vacuum rotary evaporator. It is estimated that in the world, 100 000 000 liters of solvent are used per year by analytical laboratory and academia. Microwave assisted-separation appears as a green process extraction saving energy and limiting losses of solvent, which is at the moment, a key challenge for the planet.

I.7. CONCLUSION AND FUTURE TRENDS

A shrewd glance on the threats of coming scenario, insist on the intense need of combining green chemistry with more environment friendly technologies. The two main issues of water and environmental pollution are directly related to the intensive use of volatile organic solvents and higher energy inputs. Use of microwave technology for extraction of biologically active compounds is the great success of modern chemistry. The main advantage of microwave assisted extraction resides in the performance of heating source. The high temperatures achieved by microwave heating dramatically reduce both the extraction time and the volume of solvent required, which automatically help in lowering environmental burden by rejecting less CO_2 in the atmosphere. Microwave extraction technology has received

increasing attention in last decade as an alternative method due to efficient extraction of several compounds from variety of matrices, with more yields, less degradation and higher purity. Until now, the role of microwave in extraction has not reached upto the level of maturity in comparison to other conventional methods. A wide range of natural compounds are still continuously extracted with these conventional methods. Detailed study of literature has proved the usefulness of microwaves in extraction technology and its potential for developing large scale commercial extraction and its capability in assisting to minimise water usage with reduce cost of waste water treatment and finally helping in creation of clean and fresh environment.

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Chapter II

Design, optimisation and implementation of a new extraction method: Microwave hydrodiffusion and gravity

♠ *Clean recovery of antioxidant flavonoids from onions: Optimising solvent free microwave extraction method*
Journal of Chromatography A 1216 (2009) 7700-7707.

♠ *A remarkable influence of microwave extraction: Enhancement of antioxidant activity of extracted onion Varieties*
(Submitted to Food Chemistry)

♠ *Solvent free microwave assisted extraction of antioxidants from sea buckthorn (Hippophae rhamnoides) food by-products*

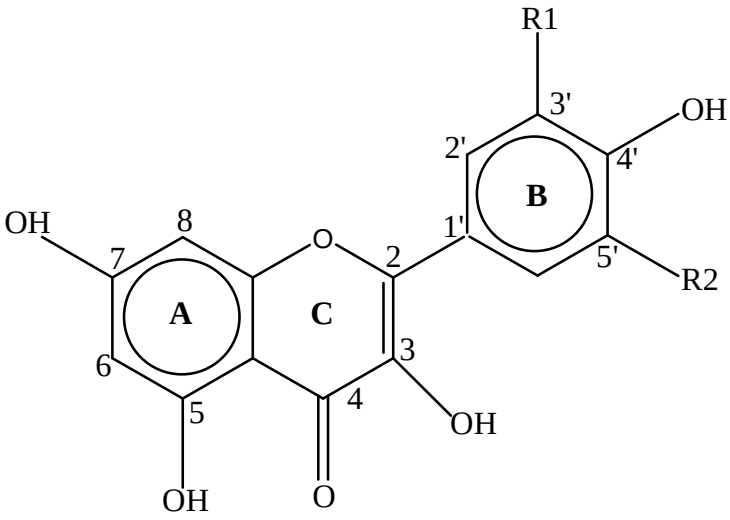
(Accepted) Journal of food and bioprocess technology

II.1. INTRODUCTION

Human biological system is vulnerable to the attack of extremely reactive oxygen species (ROS), which are produced continuously as a result of endogenous enzymatic reactions and also by exogenous sources [1, 2]. The formation and activity of these ROS are believed to be responsible for degenerative diseases and their associated complications like cancers, cardiovascular diseases and accelerated aging of organisms [3]. Increased consumption of diets rich in fruits and vegetables are associated with low prevalence of degenerative diseases as they provide a great amount of antioxidant phytochemicals and literature proved antioxidants as one of the defence mechanisms within the organism against ROS [4]. These phenolic antioxidants act as free radical scavengers and offer protection against cellular damage by retarding oxidative stress. Among vegetable polyphenols the flavonoids group generally dominate and found relatively in higher concentration as sugar conjugates as studied by Miean and Mohamed [5] in 62 edible plants.

In many in vitro experiments, dietary flavonoids having a catechol (1,2-dihydroxybenzene) group have been shown to inhibit oxidation of biomolecules by: (i) acting as free radical scavengers via donation of hydrogen atoms or electrons (ii) binding proteins and enzymes involved in the generation of reactive oxygen species (ROS) (iii) complexing transition metal ions able to catalyse ROS formation by redox cycling and (iv) regenerating potent endogenous antioxidants such as α-tocopherol [6]. Moreover, uncontrolled oxidation of biomolecules (e.g., nonenzymatic lipid peroxidation) has been reported to take place during cell degeneration, tumour promotion, coronary heart diseases and some forms of cancer [7, 8]. Importantly, it is now increasingly recognized that flavonoids may exert favourable health effect by regulating the expression of genes involved in the inflammatory response, the metabolism of carcinogens and the antioxidant defence [9].

Onion (*Allium cepa* L.) a versatile vegetable of Allium family is appreciated worldwide not just for its distinctive taste and flavour but also as a significant source of many beneficial compounds. Several studies revealed the presence of various dietary flavonoids in different varieties of onions along with other bioactive compounds [10]. Quercetin is the most common flavonol aglycone. It is present (usually as glycosides) in a wide range of fruits and vegetables and particularly abundant in onion, which is a major source of antioxidants (sulphur compounds, flavonoids) [11–13]. Two major groups of flavonoids found in onions are; anthocyanins (cyanidin and peonidin glycosides) [14] and flavonols (quercetin, isorhamnetin, kaempferol and their glycosides) [15] Figure 7. The most abundant flavonols in



Myricetin R1=R2=OH (3,3',4',5',5,7)

Quercetin R1=OH, R2=H ((3,3',4',5,7)

Kaempferol R1=H, R2=H (3,4',5,7)

Figure 7: Structure of the flavonol molecules

onions are quercetin 4'-O-β-D-glucoside and quercetin 3,4'-O-β-D-diglucoside, which account for more than 85% of the total flavonoid content [12, 16]. Moreover, red onions are richer in flavonols than yellow, and pink onions [17]. Red onions also display anthocyanins, which not only impart red colour but also participate in their strong antioxidant activity. In contrast to red-skinned onion, white onions contain only trace levels of flavonols [18]. Quercetin is usually found in higher concentration in the outer dry skin, where it exists mostly in the aglycone form [19] and imparts a yellow or brown colour. An antifungal component, 3,4-dihydroxybenzoic acid was shown to be produced in the outer dry brown skin of onions by hydrolysis of quercetin glucosides and subsequent oxidation of the aglycone [20]. In the edible portion of onions, quercetin glucosides largely prevail and the sugar moieties strongly lower the antioxidant capacity of the aglycone due to the conjugation of the OH groups at C3 and C4′, which are critical to the H-donating activity. While considering the usefulness of antioxidants against cardiovascular disease and colorectal cancers, it's necessary to examine their extraction processes from different vegetables for obtaining maximum health effects.

Efficiency of extraction process and mass of release components depend on degree of vegetal cell disintegration which have been achieved previously by conventional solid-liquid extraction, with assistance of processes like heating, boiling, pressing, blending, maceration and mechanical fragmentation of plant material [18, 20]. Quercetin is typically obtained by acid hydrolysis of quercetin glycosides extracted from onion tissues by conventional solvent extraction method. Leaching or organic solvent extraction is the most extensively used process for obtaining plant phenolic components from many decades. Current literature also reports extraction by steam distillation from onion sprouts [21] and Soxhlet extraction of

onion peel [3]. Phenolic extracts from onion and lettuce are purified and concentrated by using ion-exchange resins [22, 23]. Overall, using these conventional methods may result in the degradation of some chemically sensitive phenols due to intensive mechanical disruption. In addition, the involvement of long extraction periods, severe heating conditions and extensive use of organic solvents favour the release of oxidative enzymes that promote degradation.

More efficient and automated methods have been developed to cope with the industrial demand for natural antioxidants. Indeed, the food, beverage and cosmetic industries are now eager to replace potentially hazardous synthetic antioxidants by natural ones, which are better accepted by consumers. The use of supercritical fluid extraction (SFE) [24] and pressurized liquid extraction (PLE) [25] as upcoming extraction techniques has been reported for the extraction of onion phenolic compounds (Table 6). These novel methods are advantageous in comparison to conventional methods because they require shorter extraction periods and consume less solvent.

Microwave hydrodiffusion and gravity (MHG) is a novel technology that has massive potential for variety of extractive applications as the extraction of essential oil have been performed from rosemary leaves [26] and from Spearmint (M. spicata L.) and Pennyroyal (M. pulegiom L.) plant [27]. But, it was the first time that we have utilized the efficiency of this innovative technology for the extraction of polyphenols with onion as model [28]. Microwaves accelerate the diffusion of secondary metabolites by increasing tissue softness and cell permeability. They also enhance cell disruption due to their high penetration capacity, thereby increasing mass transfer within and outside the plant tissues [29]. MHG not only appears as efficient and economical but also as environment-friendly, as it does require less energy and no solvent and simply combines microwaves and earth gravity at atmospheric pressure [30].

In the first optimization part, we have used common yellow onion as extractive vegetal material, a vegetable of huge economic importance grown all over the world, which loses its water content more rapidly when treated with MHG under controlled temperature. This innovative method proved itself as an ideal alternative extraction method by extracting onion juice with retention of fresh organoleptic qualities and also by retaining increase content of valuable phenolic components. we have optimized different extraction parameters (microwave power, extraction period) and investigated the heating effect of microwaves in different parts of plant material and also in the reactor along with focussing on nutritional

attributes in terms of total phenol and flavonol contents (total quercetin (TQ), quercetin aglycon (QA) quercetin-3,4′-diglucoside (QDG), quercetin-4′-monoglucoside (Q4G), quercetin-3-monoglucoside (Q3G), kaempferol (KMF) and myricetin (MRT). Finally, cytological observations have been performed to evaluate the effects of MHG extraction on scale tissues.

In the generalization part we have performed extraction of different representative onion varieties (red, yellow, white and grelot onion) and a food byproduct (sea buckthorn) by MHG and compared them with conventional solvent extraction. Onion extracts are characterized not only by their total content of reducing compounds (TCRC) but also by their antioxidant capacity in three tests: the reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH), the ORAC (oxygen radical absorbance capacity) test and the inhibition of the AAPH-induced peroxidation of linoleic acid in SDS micelles.

Table 6: Previous literature on use of novel techniques for extraction of onion polyphenols

Extraction techniques	Plant material	Extracted flavonoids	Solvent	Comments	Ref
SFE	Skins of red & yellow onions	Quercetin aglycone	Ethanol (5%) modified CO2	Viable alternative of conventional methods due to controlled pressure and temperature conditions but the high capital cost and use of high percentage of organic modifiers with long extraction period (2.5h) limited its use	[24]
PLE	Edible portions of onion, carrot, potato & white cabbage	Quercetin & Isorhamnetin glycosides	Methanol (65%)	Highly automated method, allow extraction of oxygen sensitive flavonoids, use of high temperature and pressure speed up extraction but have limited application in food industry because of the use of solvents	[25]
SWE	Wastes of red & yellow onions	Quercetin, Isorhamnetin & kaempferol glycosides	Water	More green procedure as water was the only solvent and the later hydrolysis reaction was catalyzed by enzymes, but the elevated temperature might cause degradation of thermolabile substances	[31]
MHG	Edible portion of yellow onion variety	Quercetin glycosides, kaempferol & myricetin	No water or solvent	A totally green, economical and rapid procedure as it works in the absence of any solvent or water in short time by utilizing very less amount of energy and resulting a good percentage of yield	[28]

SFE (supercritical fluid extraction), SWE (subcritical water extraction), PLE (pressurized liquid extraction), MHG (microwave hydrodiffusion and gravity)

II.2. EXPERIMENTAL

II.2.1. Raw Material

Different raw onion (Allium cepa L.) varieties (red, yellow, white, grelot onion), were purchased from a local supermarket in Avignon province (South France). Onion bulbs which were apparently free of external damages was selected and peeled manually for their following processing. Sea buckthorn (*H. rhamnoides*) berries were obtained from shrubs grown in Alpes de Haute Provence, France. Sea buckthorn pomace (by-products) was collected after pressing of fruits in a vertical laboratory hydraulic press (REUS, P: 200 bars) (Fig. 17). The press cake was then directly used for extraction.

II.2.2. Chemicals

All solvents used for chromatographic purposes were HPLC grade. Methanol and Formic acid were from Merck (Darmstadt, Germany) and Acetonitrile was from Fisher Scientific Ltd. (Bishop Meadow Road, Loughborough, UK). The HPLC grade flavonol standards quercetin-3,4′-diglucoside and quercetin-4′-glucoside (spiraeoside) were purchased from Extrasynthese (Lyon, France). Quercetin and quercetin-3-glucoside were purchased from Sigma Chemicals Chimie (Fallavier, France). DPPH (2,2-diphenyl-1-picrylhydrazyl), AAPH (2,2´-azobis (2-methyl propionamidine) dihydrochloride), SDS (sodium dodecylsulphate), linoleic acid all were from Sigma-Aldrich (Steinheim, Germany) and Trolox and flouresceine were purchased from Acros Organics (Slangerup, Denmark) and Acros Organics (Morris Plains, USA), respectively. Phosphate buffer was prepared with HPLC grade water to avoid metal traces.

II.2.3. Moisture Content Determination

Moisture content determination of onions and by-products (peels, seeds, residual pulp) of sea buckthorn obtained after its pressing for juice production was carried out firstly by conventional Dean-Stark distillation apparatus according to the official method of the American Oil Chemical Society (AOCS) [32] and also by dehydration in an electric oven at 80°C. The average moisture content measured by both processes for onions was 88.5 ± 0.5% and 57% for byproducts of sea buckthorn.

II.2.4. MHG Apparatus and Procedure

Microwave hydrodiffusion and gravity has been performed in a Milestone EOS-G microwave laboratory oven illustrated in figure 8. This is a multimode microwave reactor 2.45 GHz with a maximum delivered power of 900W variable in 10W increments. Time, temperature, pressure and power can be controlled with the software package. The extraction vessels are made from Pyrex and have a capacity of 1000 mL. During experiments temperature was monitored by temperature sensor optic fibers which were inserted in the centre and outer layer of sample and also in the sample reactor. Temperature variations in different parts of plant material and reactor were measured continuously and data was saved automatically. This feedback helped in controlling the temperature by microwave power regulator.

MHG procedure was performed at atmospheric pressure; fresh entire onion bulbs was heated using a fix power density $1Wg^{-1}$ without the addition of solvents or water The direct interaction of microwaves with biological water favours the release of compounds trapped inside the cells of plant material. These compounds thus move naturally by diffusion along with hot water or crude juice out of the cells of plant material and move thus naturally downwards under the effect of earth gravity on a spiral condenser outside the microwave cavity where it condensed. The crude juice was collected continuously in a graduated cylinder. The extraction was continued until no more juice was obtained or overheating was detected. Extracted crude juice was collected and freeze-dried.

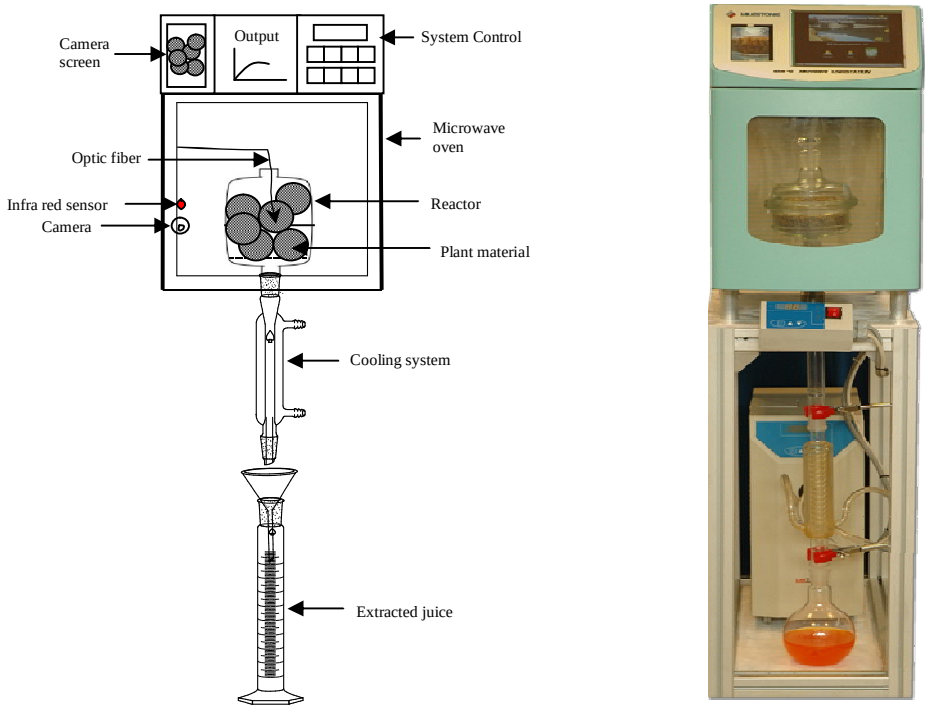


Figure 8: Explanatory diagram of Microwave hydrodiffusion and Gravity apparatus

II.2.5. Conventional Solid-liquid Extraction

In conventional solid-liquid extraction fresh onion scales were used, onion bulb was peeled and cut manually and 5g onion scales were homogenised with 50mL of 80% methanol in an ultrahomogeniser at 8000 rpm for 5min. After that the mixture was filtered and supernatant was collected, methanol was removed from the extracts by evaporation under vacuum at 40°C with rotary evaporator. After evaporation samples were freeze dried to remove water and finally dry extract yield was calculated and expressed in percentage.

II.2.6. HPLC analysis

HPLC analyses were performed using a Waters (Milford, MA) HPLC system consisting of a Waters 600E pump, a Waters 717 manual injector rheodyn, a Waters 2996 photodiode array detector. The HPLC pumps, manual injector rheodyn, column temperature, and diode array system were monitored and controlled by using Waters Empower 2 Chromatography Data software program. The wavelength used for the quantification of the onion flavonoids with the diode detector was 360 nm. The chromatographic separation was carried out on a Purospher Star RP-18 end-capped column (250 mm × 4 mm I.D.; 5 µm particle size from VWR), with a RP18 guard column (4 mm×4mm I.D.; 5µm particle size also from VWR). The end-capped column and guard column were held at 37°C and the flow rate was set at 1mL/min. The mobile phase consisted of two solvents: (A) acidified water (0.5% formic acid) and (B) 100% acetonitrile. The solvent gradient used was the following: 0 min, (A) 95% and (B) 5%; 20min, (A) 60% and (B) 40%; 30min, (A) 0% and (B) 100%; 45min, (A) 95% and (B) 5%. The injection volume was 20 µL and all analyses were performed at least three times and only mean values were reported. Identification of flavonoids was done by comparing the elution order and UV-visible spectra. Quantification was carried out by using the external standards of known concentration. Peak areas were used to quantify the compounds in the sample. A linear regression analysis was carried out on the data of the peak area versus concentration. Linear calibration curves of the standards ranging from 10 to 100 mg/L were obtained with good linearity and R2 values which were more than 99.5% accurate for all the standards. Final concentrations of different flavonoids were calculated in mg/100g DW.

II.2.7. Determination of the Total Content of Reducing Compounds (TCRC)

TCRC was estimated colorimetrically using the Folin-Ciocalteu method [33], with a kit (SEPPAL (Isitec-lab), France) especially suitable for TPC (total phenolic content) measurement of food products. This kit includes reagent A (modified Folin-Ciocalteu reagent), reagent B (alkaline buffer) and a gallic acid solution (3 g/L). A small volume (20 µL) of H2O (blank), gallic acid solution (standard) and 200 µL the extract (sample) was mixed with reagent A (2 mL). After 1 min, 1 mL of reagent B was added in both water and gallic acid standard and 850 µL in sample. The mixtures were allowed to stand for 30 min in the dark at room temperature. Then, their absorbance was measured at 760 nm with a diode-array Hewlett-Packard 8453 spectrophotometer) [34]. TCRCs were calculated by using the following formula: TCRC = 3 × (sample absorbance – blank absorbance) / (standard absorbance – blank absorbance). TCRC measurements were performed thrice and mean values, expressed as mg gallic acid/g of dry weight (mg GAE/g DW), were reported.

II.2.8. Antioxidant Tests

It is recommended to use various methods for evaluating the antioxidant capacity of complex heterogeneous systems like foods, as no general standardised protocol is currently available due to the strong dependence of the antioxidant activity on the antioxidant's chemical environment.

II.2.8.1. DPPH Assay

DPPH is a stable highly coloured free radical that can abstract labile hydrogen atoms from phenolic antioxidants with simultaneous formation of a colourless hydrazine (DPPH-H) [35]. The radical scavenging activity of extracts was evaluated according to the method described by Monzocco et al. [36] with some modifications. About 200 µL of a solution of extract in MeOH (50%) and water (50%) were added to 2 mL of a DPPH solution (6x10-5 M) in MeOH and the mixture was left in the dark at room temperature for 30 min. The decrease in absorbance was measured at 517 nm. The percentage of DPPH consumed was calculated from the following equation: I % = 100 x (A0 _ A1) / A0, where A0 was the initial absorbance (no antioxidant) and A1 the absorbance in the presence of the extract or standard at different concentrations. The IC50 of each extract or standard (concentration giving I % = 50) was calculated by plotting I % as a function of the extract concentrations. All experiments were performed in triplicate.

II.2.8.2. ORAC Assay

In this method, the hydrophilic peroxyl radicals (ROO•) generated by the thermal decomposition of the diazo compound AAPH oxidize the fluorescent probe fluorescein (FL), thus causing a fluorescence quenching. Hence, inhibition of this quenching by an antioxidant is a measurement of its ability to reduce ROO• [37]. The ORAC method employed was adapted from a method previously described by Ou et al. [38]. All reagents were prepared in a 75 mM phosphate buffer at pH 7.4. Trolox (0-75 µM) was used as the standard. A mixture of FL (1825 µL of a 69 nM solution in phosphate buffer) and extract (100 µL of a 5 g/L solution in MeOH) was pre-incubated for 10 min at 37°C. Then, 150 µL of a 510 mM AAPH solution in the phosphate buffer was added. The fluorescence intensity was measured with a Xenius spectrofluorometer (SAFAS) every second during 20 min with excitation and emission wavelengths set at 493nm and 514nm, respectively. Its decay reflects FL oxidation by the AAPH-derived peroxyl radicals. The ORAC value is calculated from the area under the curve expressing the quenching of FL fluorescence as a function of time in the presence of the extract in comparison with a calibration curve constructed with known trolox concentrations (good linearity, $R2 > 0.99$). The measurements were taken in triplicate. The area under the curve (AUC) was calculated as $AUC = (1 + f2/f0 + f4/f0...+ f40/f0)$ where fi is the fluorescence reading at time i. The net AUC was obtained by subtracting the AUC of the blank (no antioxidant). The results were expressed as micromoles of trolox equivalents (TE) per gram of extract on a dry weight basis (µmol TE/g DW).

II.2.8.3. Inhibition of Linoleic Acid Peroxidation

Two mL of a freshly prepared pH 7.4 phosphate buffer containing linoleic acid (2.55 mM) and SDS (0.1 M) were placed at 37°C in the spectrometer cell. At time zero, 25 µL of a freshly prepared aqueous solution of AAPH (80 mM) was added, followed by 25 µL of the antioxidant solution after 15 min. Quercetin solutions were prepared in MeOH. Onion extracts were not soluble in MeOH due to their high sugar content and were thus dissolved in water. The experiments were repeated with different concentrations of standard (1 mM and lower) and extracts. The initial level of linoleic acid hydroperoxides (molar absorption coefficient at 234 nm = 26100 M-1 cm-1) [39] was below 2% in all experiments. The uninhibited and inhibited peroxidation rates were calculated from the slope of the lines expressing the absorbance at 234 nm vs time before and after antioxidant addition using fixed time intervals.

Each experiment was run in triplicate. Finally, the IC50 values (concentration providing 50% inhibition) of each extract and standard were calculated graphically by plotting the ratio between inhibited (RP) and uninhibited peroxidation rates (RP°) vs the extract concentration.

II.2.9. Cytology

Scale fragments (approximately 5x5mm) were excised from the two peripheral bulb scales before (control) and after MHG-treated onions. They were immediately fixed in FAA (formalin, acetic acid, ethanol: 2/2/8, V/V/V) for 48h at 4°C. Samples were then rinsed in tap water for 2h, dehydrated in ethanol series (70-100%) and embedded in methacrylate resine. Sections of 3µm thickness were cut using an automatic microtome (Leica 2025), collected on microscope slides and stained with PAS (periodic acid-Schiff's reagent) and amido blue black procedures that specifically stain polysaccharides in pink and proteins in blue. Observations and photographs were made using a light microscope (Leica DM 2000) equipped with a digital camera (Leica DFC 30F). Ten scale fragments from treated and control onion bulbs were sectioned and microscopically analyzed.

II.3. RESULTS AND DISCUSSION

II.3.1. Optimisation of MHG for Flavonoids Extraction

Efficient performance of MHG for onion antioxidants extraction depends on different factors like moisture of plant material, irradiation power, temperature and time. To determine optimal reaction conditions for obtaining significant results a preliminary study consisting of various experiments was carried out.

II.3.1.1. Microwave Heating

Figure 9 shows the heating proceed in the centre of onions at different powers from 300W – 900W detected by temperature sensor optic fibre. Different phases in development of temperature can be distinguished (figure 9). The first phase corresponds to the heating phase (A), a rapid increase in temperature was observed from initial temperature (20°C) of onions to the boiling point of water (100°C). Heating rates observed in this phase were proportional to the different applied powers: 8.5°C/min (300W), 13.5°C/min (400W), 29.7°C/min (500W), 32.2°C/min (600W), 36°C/min (700W), 40°C/min (800W) and 45.9°C/min (900W). During this phase, in situ water of plant material was heated up, when irradiated with microwaves and

diffused out of plant material and moved downward under the influence of earth gravity. This phase was ended with appearance of first drop of water outside the microwave cavity.

At this point, temperature maintained to a plateau region and remained in this steady state at 100°C until the complete extraction of non bounded water. It corresponds to the extracting phase (B) of process, and when there was only tightly bound water remained, the temperature increased very quickly and led to the burning phase (C) which leads to the end of extraction.

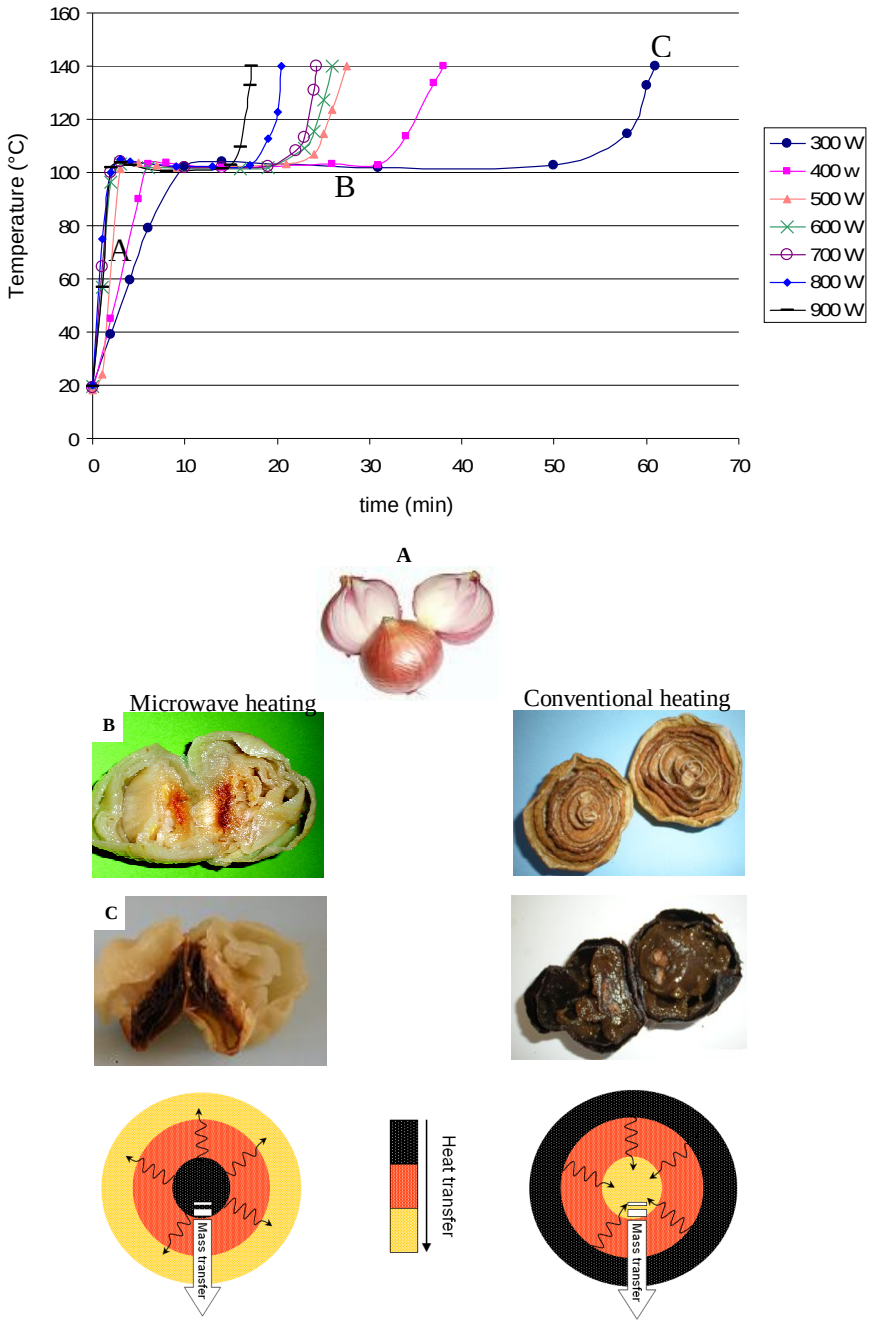


Figure 9: Heating phenomenon of microwaves in onion sapmles at different powers in comparison to conventional heating. A=heating phase; B=Extracting phase; C=Burning phase.

Heating rates of burning observed at different powers during this phase were: 5.14°C/min (300W), 6°C/min (400W), 8.5°C/min (500W), 9.6°C/min (600W), 10.3°C/min (700W), 12°C/min (800W) and 16.4°C/min (900W). Here, we can observe that the initial heating rates were more rapid than the heating rates of burning. Perhaps, it was due to the less free water content, inside the onion during the last phase of heating.

Similar phases (heating A, extracting B and burning C) were also detected by using optic fibers in outer layer of onion and also in reactor but the heating efficiency were in descending order from centre to outer layer of onion and then the reactor. Heating rates of phase A and phase C observed in the outer layer of onion and also in reactor were proportional to the applied powers but were less quick in comparison to the rates observed in centre of onion (figure 10).

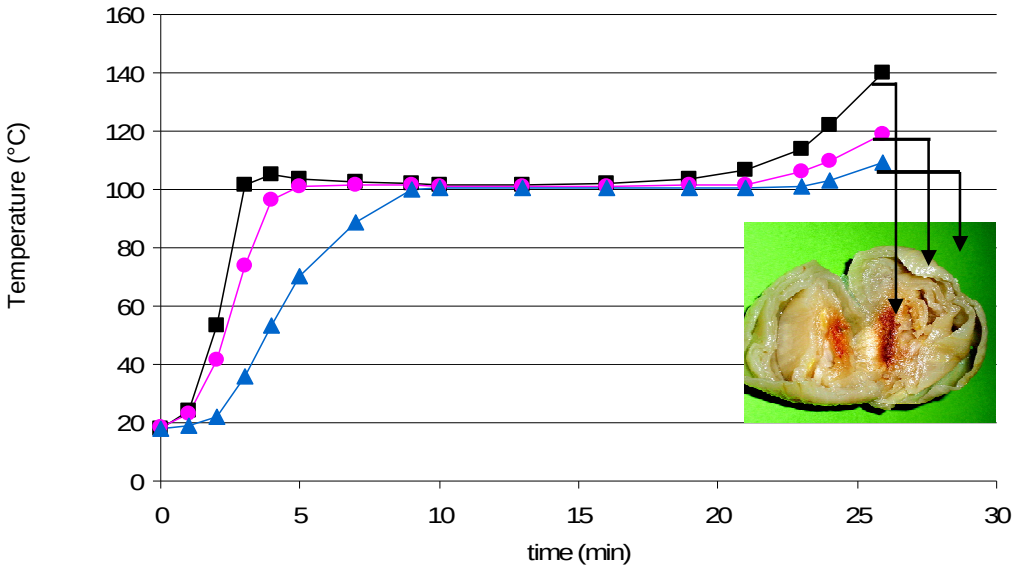


Figure 10: Heating phenomenon of MHG in onion showing: temperature in centre of onion (—■—); temperature in outer layer of onion (—●—); temperature of reactor (—▲—).

This microwave heating phenomenon shows a fundamental difference between microwave and conventional heating: in conventional heating heat transfers occur from the heating device to the medium, while in microwave heating heat is dissipated inside the irradiated medium. In contrast with conventional heating, microwave heat transfer is not limited by thermal conduction or convection currents. In practice this means that a much faster temperature increase can be obtained. Furthermore, the maximum temperature of the material heated by microwaves is only dependent upon the rate of heat loss and power

applied. In our microwave heated reactor, the average temperature of the onion is significantly higher than the surrounding temperature. This is due to the fact that the microwave power is dissipated over the whole volume of the onion (which contains 88% water).

In conventional solvent extraction, mass transfer occurs from the inside to the outside while heat transfer occurs from the outside to the inside. For solvent free microwave, the two transport phenomena (heat and mass transfer) are in the same direction from the inside of the extracted material to the bulk reactor [40]. The extraction action is realised without solvent under microwaves is due to a synergy combination of the two transfer phenomena mass and heat acting in the same direction. In Microwave Hydrodiffusion and Gravity, heat is dissipated volumetrically inside the irradiated medium; while in conventional solvent extraction heat is transferred from the heating medium to the interior of the sample. Microwaves are volumetrically distributed, and heat transfers occur from the sample to the colder environment. This causes an important difference between conventional and microwave heating. In conventional heating, heat transfer depends on thermal conductivity, on the temperature difference across the sample, and for fluids, on convection currents. As a result, the temperature increase is often rather slow. By contrast, in microwave heating, due to the volumetric heating effect, much faster temperature increases can be obtained, depending on the microwave power and the dielectric loss factor of the material being irradiated. Since evaporation is very limited and needs more energy, antioxidants and */in-situ/* water are extracted by this physical phenomenon, known as hydro-diffusion, allows the extract diffused outside the plant material to drop by earth gravity out of the microwave reactor.

II.3.1.2. Extraction Kinetics

In order to carry out the study on extraction kinetics of onion extracted juice, volume of onion juice obtained at different powers was plotted as function of time. Extraction curves obtained at different powers in figure 11 shows three diverse stages of extraction.

Stage 1 corresponds to the induction time, during this phase no recovery of water was occurred. It ends with emergence of first water drop.

Stage 2 represent the constantly increasing flow rate of extract as illustrated in figure 11 by linear curves at different powers. All the easily exchangeable water of onion was extracted in this phase. During this phase the gradient of curves increases with increase of power.

Stage 3 marks the end of extraction process as represented by horizontal line on graph. At initiation of this phase, onions were almost dry with no more further extraction. At this point, burnt smell was generated as a result of prolonged heating.

Finally, the extracted crude juice was collected freeze dried and yield was expressed in percentage (%).

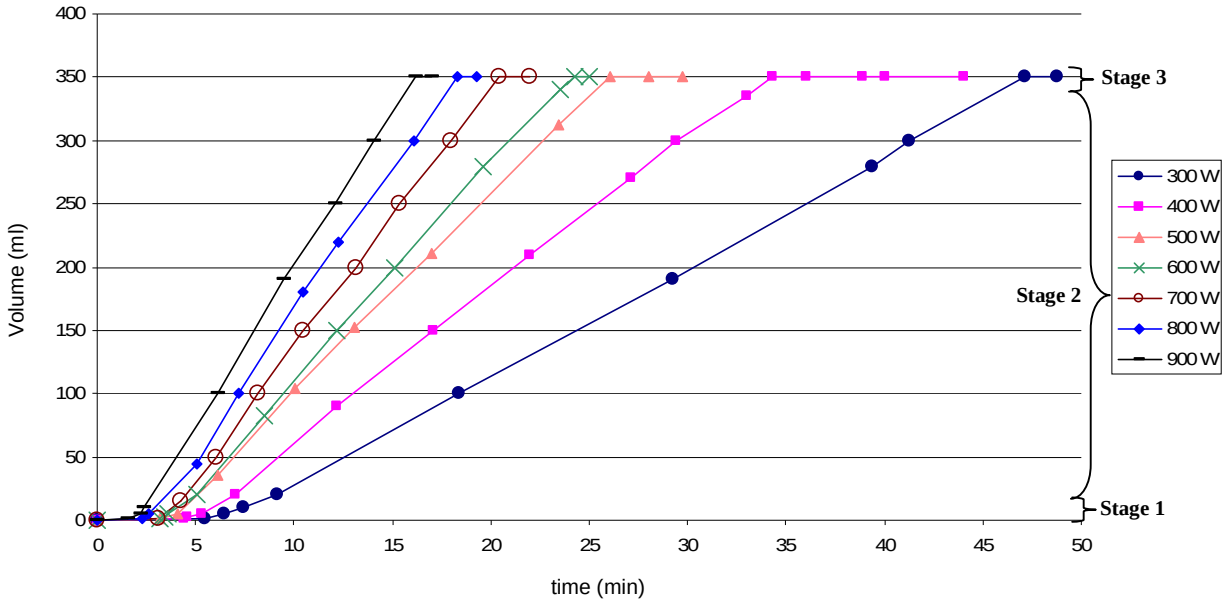


Figure 11: Extraction curves obtained at different powers showing different stages of extraction

II.3.1.3. Extraction Yield

The aim of this part of study was to examine the impact of MHG on extraction yield of crude onion juice at various powers. Actual yield of four medium sized onions was taken until the time at which moisture collection was completely stopped due to overheating. No remarkable difference in onion juice yield was observed at different powers. A slight decrease in juice volume was observed while moving from lower (300W) to higher (900W) power. The percentage of crude juice yields calculated at different powers was: 82.7% (300W), 82.1% (400W), 81.5% (500W), 81.1% (600W), 80.8% (700W), 80.3% (800W) and 79.7% (900W), which is close to the actual moisture percentage (88.52%) of fresh onion.

The water content of onion is not an alone factor for determining the final yield. The onion crude juice also holds some soluble compounds like sugar, acids and polyphenols. The dry extracts weight which was taken, after removal of water content of crude onion juice by

the process of freeze drying, at different powers was also in descending order from lower to higher powers. The weight of dry extracts yields we obtained at different powers, 4.70% (300W), 4.40% (400W), 4.28% (500W), 3.41% (600W), 2.87% (700W), 2.19% (800W) and 1.95% (900W), proved that the efficient extraction of soluble solids significantly depends on applied powers. As it vary remarkably among very lower and very high power.

II.3.1.4. Structural Changes in Onion Tissues during Extraction

Figure 12 summarises microscopic aspects of MHG-processed scale tissues in comparison with control. Sections from control scales exhibited a general structure typical of leaf anatomy with an important mesophyll enveloped in epidermal layers. All tissues showed fully differentiated cells that are compactly arranged with intercellular spaces confined to the angles between cells (figure. 12A). There is a gradual increase in cell size from the outer to the inner layers but in cytological terms these cells displayed a large vacuole occupying the essential cell volume, reducing the cytoplasm to a thin layer pressed against the cell wall (figure. 12B). Sections from MHG-treated scales showed drastic structural alterations particularly in cell walls and vacuoles compartments. Epidermal, sub-epidermal and mesophyll cell walls showed profound destructuration (figure. 12C, D and E) as attested by loosed appearance of the constituting polysaccharides and the presence of fibrillar material in place of the middle lamella (figure. 12E). This gives rise to the loss of cell cohesion and cell architecture (figure. 12C and D). Throughout scale tissues the vacuoles presented a crushed-like appearance with discontinuous contours indicating a loss of their contents. Together, the MHG-induced cell wall and vacuole alterations that culminate with cell plasmolysis provide evidence for the above reported effectiveness of this method since flavonoids such as quercetin are thought to accumulate in the vacuoles [41].

II.3.1.5. Total Phenolic Content Obtained at Different Powers

The amount of total phenolic content (TPC) varied in the onion extracts obtained at different powers as shown in Table 7. Highest phenolic content (58.29 mg GAE/g DW) was found at power of 500W and lowest content was observed at 900W (29.94 mg GAE/g DW). Initially TPC increased with increase of power from 300W (47.54 mg GAE/g DW), and a maximum amount was detected at 500W but with further increase of power, phenolic content concentration started to decrease and lowest concentration was observed at very high power (900W). TPC results obtained at 500W were not only significant in comparison with

conventional solvent extraction (64.81 mg GAE/g DW) but also correlate with previous data. Our detected range of TPC falls in the range (4.6-74.1 mg GAE/g DW) observed in different varieties and layers of Allium cepa, including varieties contain very high level of phenolic

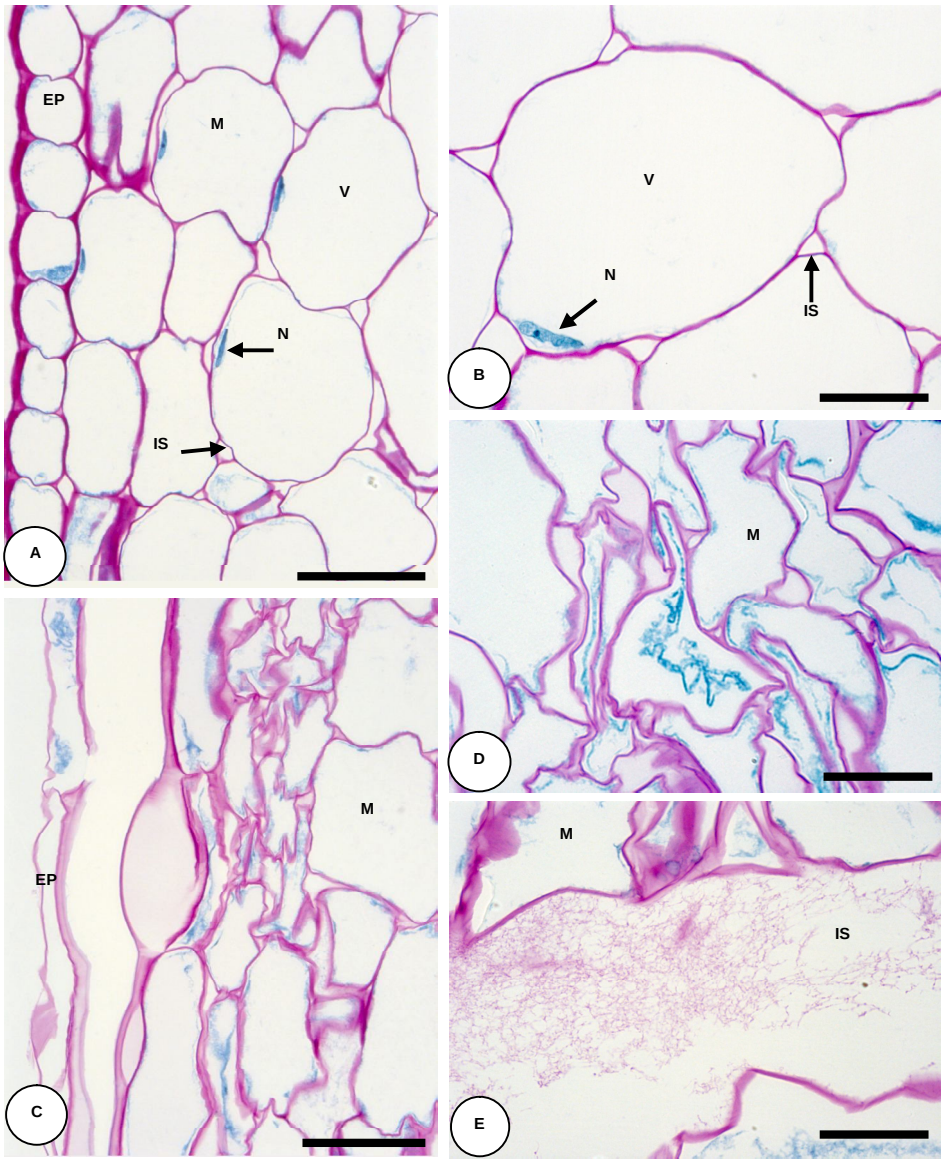


Figure 12: Cytological aspects of MHG-treatment of onion. Polysaccharides: pink, proteins: blue (EP = epidermis, IS = intercellular spaces, M = mesophyll, N = nucleus, V = vacuole). A and B: sections of untreated bulb scales showing the general structure with the mesophyll and epidermis. Note the voluminous appearance of vacuoles. C and D: portions of MHG-extracted scales illustrating alterations of wall and vacuole compartments. Epidermis and mesophyll cells are shrunken with collapsed vacuoles. E: Detail showing middle lamella disintegration. Bars = 10µm in A and C; 5µm in B, D and E.

content (red onion) to very low level (white onion) [17]. These results are also found in good concentration in comparison to the TPC values studied by Nuutila et al. [42] in the dry outer skin of red (80.0 mg GAE/g) and yellow onions (26.0 mg GAE/g). TPC values of microwave extracted (at 500W) residue was also observed after its conventional solvent extraction, in order to check the remaining amount of phenolic contents in residue, which was 21.60 mg GAE/g DW, these results shows that with MHG we have extracted a good percentage of phenolic compounds along with "in situ" water content of plant material.

II.3.1.6. Flavonoid Content of Onion Extracts Obtained at Different Powers

II.3.1.6.1. Total Quercetin and Major Flavonoids

Total quercetin presented in Table 7 is the sum of concentration of free quercetin and different forms of quercetin present in conjugation with carbohydates mainly as glucosides like QDG, Q4G and Q3G. QDG and Q4G provide a good estimation of level of total quercetin in the sample as they are representing about 90% of overall flavonol content [43]. QDG was detected in highest concentration in comparison to other quercetin glucosides followed by Q4G identified as second major flavonol compound. Quantification of all these compounds has been done by comparing the retention time and absorbance of peaks at 360nm with the use of authentic standards.

In preliminary study, extraction efficiency of different powers for flavonol contents was tested. Higher levels of total quercetin (326.5 mg/100g DW) was calculated at 500W (Table 7) compared to other applied powers, which correspond well to the previous published data (414 mg/100g DW found by Aoyama andYamamoto [18] in yellow onion; 348mg/100g DW quercetin content in yellow onion illustrated in Danish results by Justesen et al. [44], 507 mg/100g DW by Hertog et al. [45] and 280 mg/100g DW by Mogren et al. [46]. All the analyzed flavonols have shown almost similar behaviour to TQ, as the highest levels of QDG (239.7 mg/100g DW) and Q4G (82.55 mg/100g DW) were also found at 500W. These results not only fall in the range reported by Cardi et al. [43] (QDG: 153-404 mg/100g DW, Q4G: 58-286 mg/100g DW) among different onion varieties but Q4G was also found in good concentration as compared to the results determined by Roldán-Marín et al. [47] (282 mg/100g DW concentration of QDG and 43.9 mg/100g DW of Q4G) in high pressure processed onion. Bonaccorsi et al. [16] have also found QDG in highest concentration in red onion variety (254-274 mg/100g DW), our results also correspond well with these results as yellow onion ranked after red onion as a good source of quercetin flavonoid contents.

Concentration of QDG (581.8 mg/100g DW) and Q4G (187.5 mg/100g DW), in fresh onion samples treated with conventional solvent extraction was also detected in higher amount in comparison to other flavonols.

TQ concentration determined by conventional solvent extraction method was 782.6 mg/100g DW which falls in the range of flavonols content of yellow onion (270-1187mg/100g DW) reviewed by Slimestad et al. [48]. For analysing the effect of microwave on stability of flavonoid content and their extraction efficiency, concentration of flavonoids retained in microwave extracted (at 500W) onion samples was also calculated by conventional solvent extraction. TQ retained in the residue of microwave extracted onions was found 440.7 mg/100g DW which is 56% of the TQ content determined by conventional solvent extraction of fresh onion. Amount of TQ observed at 500W is 42% of the concentration determined by conventional method. Our results showed 2% losses of TQ by microwave extraction in comparison to conventional fresh onion extraction. Concentration of major flavonols, QDG (342.4 mg/100g DW) and Q4G (95.2 mg/100g DW), retained in residue of onions was found almost comparable with microwave extracted content along with water content of onions.

These results showed that there was no remarkable loss or degradation of flavonoid compounds occurred at 500W and onion bulbs still retained a good amount of these major compounds after microwave extraction of water content. Similar to the findings of Rodrigues et al. [49], as they have been determined no loss of QDG and Q4G at mild (450W) microwave heating in comparison to untreated onion but 16% (QDG) and 18% (Q4G) losses were detected with increase of power (750W). Similarly, losses of these compounds were also observed with increase of power, in comparison to the highest concentration observed at 500W. A rapid decrease in concentration was observed at intense powers as shown in Table 7 and finally lowest amount of QDG was determined at 900W (101.6 mg/100g DW) with complete absence of detectable amount of Q4G, as more intense treatments resulted with loss of quercetin components. But very low power (300W) was also not an effective and efficient power for flavonoids extraction, along this it also consumes more time for complete extraction of onion water content. QDG exhibited the lowest loss (degradation or conversion into quercetin aglycon) as it is still present at very drastic condition at 900W in comparison to Q4G, Q3G and free quercetin which were not detected at 900W. In QDG glucose is attached at 3 and 4′ and due to blockage of the two positions it showed much greater stability then

Q4G in which position 3 is not conjugated. Makris and Rossiter [50] have also observed the lowest loss in QDG concentration (8.4%) and Q4G and QA content declined by 37.6% when subjected to heating treatments. Our results are also supported by the work of Kana et al. [51] who has selected microwave roasting without water as a better cooking method to retain flavonoids in onion tissue. But the concentration of TQ varied with power. This shows that microwave with a mild power is an efficient method for extraction of quercetin components without remarkable degradation. With MHG we can extract more than 40% of the flavonol components along with the "in situ" water content of onions also possessing fresh organoleptic properties.

II.3.1.6.2. Minor Flavonoids

Free quercetin was also quantified in conventional and microwave extracted samples at retention time of 23.81min by HPLC. The content of free quercetin showed a low percentage in comparison with QDG and Q4G (Table 7). Any measurable quantity of QA was not detected at low powers 300W and 400W. At 500W just traces of QA were observed, similar to the results obtained by Patil et al. [52] in four yellow onion varieties and one pink and red onion variety. They have detected not more than 0.4 mg/100g DW of free quercetin in all the onions Zielinska et al. [53] also mentioned only 1.1% of free quercetin in yellow onion bulbs. At 600W a good concentration (5.25 mg/100 g DE) of QA was detected but then a fall in concentration was observed with increase of power. In fresh onions normally there is always a low concentration of QA but as a result of some treatment or processing breakdown of glycosidic bonds in QDG and QMG, a good concentration of QA can be detected. Perhaps similar is the case here with decrease of qercetin glycosidic forms, an increase in concentration of QA observed at high powers of 600W and 700W. But with further increase of power, phenomenon of degradation was more profoundly expressed in free quercetin which has both sides exposed. With conventional solvent extraction method (80% methanol) a very less concentration of QA was observed (1.32 mg/100 g DW) and in the residues we have also detected just traces of QA.

Along with QA the other minor components which are representing almost less than 5% of total amount of flavonols were analysed. Very small peaks of Q3G and kaempferol have been identified by comparison with their standards. Similar to the already discussed major compounds highest concentration of Q3G have been detected at 500W (4.22mg/100g DE) which is almost 1.27% of the total amount of analysed flavonols. This value is in good

agreement with the results of Zielinska et al. [53] who have determined 1.4% of Q3G in Sochaczewska onion variety which is a typical onion with a yellow-brown bulb colour. Similar to other flavonoids, it was also detected in minor amounts at vary high powers.

Kaempferol presence in comparison to quercetin content in different varieties of onions has been reported in minor quantities [52]. Kaempferol content identified and quantified at 400W (4.01mg/100g DE) and 500W (3.99mg/100g DE) were not significantly different from each other (Table 7), and these values were similar to the previously reported values of 3-7mg/100g DW by Bilyk et al. [54] in the outer and inner skins of bulbs of different varieties. A slightly higher content of kaempferol has been reported by Sellappan and Akoh [55] in five varieties of onion which ranged from 15.4 - 19.8mg/100g DW. In contrast to the findings of Sellappan and Akoh [55], myricetin was not detected in yellow onion variety which we have extracted and analysed.

Table 7: Flavonoids and total phenolic contents (TPC) obtained at different powers of MHG and by conventional extraction

MHG Power	QA* (mg/100g DW) **23.81min**	QDG* (mg/100g DW) **16.18 min**	Q4G* (mg/100g DW) **19.85 min**	Q3G* (mg/100g DW) **17.88 min**	Total quercetin (mg/100g DW)	KMF* (mg/100g DW) **26.80min**	MRT* (mg/100g DW) **20.65min**	TPC (mg GAE/g DW)
300W	ND	177.4 ± 11.5	4.41 ± 0.1	3.65 ± 0.37	185.5 ±11.97	ND	ND	47.54 ± 0.7
400W	ND	184.9 ± 14.3	56.62 ± 7.4	4.08 ± 0.64	245.6 ±22.34	4.01 ± 0.21	ND	56.46 ± 1.0
500W	Traces	239.7 ± 16.2	82.55 ± 8.7	4.22 ± 0.89	326.5 ±25.79	3.99 ± 0.17	ND	58.29 ± 1.0
600W	5.25 ± 1.0	159.8 ± 13.8	49.13 ± 9.8	3.70 ± 0.29	217.9 ±24.89	2.90 ± 0.08	ND	43.23 ± 0.8
700W	4.23 ± 0.4	141.7 ± 15.4	17.66 ± 4.6	3.54 ± 0.41	167.1 ±20.81	1.59 ± 0.04	ND	40.10 ± 0.9
800W	Traces	123.5 ± 13.8	6.91 ± 0.2	Traces	130.4 ±14	ND	ND	38.54 ± 1.1
900W	ND	101.6 ± 11.6	ND	ND	101.6 ±11.6	ND	ND	29.94 ± 1.3
Conv	1.32 ± 1.09	581.8 ± 22.4	187.5 ± 17.3	11.98 ± 2.1	782.6 ±42.89	5.18 ± 0.19	ND	64.81 ± 1.5
Residue	Traces	342.4 ± 23.1	95.2 ± 15.8	3.10 ± 0.38	440.7 ±39.28	traces	ND	21.60 ± 0.3

*QA=Quercetin aglycon; QDG=quercetin-3,4′-diglucoside; Q4G=quercetin-4′-monoglucoside; Q3G=Quercetin3-glucoside; KMF=Kaempferol; MRT=Myricetin; Conv=conventional solvent extraction; TPC=total phenolic content.

The presence of different flavonoids varied among different varieties and their concentration also depends on different factors like climatic conditions and stages of maturity.

II.3.1.6.3. Flavonoid Contents at Optimized Power

Impact of different microwave irradiation powers, from 300W-900W, were examined in terms of heating and burning rate, yield, extraction rate, flavonoid contents. With increasing power no better results were obtained, as at high powers (600W-900W), high speed of extraction was observed but resulted with less total dry extract yield due to degradation of phenolic compounds. On the other hand low power resulted more yield but with slower extraction rate and are also inefficient for complete extraction of flavonoids. An irradiation power of 500W was selected as an optimum power for later experiments. At optimized power, the yield and flavonoids composition were examined after each five minutes (A=0-3min, B=3-8min, C=8-13, D=13-18, E=18-23, F=23-27.5).

Initially, in situ water was heated at the rate of 27.9°C/min and it takes almost 3mins (A) to reach the extraction temperature 100°C, resulted with appearance of first drop. Almost comparable yields of water content were obtained after each five minutes: 14.7% (B), 17.6% (C), 16.9% (D), 16.4% (E), and 15.9% (F), until the end of extraction process completed in 27.5mins. But the dry extract yield obtained after lyophilisation was in inverse proportion to the time, as with increase of time, the percentage of extracted component decreased (Figure 13).

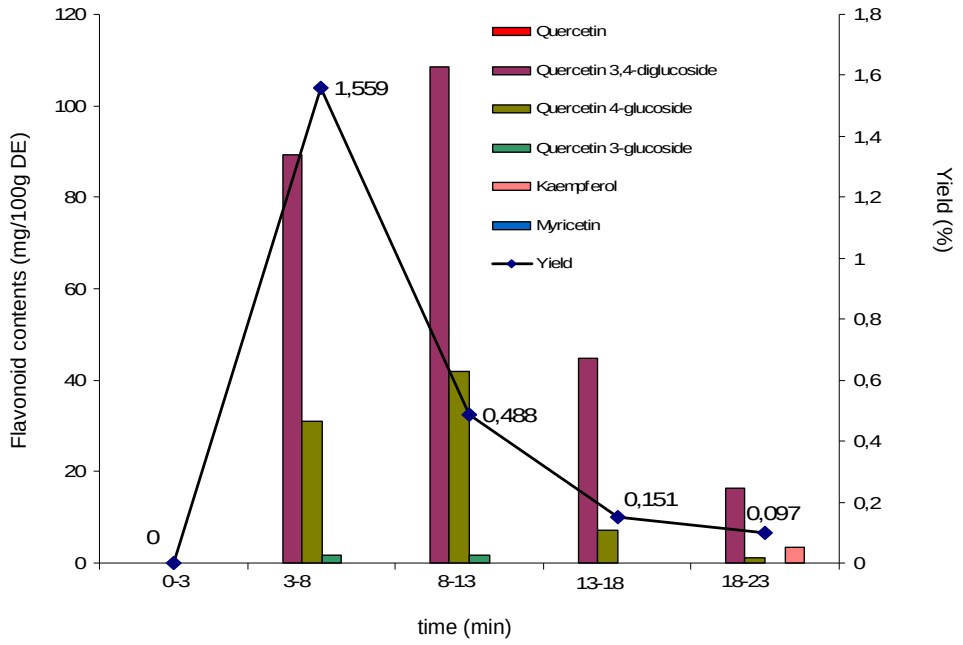


Figure 13: Yield and distribution of flavonoid contents in different intervals of time at 500W together represents 100% of flavonoid content

Highest yield of flavonoid components obtained in part C: QDG (108.5mg/100g DE), Q4G (41.9mg/100g DE), Q3G (1.80mg/100g DE). Traces of QA were also detected only in part C which also showed the highest content of total phenols (20.1mg GAE/g DE) in comparison to other parts of extraction process: 18.9mg GAE/g DE (B), 16.1mg GAE/g DE (D) and 15.9mg GAE/g DE (E). Highest dry extract yield was observed in part B as shown in figure 13. but the lower content of flavonoids observed in comparison to part C; QDG (89.18mg/100g DE), Q4G (30.9mg/100g DE), Q3G (1.74mg/100g DE). After part C the next extracted parts D and E resulted with further decrease in dry extract yield with minimum content of flavonoids. In part D only the two major components: QDG (44.6mg/100g DE) and Q4G (7.16mg/100g DE) were detected. Kaempferol was detected in part E of extraction along with minimum quantity of these above mentioned major flavonoid components QDG (16.4mg/100g DE) and Q4G (1.12mg/100g DE). Onion juice extracted in first 23mins just before the onset of burning was analysed with HPLC (figure. 14) and rejected the last part of juice which was extracted during burning, as the content of flavonoids have already been decreased.

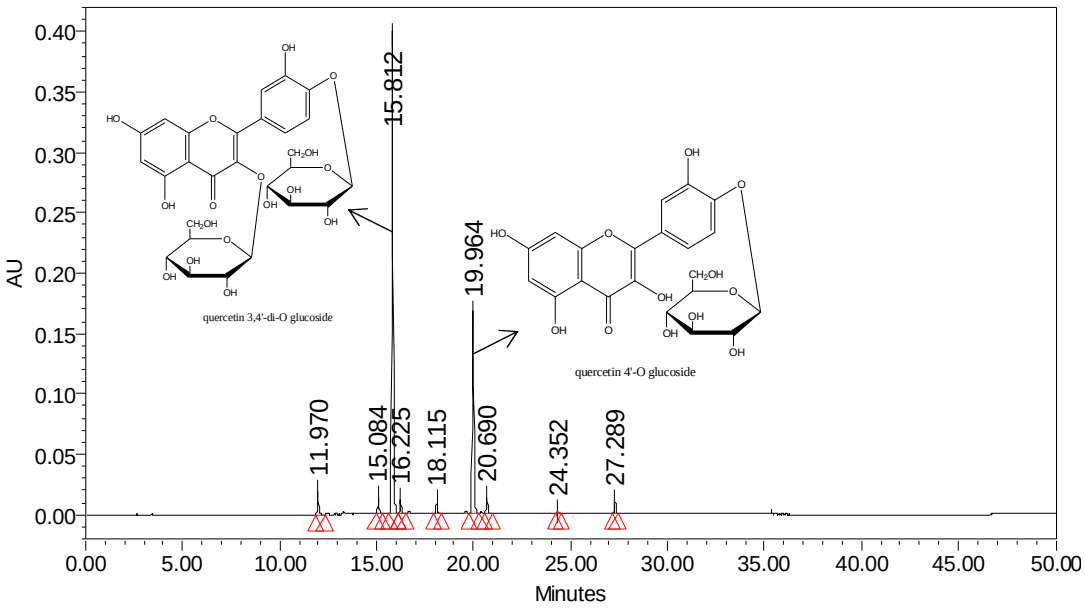


Figure 14: HPLC chromatogram of onion sample at optimized power (500W)

II.3.2. Generalization of the technique (MHG)

II.3.2.1. Antioxidant Activity Evaluation of different onion varieties

After power optimization for extraction of polyphenols (500W for 500g of plant material (1W/g), we have generalized this technique by extracting four (red, yellow, white and grelot onion) varieties of *Allium cepa*, a rich source of quercetin (flavonol) glycosides and were studied for their total content of reducing compounds (TCRC), flavonol content and antioxidant activity evaluation. Extracts obtained by solvent free microwave hydrodiffusion and gravity (MHG) technique and conventional solvent extraction (CSE) were analysed with HPLC for quantification of flavonoids. Three different methods were selected for evaluating the antioxidant capacity of the different onion varieties (after the determination of their phenolic content by the Folin-Ciocalteu method): the reduction of the stable DPPH (2,2-diphenyl-1-picrylhydrazyl) radical, the ORAC (oxygen radical absorbance capacity) method, and the inhibition of the AAPH-induced peroxidation of linoleic acid in SDS micelles.

II.3.2.1.1. Quantification of Flavonoid Content

Total quercetin contents of onion samples analyzed by HPLC varied from 1.2 to 134.7 mg QE/100g DW for MHG samples and from 1.9 to 319.7 mg QE/100g DW for CSE samples depending on the variety of onion (Table 8). Considering that MHG does not involve any solvent, it can be claimed that this process permits the extraction of substantial concentrations of flavonols, which are only ca. twice as low as in the standard extraction process with methanol. This difference in flavonoid contents in MHG vs. CSE is consistent with our previous results [28]. The quercetin content in red onion was higher than in yellow onion while white and grelot onions appeared very poor in flavonols. Similar results were reported in the literature [18, 52, and 42]. In red and yellow onions, both MHG and CSE samples contained quercetin as 3,4′-diglucoside, 4′-glucoside and 3-glucoside. Quercetin-3,4′-diglucoside represents at least 90% of the total flavonol content in onions. In white and grelot onions, this is the sole form detected. These results are consistent with the literature [16, 43, and 56]. Free quercetin was not detected in any onion extract. It should be mentioned that only the edible portion of onion bulbs was analyzed whereas free quercetin is mostly present in the outer dry layer [17]. Genetic and environmental factors may also slightly influence the content and distribution of flavonols in onions.

Table 8: Flavonol contents and total contents of reducing compounds (TCRC) of different onion extracts obtained by MHG at 500 W and by conventional solvent extraction (CSE)

Onion varieties	QA* (mg/100g DW) 23.81 min	QDG* (mg/100g DW) 16.18 min	Q4G* (mg/100g DW) 19.85 min	Q3G* (mg/100g DW) 17.88 min	Total quercetin (mg QE/100g DW)	TCRC (mg GAE/g DW)
Red (MHG)	ND	224.7± 17.1	37.2 ± 7.8	3.3 ± 0.59	134.7	25.07 ± 0.4
Red (CSE)	ND	555.7 ± 24.5	74.5 ± 8.6	4.8 ± 0.83	319.7	23.09 ± 0.7
Yellow (MHG)	ND	197.4 ± 16.3	25.3 ± 5.1	3.6 ± 0.34	114.0	17.90 ± 0.1
Yellow (CSE)	ND	378.9 ± 23.7	45.8 ± 6.6	3.3 ± 0.40	214.7	14.69 ± 0.2
White (MHG)	ND	5.09 ± 0.89	ND	ND	2.4	7.58 ± 1.3
White (CSE)	ND	10.93 ± 1.9	ND	ND	5.2	6.62 ± 0.9
Grelot (MHG)	ND	2.55 ± 0.09	ND	ND	1.2	11.3 ± 0.7
Grelot (CSE)	ND	4.06 ± 0.34	ND	ND	1.9	5.03 ± 0.3

*QA=quercetin aglycone; QDG=quercetin-3,4′-diglucoside; Q4'G=quercetin-4′-glucoside; Q3G=quercetin-3-glucoside

II.3.2.1.2. Antioxidant Activity

The extracts of the different onion varieties obtained by MHG were compared to those obtained by conventional solvent extraction for their total content of reducing compounds, their radical scavenging capacity (DPPH and ORAC tests) and their ability to inhibit linoleic acid peroxidation. These popular methods are based on the ability of phenols to transfer electrons or H-atoms to metal ions and radicals, including peroxyl radicals (ORAC, inhibition of lipid peroxidation). Those mechanisms are relevant to the protection of lipid-rich foods against autoxidation and potentially to the prevention of oxidative stress in humans, particularly in the GI tract, which is probably the sole biological site where native dietary polyphenols are expected to accumulate in concentrations high enough to exert a substantial antioxidant protection [57].

Table 9: DPPH reducing capacity of different onion varieties, their inhibition action against linoleic acid peroxidation in SDS micelles expressed as IC50 and ORAC values

Onion varieties	DPPH test			Inhibition of lipid peroxidation			ORAC test	
	[A]IC_{50}	R^2	[B]IC_{50}	[A]IC_{50}	r^2	[B]IC_{50}	µmol TE/g	mmol TE/mmol GAE
Red (MHG)	17.09	0.9931	2.5	7.00	0.9993	1.03	201	1.363
Red (CSE)	18.00	0.9902	2.4	8.65	0.9955	1.17	148	1.090
Yellow (MHG)	36.35	0.9961	3.8	10.03	0.9929	1.06	121	1.149
Yellow (CSE)	58.61	0.9947	5.1	16.06	0.9967	1.39	102	1.180
White (MHG)	74.54	0.9956	3.3	24.82	0.9976	1.11	76	1.704
White (CSE)	82.76	0.9940	3.2	48.45	0.9966	1.89	59	1.515
Grelot (MHG)	63.58	0.9942	4.2	21.76	0.9945	1.78	37	0.557
Grelot (CSE)	85.18	0.9998	2.5	74.19	0.9932	2.20	29	0.980

[A] g of onion extract per L; $IC_{50,}$ concentration for 50% inhibition
[B] mmol of gallic acid equivalent per L

II.3.2.1.2.1. TCRC and DPPH Assays

The Folin-Ciocalteu method allowed a good discrimination between the different varieties of Allium cepa (Table 8). The highest content of reducing compounds was found in the red onion extract obtained by MHG (25.07 mg GAE/g DW). The lowest content was measured in the grelot onion extract obtained by CSE (5.03 mg GAE/g DW). Intermediate values were found in yellow onion extracts, 17.90 mg GAE/g DW (MHG) and 14.69 mg GAE/g DW (CSE). Interestingly, the TCRC values are much higher than the total quercetin contents, thus suggesting that most of the reducing activity of onion extracts is not due to flavonols. Interestingly, while MHG afforded lower concentrations of quercetin glycosides than CSE, MHG proved slightly more efficient than CSE at extracting the overall pool of reducing compounds. The TCRC values reported in this work are consistent with the literature [17, 58].

TCRC values generally correlate with the DPPH-scavenging capacity with IC50 values ranging from 17.09 mg/ml to 85.18 mg/ml (Table 9). The red onion extracts had the lowest IC50 values followed by the yellow, white and grelot onions, which showed moderate to low DPPH-scavenging activities as already reported [59]. In the DPPH method, all samples gave a linear response (R2 > 0.99) in the concentration range 1-200 mg/ml for the extracts. Consistently with the TCRC determination, MHG extracts had lower IC50 values than their CSE counterparts for each onion variety. For instance, the yellow onion extract obtained by MHG has a IC50 of 36.35 mg/ml vs. 58.61 mg/ml by CSE. White and grelot onion extracts displayed very poor DPPH-scavenging activities.

II.3.2.1.2.2. Peroxyl Radical Scavenging Capacity

Inhibition of the AAPH-induced peroxidation of linoleic acid in SDS micelles. In this method, the thermal decomposition of diazo compound AAPH (R-N=N-R) produces hydrophilic peroxyl radicals (ROO•) that can abstract labile bis-allylic H-atoms from linoleic acid (LH), thereby initiating a radical chain reaction with the lipid peroxyl radical (LOO•) as the propagating radicals. The experiments are monitored by UV/VIS spectroscopy at the wavelength of maximal absorption of the lipid hydroperoxides (LOOH), i.e. 234 nm. In the absence of antioxidant, LOOH accumulate rapidly at a constant peroxidation rate (RP°, see blank in Figure 15). When an antioxidant is introduced, the reaction slows down (inhibited peroxidation rate RP < RP°) until the antioxidant is consumed. The duration of this inhibited phase depends on the initial antioxidant concentration and on the antioxidant stoichiometry (number of peroxyl radicals reduced per antioxidant). The RP/RP° ratio was plotted vs sample concentration and the sample concentration that leads to 50% inhibition (IC50) was estimated.

Initiation	$R\text{-}N{=}N\text{-}R + 2\,O_2 \longrightarrow 2ROO^{\bullet} + N_2$	(1)
	$ROO^{\bullet} + LH + O_2 \longrightarrow ROOH + LOO^{\bullet}$	(2)
Propagation	$LOO^{\bullet} + LH + O_2 \longrightarrow LOOH + LOO^{\bullet}$	(3)
Inhibition of initiation	$ROO^{\bullet} + AH \longrightarrow ROOH + A^{\bullet}$	(4)
Inhibition of propagation	$LOO^{\bullet} + AH \longrightarrow LOOH + A^{\bullet}$	(5)
Termination	$LOO^{\bullet} + LOO^{\bullet} \longrightarrow$ Non-radical products	(6)

Scheme: Mechanism of the inhibited lipid peroxidation

Remarkably, the red onion extracts have the lowest IC50 values (7.0 mg/ml and 8.65 mg/ml for MHG and CSE respectively) in comparison to the other colored onion extracts. Yellow onion extracts exhibited medium antioxidant activity with IC50 values of 10.03 mg/ml for MHG and 16.06 mg/ml for CSE. White and grelot onion extracts were less efficient or inactive (Table 9, Figure 15).

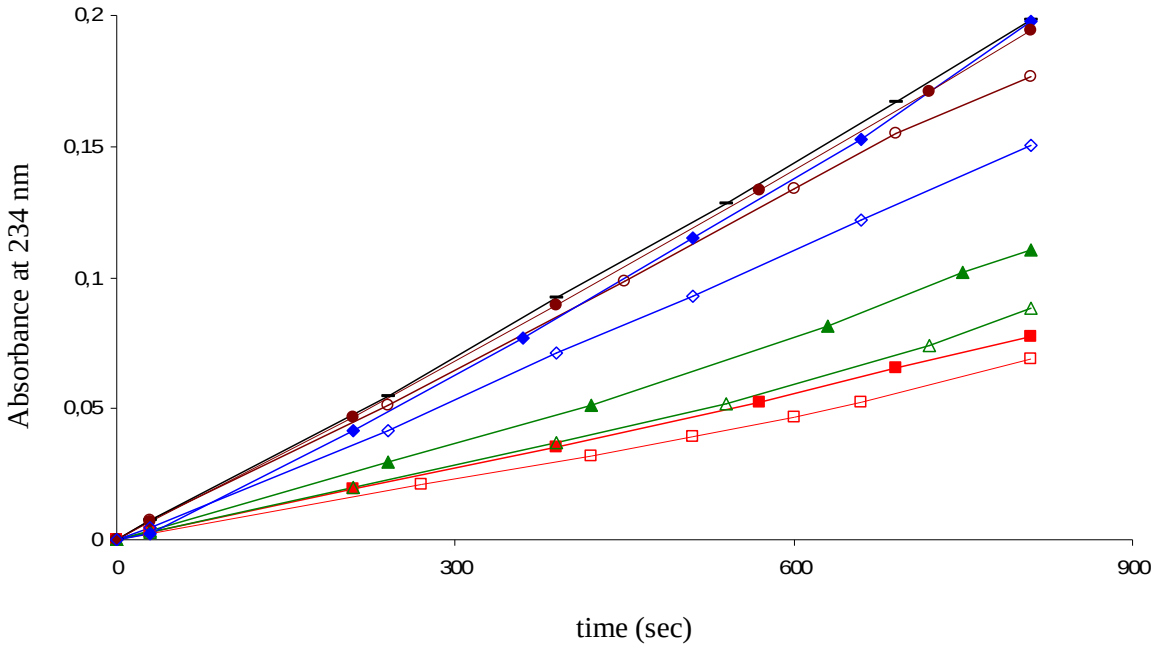


Figure 15: Relative accumulation of hydroperoxides at 234nm generated from peroxidation of linoleic acid (2.55 mM) in SDS micelles (0.1 M) initiated by AAPH (1 mM). Samples conc. (8.1mg/ml). = ME red onion, = SE red onion, = ME yellow onion, = SE yellow onion, = ME white onion, = SE white onion, = ME onion grelot, = SE onion grelot, = Blank

In the inhibition of lipid peroxidation, the hydrophilic onion antioxidants are expected to act in the aqueous phase and/or at the interface by reducing the initiating peroxyl radicals and not in the lipid phase where the propagating lipid peroxyl radicals are located [39]. Consistently, no lag phase (typical of chain-breaking antioxidants) is observed and the peroxidation is simply retarded (Figure 15).

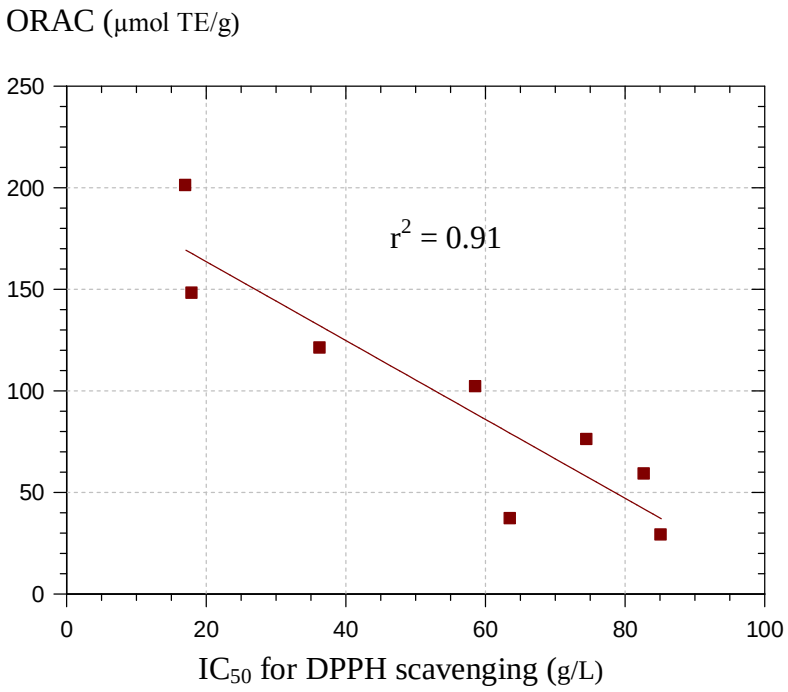
ORAC test. In the ORAC test, antioxidants inhibit the AAPH-induced oxidation of fluorescein by reducing the AAPH-derived peroxyl radicals (ROO•). It was possible to rank the extracts (Table 9) as follows: MHG red onion (201 µmol TE/g)> CSE red onion (148

µmol TE/g)> MHG yellow onion (121 µmol TE/g)> CSE yellow onion (102 µmol TE/g)> MHG white onion (76 µmol TE/g)> CSE white onion (59 µmol TE/g)> MHG grelot onion (37 µmol TE/g)> CSE grelot onion (29 µmol TE/g). Our ORAC values are higher than those of Boivin et al. [60] (11.5 µmol TE/ml for yellow onion, 10.6 µmol TE/ml for green onion). However, our results about white onion extracts are comparable to those by Ou et al. [61] (85 µmol TE/g as mean value of 33 white onion samples). A possible reason for discrepancies might be variations in the concentration of free quercetin in various layers of different onion varieties. In fact, free quercetin exists mostly in the outer dry layer of red and violet onions [17], which consequently has a high antioxidant activity. On the other hand, quercetin is found as glucosides in the edible portions of onion bulbs and only rarely as aglycone [11]. These results are also supported by our quantification of flavonols by HPLC (Table 8). All the varieties investigated contained quercetin glucosides but free quercetin was undetectable. The highest antioxidant activities were found in the most strongly colored variety, red onion, and the lowest activities in white and grelot onions.

Since both the ORAC and DPPH assays measure radical-scavenging activities in homogenous aqueous or MeOH solutions, a close correlation between the data was expected and actually observed (Figure 16A). No such correlations were observed with the inhibition of lipid peroxidation (Figure 16B). In fact, when the radical-scavenging activity is increased (lower IC50 values in the DPPH assay reflecting a higher antioxidant concentration), the inhibition of lipid peroxidation first becomes more efficient (lower IC50 values), then remains essentially constant. This biphasic behaviour could reflect saturation of the water-lipid interface of the micelles by the polar antioxidants.

The OH groups of quercetin that are most critical to its potent H-atom donating activity (lowest bond dissociation energy) are the OH groups at the 3 and 4’ positions, i.e. precisely those that are conjugated to Glc residue in onions (Table 8). Hence, it can be claimed that the quercetin glucosides found in onions are only poor antioxidants. Moreover, the total contents of reducing compounds are much higher than the total concentrations of quercetin glucosides. Thus, it can be assumed that the quercetin glucosides only make a marginal contribution to the overall antioxidant activity of onion extracts. As an example, red onion (MHG extract) is 50-60 times more concentrated in quercetin glucosides than white onion (134.7 vs. 2.4 mg quercetin equiv. / 100 g DW) while being only 3-4 times more antioxidant as demonstrated by the DPPH and lipid peroxidation tests.

A



B

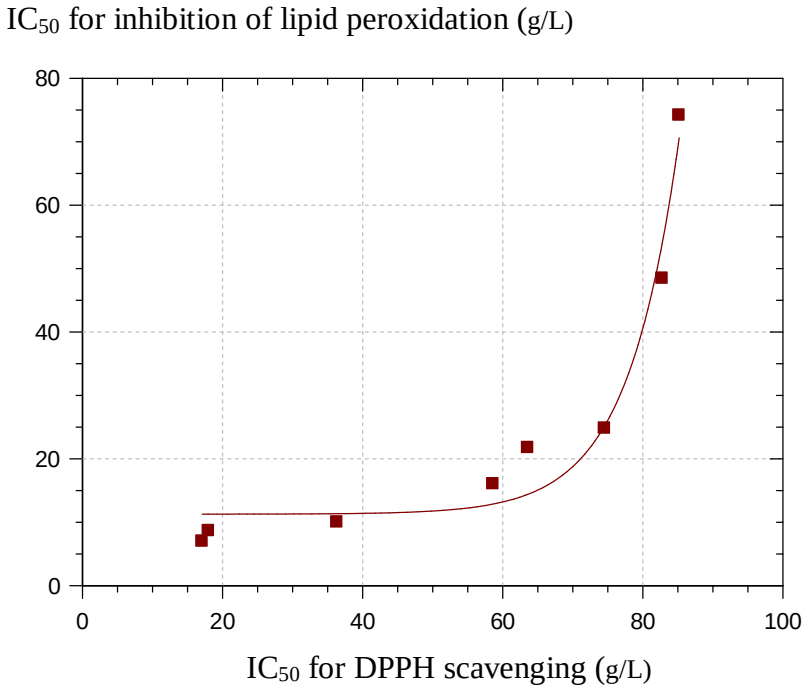


Figure 16: Correlations between the data of the three antioxidant assays (experimental points are from Table 9).

On the other hand, when the antioxidant tests are expressed by reference of the total contents of reducing compounds, only weak differences (at most, a factor 2) emerge between extracts. This is a strong indication that the distribution of reducing compounds is similar in all extracts and that the overall pool of reducing compounds is responsible for the antioxidant

and radical scavenging activities. In particular, the fact that red onion extracts are more potent than white onion extracts is simply due to the higher content in reducing compounds (ca. a factor 3.3) of the former. The pool of reducing compounds may include phenols distinct from quercetin glucosides, reducing sugars and sulphur-containing compounds (S-alk(en)yl-L-cysteine and sulfoxides, cysteine-containing peptides) [62–64].

II.3.2.2. Antioxidant Activity Evaluation of food by-products Sea buckthorn (Hippophae rhamnoides)

Sea buckthorn is a bush that grows widely in various regions of Asia, Europe, and North America. It is a hardy plant, drought and cold tolerant, useful for land reclamation and farmstead protection. Different parts of Sea buckthorn have been used for the treatment of diseases in traditional medicines in various countries in the world [65]. These beneficial effects have made sea buckthorn products, especially oil, desirable for medicinal and cosmetic purposes [66]. Sea buckthorn products available in the market range from oil, juice, and food additives to candies, jellies, cosmetics, and shampoos. Its fruit are berries of orange to red colour and have an acid, lightly bitter taste. It flowers in April, and the fruits are collected through August to October. Sea buckthorn berries are a rich source of many different bioactive compounds that may contribute to the claimed and proven health benefits of sea buckthorn juice and oil. Juice extraction from sea buckthorn berries leads to a residual press cake whose pulp is reported to contain mainly flavones [67]. Sea buckthorn pomace is a by-product which produced during sea buckthorn juice extraction, consisting of pulp, seed and skin (figure 17).

Utilization of these by-products as feed stock of some other process for extraction of beneficial components has been increased over the last few decades. Along with reducing the wastes, the purpose behind their utilization is also the extraction of beneficial antioxidants. In recent years, intensive research on natural antioxidants has been developed because synthetic antioxidants such as butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) have shown serious side effects [68]. Polyphenols are the major plant compounds which are considered as major preventive agents against several degenerative diseases and protect the body tissues against oxidative stress. Special attention is focussed on their extraction from inexpensive or residual sources with more clean and green extraction techniques which typically use less solvent and energy, such as microwave extraction, supercritical fluid

extraction, ultrasound extraction, ultrafiltration, flash distillation, controlled pressure drop process, and subcritical water extraction. These novel methods have been used for extraction of different compounds form various parts of sea buckthorn (Table 10).

Table 10: Previous articles on extraction of sea buckthorn by different novel techniques

Extracted parts of sea buckthorn	Analytes	Comments	References
Leaves	flavonoids	30 min of UAE gave the same result as soxhlet for 240 min	[69]
Seeds, Fruits, Pulp, Leaves	flavonoids	For the same part of the vegetable the compound extract differs with the extraction method (Microwave, UAE, Soxhlet)	[70]
Seeds	β-Sitosterol	SFE represents an efficient and environmentally friendly technique for isolation of phytosterols	[71]

UAE (ultrasound assisted extraction), SFE (supercritical fluid extraction)

In this paper, MHG is applied to sea buckthorn by-products to extract specific antioxidants (figure 17). The extracts were identified for flavones content by HPLC, and then analysed for their total phenolic contents (TPC) and antioxidant activity by the reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical.

After pressing sea buckthorn for juice production, by-products (peels, seeds, residual pulp) contain around 57 % of moisture. It is generally bounded water contained inside the cells which cannot be extracted by mechanical press. Using MHG, it is possible to extract this “in situ” or bounded water contained in the cells with other bioactive compounds. After microwave treatment, the moisture of residual solid is about 31 %. MHG had extracted (66 %) of bounded water with antioxidants which cannot be done with conventional procedures. The dry extract yield obtained for microwave extracts was 3% of the dry contents of extracted plant material against the conventional solvent extraction method (CSE), which has resulted 17.6% dry extract yield.

The compound detected in higher concentration in the by-product of sea buckthorn was Isorhamnetin 3-*O*-rutinoside (123 mg/100g DW in MHG extract and 187 mg/100g DW in CSE extract), followed by Isorhamnetin 3-*s*-glucoside. With CSE we have got a little higher content of these compounds in comparison to MHG extract. These results are also found in good relation with the content of these compounds detected by Chu Chen [72] in 34

samples of different varieties of sea buckthorn. These major compounds were followed by Quercetin 3-*O*-Glucoside and Isorhamnetin in little higher concentration in microwave extracts as shown in Table 11.

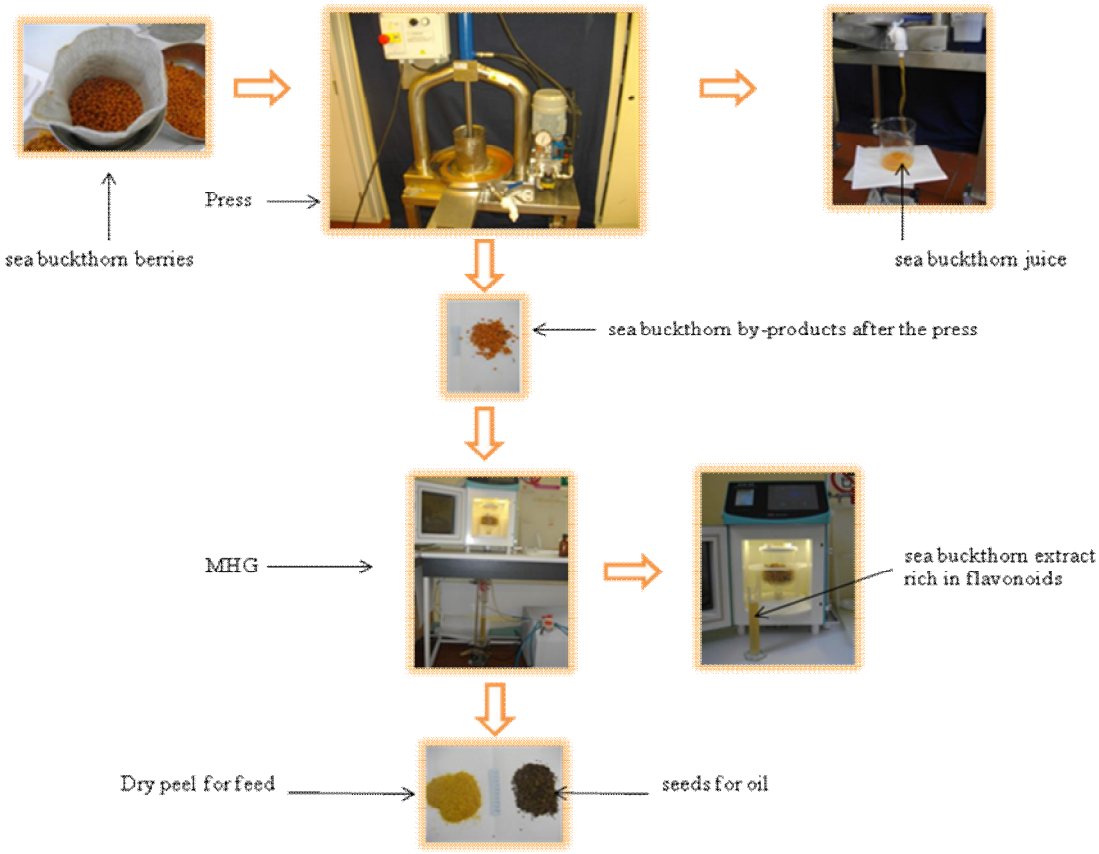


Figure 17: Processing of sea buckthorn berries

Table 11: Flavonoids contents obtained at 400 W of MHG and by CSE method (RF).

Flavonoids (retention time)	MHG (mg/ 100g DW)	CSE (mg/100 g DW)
Isorhamnetin 3-*O*-rutinoside (30.2 min)	123 ± 5.17	187 ± 4.01
Isorhamnetin 3-*O*-glucoside (31.5 min)	97 ± 3.21	162 ± 3.04
Quercetin 3 O Glucoside (27 min)	25 ± 0.14	16 ± 0.32
Isorhamnetin (45.9 min)	0.84 ± 0.02	0.64 ± 0.01

The Folin-Ciocalteu method allowed a good discrimination between the MHG and CSE extracts (Table 12). MHG has extracted a much higher concentration of phenolic contents in comparison to CSE extracts. This much higher concentration of phenolic contents in MHG extract against the lower yield of flavonol content by HPLC (Table 11) has shown clearly that flavonols are not responsible for the total reducing activity of sea buckthorn extracts. As there are many other compounds present in sea buckthorn like vitamin C, anthocyanin contents, fatty acids (palmitic, palmitoleic, stearic, oleic and linoleic) [73] or phenolic acids like gallic acid, protocatechuicacid, p-hydroxybenzoic acid, vanillic acid, salicylic acid, p-coumaric acid, cinnamic acid, caffiec acid and ferulic acid as determined by Arimboor [74] in the berries and leaves of sea buckthorn and MHG proved itself more efficient than CSE by extracting the overall pool of these reducing compounds. Our detected range of phenolic contents was found in good correlation with the previous literature [74].

TPC values of both the extracts also correlate with the IC50 values obtained by DPPH-scavenging capacity assay (Table 12). MHG extract with lower IC50 value (0.71g/L) against CSE extract (with higher IC50 value 1.23g/L) has shown its competence for extraction of maximum content of reducing compounds without using any solvent against conventional solvent extraction methods. DPPH results have confirmed the TPC results and we have also expressed the DPPH values in mmol of gallic acid equivalent per L in Table 12.

Table 12: Total phenolic content and IC50 values of MHG and conventional extracts obtained by DPPH test

Samples	DPPH test			TPC(mg GAE/g DW)
	[A]IC_{50}	R^2	[B]IC_{50}	
MHG	0.71	0.9981	4.78	1147 ± 11.9
CSE	1.23	0.9943	5.36	741.9 ± 7.6

[A] g of onion extract per L; $IC_{50,}$ concentration for 50% inhibition
[B] mmol of gallic acid equivalent per L

These results expressed in Table 12 has clearly shown that the application of microwaves for extraction of sea buckthorn, has extracted the phenolic contents in higher concentration in comparison to conventional methods without causing considerable changes in the composition, phenomenon which was already described by Paré and Bélanger [75] and Bélanger and Paré [76]. The influence of microwave energy on extraction is strictly thermal

and the microwave energy quantum is given by the usual equation W = h.v. Within the frequency domain of microwaves and hyper-frequencies (300 MHz - 300 GHz), the corresponding energies are respectively 1.24 10-6 eV - 1.24 10-3 eV. These energies are much lower than the usual ionisation energies of biological compounds (13.6 eV), covalent bond energies like OH (5 eV), hydrogen bonds (2 eV), Van der Waals intermolecular interactions (lower than 2 eV) and even lower than the energy associated to Brownian motion at 37°C (2.7 10-3 eV). From this scientific point of view, direct molecular activation of microwaves should be excluded. Some kind of step by step accumulation of the energy, giving rise to a high-activated state should be totally excluded due to fast relaxation.

II.4. CONCLUSION

MHG by successively extracting the flavonol contents, of different onion varieties and by-products of sea buck thorn, in the complete absence of any solvent or water has shown its aptitude toward fulfilling the high demand of consumer for healthier food, pharmaceutical and cosmetic products. In this work, extraction of flavonols has been carried out with MHG (vs CSE) after optimization of different extraction parameters and the extracts were analyzed for their phenol content and antioxidant activity. The results of these assays showed that MHG extracts posses more antioxidant activity than the CSE extracts. Along with this the cytological study of MHG treated onion tissues has also shown the effectiveness of microwaves in opening of cellwalls. On one side this technique is playing a great role in the development of cleaner environment, as it consume no solvent and generate less waste, and on other side it has also reduced the cost and energy consumption on extraction and treatment of wastes.

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Chapter III

Vacuum microwave hydrodiffusion and gravity technique: an idea towards improvement with application of vacuum system

♠ *A novel idea in food extraction field: Study of Vacuum microwave hydrodiffusion technique for onion by-products extraction*

(Submitted to Journal of food engineering)

III.1. INTRODUCTION

Replacement of chemical synthetic compounds with natural substitutes has attracted considerable scientific interest to extract diverse range of components from plant sources, which have wider application in food, pharmaceutical and neutraceutical products having direct interaction with human health. Separation of highly purified products has diverted the attention of extraction chemist towards finding technological solutions by developing new techniques or improving existing methods for eliminating the excessive organic solvents, energy and time usage in extraction processes.

Microwave assisted extraction has been utilized efficiently in the field of extraction as it offers rapid transfer of energy and resulted with subsequent heating of the solvent and extraction matrix. Several classes of compounds such as essential oil [1], aromas [2], pigments [3], antioxidants [4], glycosides and terpenes [5] have been extracted successively by different microwave extraction techniques. However, due to presence of air oxidation of some light sensitive and degradation of some thermosensitive compounds due to high temperature, occur in microwave assisted extraction techniques. Application of vacuum system during extraction process, inhibit the oxidation process and possibly favour the extraction of high coloring nutrient and antioxidant contents. Jun-Xia [6] and Xiao-Hua [7] has recently designed vacuum microwave-assisted extraction and applied it for extraction of polyphenolic compounds and pigments from Chinese herbs. The application of vacuum microwave assisted extraction (VMAE) resulted with better extraction yield at reduced temperature possibly because of no or less degradation of thermally sensitive polyphenolic compounds.

Microwave hydrodiffusion and gravity (MHG) is a recently developed technology with massive potential for variety of extractive applications. Along with other components like essential oil the efficiency of this innovative process has been checked for the extraction of polyphenols from onions as model [8], which is a major source of antioxidants (sulphur compounds, flavonoids) [9 – 11]. Two major groups of flavonoids found in onions are; anthocyanins (cyanidin and peonidin glycosides) [12] and flavonols (quercetin, isorhamnetin, kaempferol and their glycosides). Quercetin and anthocyanins found mainly in yellow and red onion respectively along with possessing strong antioxidant activity, mainly known as colouring pigment. MHG appears as an efficient, economical and environment-friendly approach as it does require less energy and no solvent and simply combines microwaves and

earth gravity. But as it works at atmospheric pressure at the boiling point of˜ *in situ*˝ moisture content, there must be possible degradation of some heat sensitive antioxidant components.

In the need towards the improvement of this novel technique to avoid or reduce the degradation of components, application of vacuum in microwave hydrodiffusion and gravity technique (VMHG) can became a mature step towards development of an effective method for extraction of heat and oxygen sensitive compounds.

In this work, we have designed this VMHG apparatus and optimized it for extraction of polyphenolic compounds by utilizing the by-products of common yellow onion as a model. After optimizing different extraction parameters (microwave power, vacuum conditions and extraction period) the extraction yields (dry and juice contents) were compared with MHG extract (at atmospheric pressure) and also with CSE, along with analysing the heating effect of microwaves in different parts of plant material both in MHG and VMHG. Along with characterizing the extracts not only by their total content of reducing compounds (TCRC) but also by their antioxidant capacity in two tests: the reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the inhibition of the AAPH-induced peroxidation of linoleic acid in SDS micelles.

III.2. MATERIALS AND METHODS

III.2.1. Raw Material

Yellow onion (*Allium cepa* L.) by-products which were rejected by local supermarket in Avignon province (South France) were purchased. These onion by-products were used completely with their outer dry layer for their following processing without any treatment.

III.2.2. Chemicals

All solvents used for chromatographic purposes were HPLC grade. Methanol and Formic acid were from Merck (Darmstadt, Germany) and Acetonitrile was from Fisher Scientific Ltd. (Bishop Meadow Road, Loughborough, UK). The HPLC grade flavonol standards quercetin-3,4′-diglucoside and quercetin-4′-glucoside (spiraeoside) were purchased from Extrasynthese (Lyon, France). Quercetin and quercetin-3-glucoside were purchased from Sigma Chemicals Chimie (Fallavier, France). DPPH (2,2-diphenyl-1-picrylhydrazyl), AAPH (2,2´-azobis (2-methyl propionamidine) dihydrochloride), SDS (sodium

dodecylsulphate), linoleic acid all were from Sigma-Aldrich (Steinheim, Germany). Phosphate buffer was prepared with HPLC grade water to avoid metal traces.

III.2.3. Moisture Content Determination

Moisture content determination of onion by-products (whole bulb of onion including outer dry layer) was carried out firstly by conventional Dean-Stark distillation apparatus according to the official method of the American Oil Chemical Society (AOCS) [13] and also by dehydration in an electric oven at 80°C. The average moisture content measured by both processes was 84.5 ± 0.5%.

III.2.4. VMHG Apparatus and Procedure

Extraction was performed by Microwave hydrodiffusion and gravity apparatus (Milestone EOS-G microwave laboratory oven) patented by Chemat [14], illustrated in figure 18. We have modified this system by introducing the vacuum; Vacuum was created in the system by a vacuum pump (KNF Neuberger, NSE 800, Germany). Vacuum pump was fitted between the condenser and the flask which were used to collect onion extract. The extraction was continued until no more extract was obtained. Extracted crude juice was collected and freeze-dried.

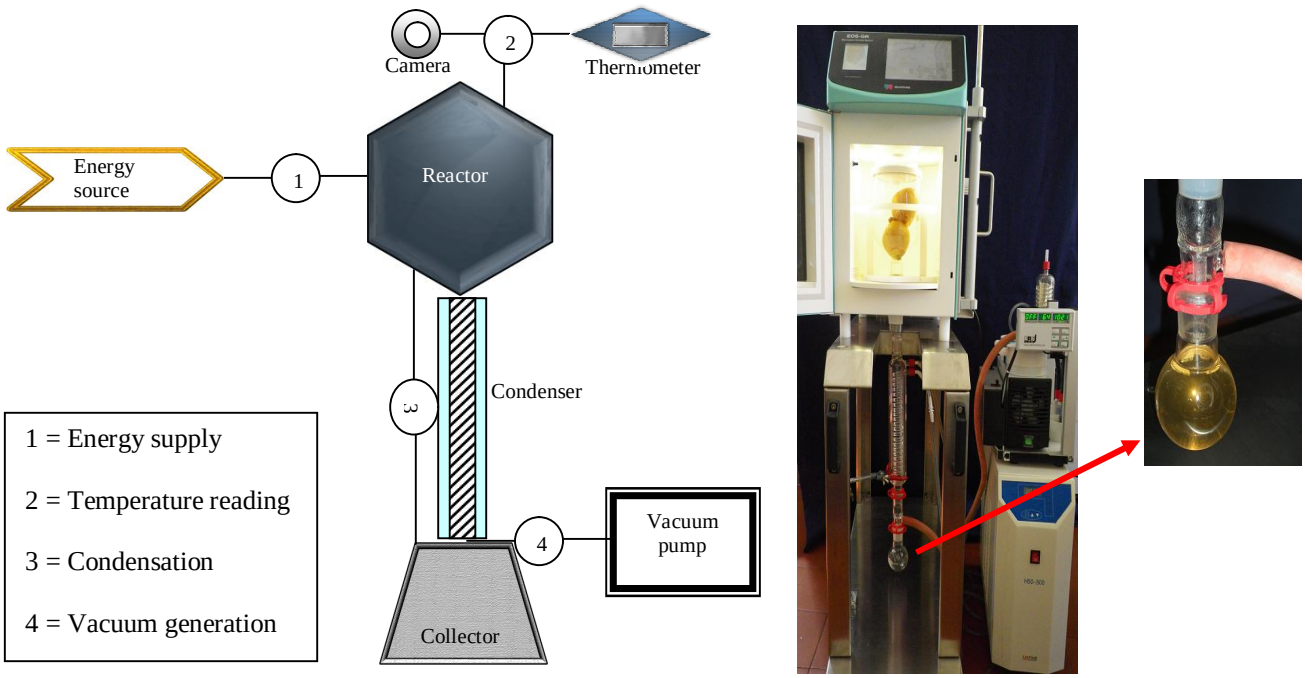

Figure 18: Explanatory diagram of Vacuum microwave hydrodiffusion and gravity apparatus

III.2.5. Conventional Solid-liquid Extraction

In conventional solid-liquid extraction onion scales were used, complete onion bulb was cut manually, 5g onion scales were homogenised with 50mL of 80% methanol in an ultrahomogeniser at 8000 rpm for 5min. After that the mixture was filtered and supernatant was collected, methanol was removed from the extracts by evaporation under vacuum at 40°C with rotary evaporator.

III.2.6. HPLC Analysis

HPLC analyses were performed using a Waters (Milford, MA) HPLC system consisting of a Waters 600E pump, a Waters 717 manual injector rheodyn, a Waters 2996 photodiode array detector. The HPLC pumps, manual injector rheodyn, column temperature, and diode array system were monitored and controlled by using Waters Empower 2 Chromatography Data software program. The wavelength used for the quantification of the onion flavonoids with the diode detector was 360 nm. The chromatographic separation was carried out on a Purospher Star RP-18 end-capped column (250 mm × 4 mm I.D.; 5 µm particle size from VWR), with a RP18 guard column (4 mm×4mm I.D.; 5µm particle size also from VWR). The end-capped column and guard column were held at 37°C and the flow rate was set at 1mL/min. The mobile phase consisted of two solvents: (A) acidified water (0.5% formic acid) and (B) 100% acetonitrile. The solvent gradient used was the following: 0 min, (A) 95% and (B) 5%; 20min, (A) 60% and (B) 40%; 30min, (A) 0% and (B) 100%; 45min, (A) 95% and (B) 5%. The injection volume was 20 µL and all analyses were performed at least three times and only mean values were reported. Identification of flavonoids was done by comparing the elution order and UV-visible spectra. Quantification was carried out by using the external standards of known concentration. Peak areas were used to quantify the compounds in the sample. A linear regression analysis was carried out on the data of the peak area versus concentration. Linear calibration curves of the standards ranging from 10 to 100 mg/L were obtained with good linearity and R^2 values which were more than 99.5% accurate for all the standards. Final concentrations of different flavonoids were calculated in mg/100g DW.

III.2.7. UV-Visible Spectroscopy

All absorbances of total phenolic contents (TPC) and DPPH assay and kinetics of linoleic acid peroxidation test were performed on a Hewlett-Packard 8453 diode array UV-vis

spectrometer equipped with a magnetically stirred quartz cell (optical path length = 1 cm). The temperature in the cell was controlled by means of a water thermostated bath [15].

III.2.7.1. Determination of the Total Content of Reducing Compounds (TCRC)

TCRC was estimated colorimetrically using the Folin-Ciocalteu method [16], with a kit (SEPPAL (Isitec-lab), France) especially suitable for TPC measurement of food products. This kit includes reagent A (modified Folin-Ciocalteu reagent), reagent B (alkaline buffer) and a gallic acid solution (3 g/L). A small volume (20 µL) of H_2O (blank), gallic acid solution (standard) and 200 µL the extract (sample) was mixed with reagent A (2 mL). After 1 min, 1 mL of reagent B was added in both water and gallic acid standard and 850 µL in sample. The mixtures were allowed to stand for 30 min in the dark at room temperature. Then, their absorbance was measured at 760 nm. TCRCs were calculated by using the following formula: TCRC = 3 × (sample absorbance – blank absorbance) / (standard absorbance – blank absorbance). TCRC measurements were performed thrice and mean values, expressed as mg gallic acid/g of dry weight (mg GAE/g DW), were reported.

III.2.7.2. Antioxidant tests

III.2.7.2.1. DPPH Assay

DPPH is a stable highly coloured free radical that can abstract labile hydrogen atoms from phenolic antioxidants with simultaneous formation of a colourless hydrazine (DPPH-H) [17]. The radical scavenging activity of extracts was evaluated according to the method described by Monzocco [18], with some modifications. About 200 µL of a solution of flavonol (standard) in MeOH or of onion extract in water were added to 2 mL of a DPPH solution ($6x10^{-5}$ M) in MeOH and the mixture was left in the dark at room temperature for 30 min. The decrease in absorbance was measured at 517 nm. The percentage of DPPH consumed was calculated from the following equation: $I\ \% = 100 \times (A_0 - A_1) / A_0$, where A_0 was the initial absorbance (no antioxidant) and A_1 the absorbance in the presence of the extract or standard at different concentrations. The IC_{50} of each extract or standard (concentration giving I % = 50) was calculated by plotting I % as a function of the extract concentrations. All experiments were performed in triplicate.

III.2.7.2.2. Inhibition of Linoleic Acid Peroxidation

Two mL of a freshly prepared pH 7.4 phosphate buffer containing linoleic acid (2.55 mM) and SDS (0.1 M) were placed at 37°C in the spectrometer cell. At time zero, 25 µL of a freshly prepared aqueous solution of AAPH (80 mM) was added, followed by 25 µL of the antioxidant solution after 15 min. Quercetin solutions were prepared in MeOH. Onion extracts were not soluble in MeOH due to their high sugar content and were thus dissolved in water. The experiments were repeated with different concentrations of standard (1 mM and lower) and extracts. The initial level of linoleic acid hydroperoxides (molar absorption coefficient at 234 nm = 26100 M^{-1} cm^{-1}) [19], was below 2% in all experiments. The uninhibited and inhibited peroxidation rates were calculated from the slope of the lines expressing the absorbance at 234 nm *vs* time before and after antioxidant addition using fixed time intervals. Each experiment was run in triplicate. Finally, the IC_{50} values (concentration providing 50% inhibition) of each extract and standard were calculated graphically by plotting the ratio between inhibited (R_P) and uninhibited peroxidation rates ($R_P°$) vs the extract concentration.

III.3. RESULTS AND DISCUSSION

III.3.1. Preliminary Study

To determine optimal reaction conditions for obtaining significant results a preliminary study consisting of various experiments was carried out. Efficient performance of VMHG for onion antioxidants extraction depends on different factors mainly degree of vacuum, time and temperature.

Effect of degree of vacuum on the dry extract yield of onion by-products has shown in the figure 19, Different degrees of vacuum from 200mbar - 900mbar were analysed at irradiation power of 500W for 500g of plant material as already optimized for complete onions in our first publication [8], in order to find optimized vacuum conditions for extraction of onion by-products. High degree of vacuum (at 200mbar) has resulted low dry extract yield (0.84%), in contrast the same degree of vacuum was good for maximum extraction of moisture content (78.9%). At such reduce pressure obviously it's possible to extract maximum *in situ* water content of plant material without burning, but due to lower capacity of inside moisture due to the low temperature, higher resistance of surface reduce cell disruption, resulted with decrease yield of solid contents. While, moving towards low vacuum the maximum recovery of solid contents was possible without burning just in 26 ± 1min of extraction. Figure 19 shows the highest soluble solid contents yield at vacuum condition of

700mbar (3.18%), at this vacuum condition cell disruption was promoted due to higher temperature of *in situ* water content. At atmospheric conditions in comparison to 700 mbar decrease amount of solid contents (2.86%) was obtained with less moisture content (68%) due to rapid set off of burning process within 23 ± 1mins of extraction duration.

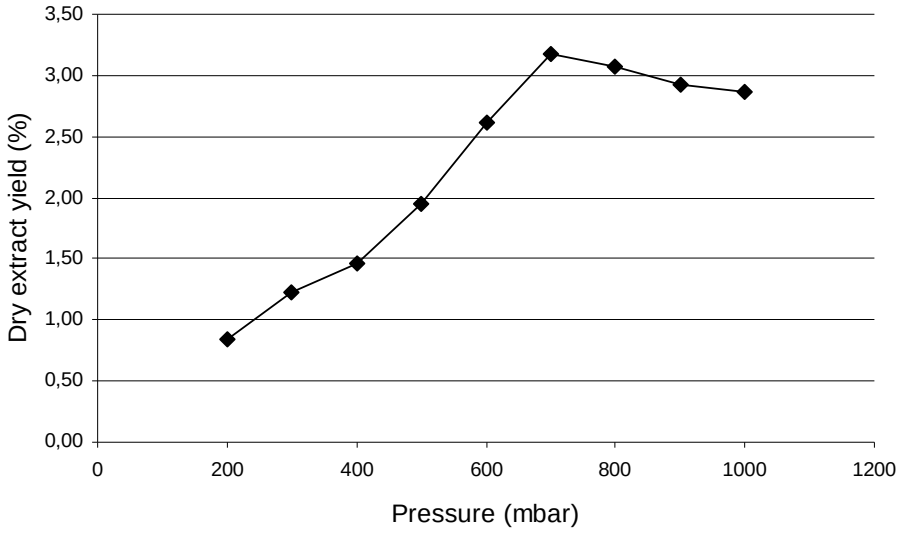

Figure 19: Effect of degree of vacuum on dry extract yield of onion by-products

III.3.2. Comparison of Extraction Procedures

III.3.2.1. Microwave Heating (VMHG vs MHG)

Three different phases (A. heating phase, B. extracting phase, C. burning phase) as already described in detail in our previous publication [8] figure 20, were studied in this VMHG system by using temperature sensor optic fibers. The VMHG system differentiate from MHG by working at less temperature, maximum temperature achieved in central part and outer layer was 87°C ± 1°C, but the reactor temperature stayed at 81°C ± 1°C. At end the burning phase was perceived more quickly (after 23 ± 1min of extraction) when irradiated by microwaves under atmospheric pressure (MHG), In contrast, with VMHG extraction method it was possible to extract until 26 ± 1min of extraction without the onset of burning. In case of VMHG the higher temperature in the central part of onion indicated the increase pressure working inside the plant material instead of the reactor. This pressure difference, which was created in the combination of microwave heating and vacuum application, resulted with internal superheating and facilitated the desorption of matrix *insitu* moisture with improved recovery of soluble solid contents by promoting cell disruption.

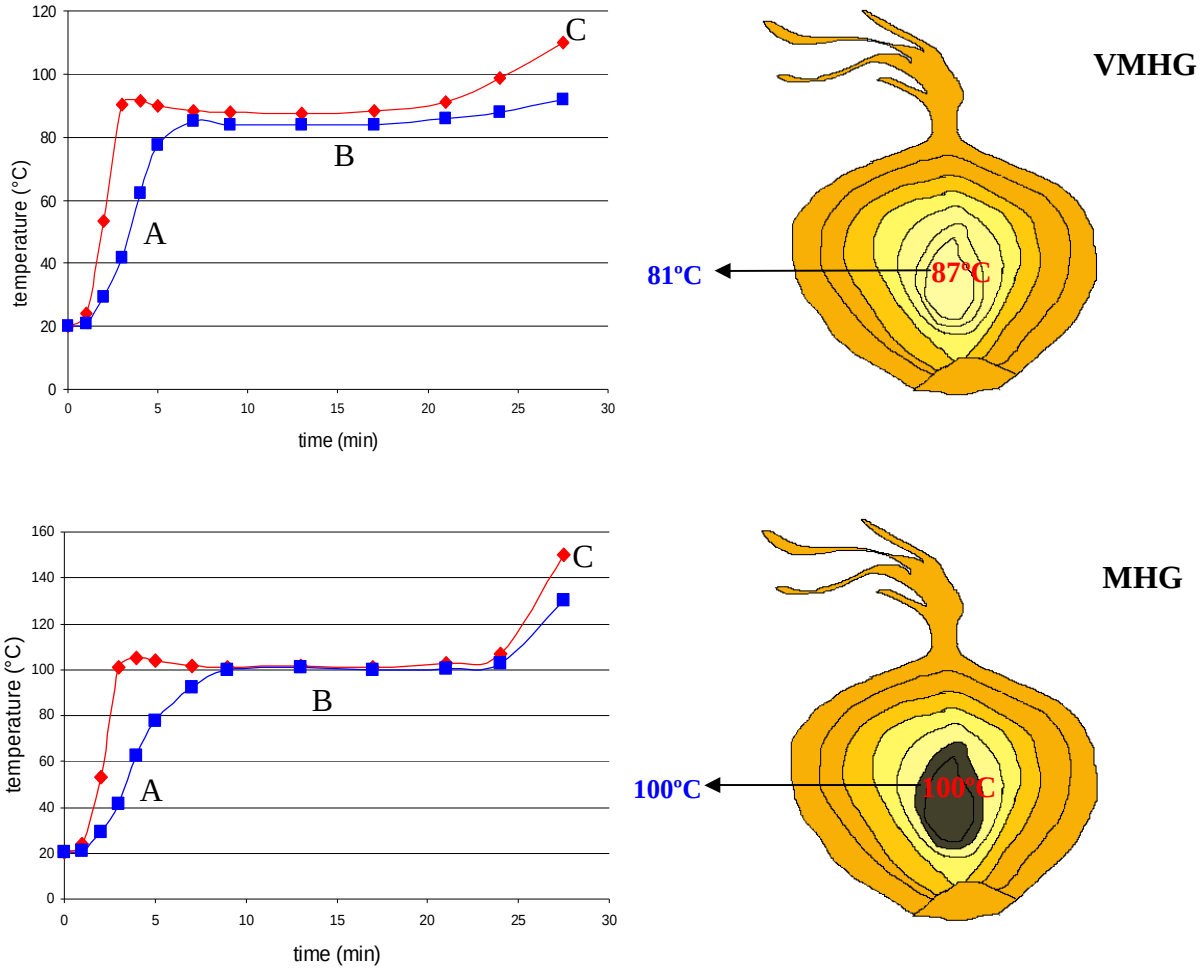

Figure 20: Temperature difference in central part of onion (—◆—) and reactor (—■—) at atmospheric pressure (MHG) and under vacuum condition (VMHG). A = heating phase, B = extracting phase, C = burning phase

III.3.2.2. Extraction Kinetics (VMHG vs MHG)

To evaluate extraction efficiency of VMHG, MHG was also applied at optimized conditions to study the extraction curve (Figure 21). Three diverse phases (phase 1, induction time period; phase 2, extraction period; phase 3, dead phase) of extraction were obtained with both the processes as already discussed in detail in our first publication [8]. With VMHG it is possible to extract almost 75% of moisture content of onion by-product without the onset of burning within optimized time period (26 ± 1min), which is close to actual moisture percentage (84.5%) of onion by-products. This moisture yield is almost 7% more than the extractable moisture amount by MHG (68 % in 23 ± 1min) with a short delay.

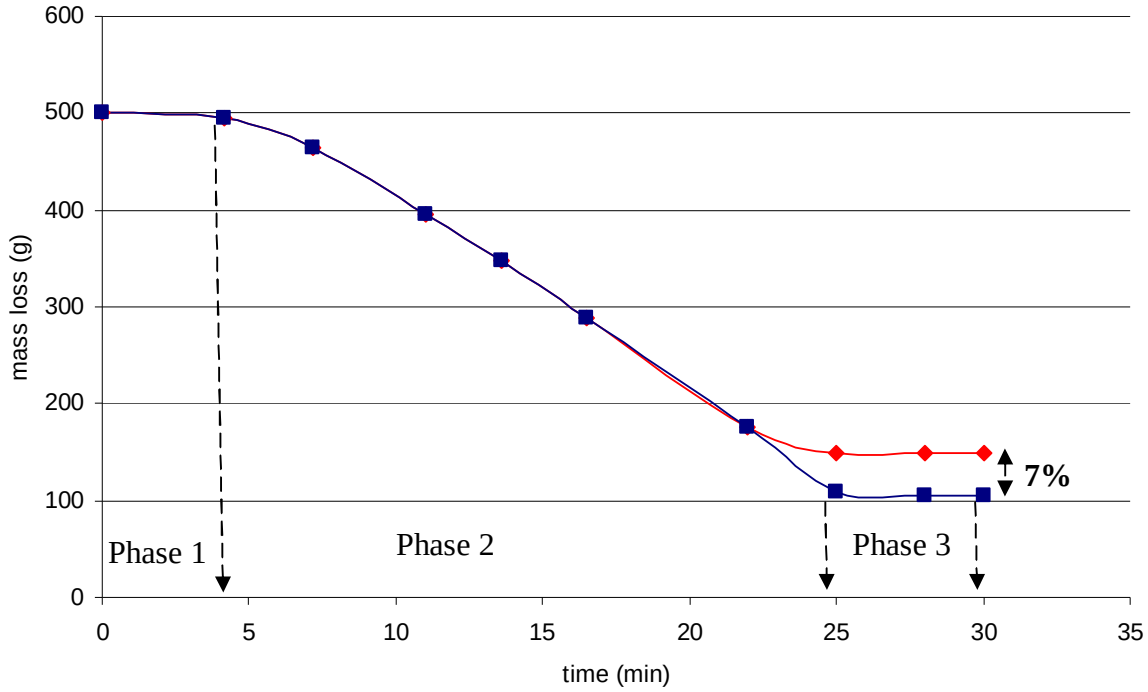

Figure 21: Extraction efficiency of VMHG (—■—) and MHG (—◆—)

III.3.3. Quantification of Flavonoid Content

In order to minimise the loss of nutritionally important components from onion by-products, we have studied their composition after extraction by VMHG, MHG and also by conventional solvent method (CSE). As the polyphenolic composition of outer dry onion layer is considerably different from the edible portion [20], analytical data obtained by HPLC revealed the presence of quercetin-3,4′-diglucoside and quercetin 4′-glucoside as predominant form along with possessing higher concentration of quercetin aglycone in all the extracts obtained by three different extraction methods (Table 13), these results are in consistence with the previous literature [21–23]. No doubt MHG without using any solvent is permitting the extraction of substantial concentrations of flavonols ‘total quercetin’ (283.09 mg QE/100g DW), which get improved upto 57% with application of vacuum system (662.27 mg QE/100g DW by VMHG). Remarkably, the VMHG extract are richer in flavonol contents in comparison with MHG extract and the higher concentration of quercetin, 4′-glucoside and 3-glucoside in comparison with their content obtained by standard extraction process reveals clearly the effectiveness of vacuum system in MHG technique. The application of vacuum condition in VMHG system has provide the advantage of higher flavonol content yield along with extracting maximum moisture content, without burning against the atmospheric extraction conditions of MHG.

Table 13: Flavonol contents and total contents of reducing compounds (TCRC) of MHG, VMHG and CSE extracts

Samples	QA* (mg/100g DW) 23.81 min	QDG* (mg/100g DW) 16.18 min	Q4G* (mg/100g DW) 19.85 min	Q3G* (mg/100g DW) 17.88 min	Total quercetin (mg QE/100g DW)	TCRC (mg GAE/g DW)
MHG	103 ± 4.5	200.7 ± 15.1	116.2 ± 3.8	11.9 ± 1.69	283.09	112.5 ± 1.9
VMHG	239 ± 16.4	489.7 ± 16.5	257.5 ± 6.6	29.8 ± 1.83	662.27	117.2 ± 2.1
CSE	395 ± 14	665.6 ± 22.3	250.2 ± 4.1	16.9 ± 0.34	890.59	114 ± 1.6

*QA=quercetin aglycone; QDG=quercetin-3,4′-diglucoside; Q4'G=quercetin-4′-glucoside; Q3G=quercetin-3-glucoside

III.3.4. Antioxidant Activity

The extract obtained by this new apparatus (VMHG) was compared to those obtained by MHG and CSE for their total content of reducing compounds, their radical scavenging capacity DPPH and their ability to inhibit linoleic acid peroxidation. These popular methods are based on the ability of phenols to transfer electrons or H-atoms to metal ions and radicals.

Generally, the antioxidant activity of onion specimens (*Allium cepa*) are linked with the flavonol fractions, instead of total phenols [24], in contrast to these findings our results does not consider the flavonol content totally responsible for reducing phenomenon. The highest content of reducing compounds was found in the VMHG extract (117.2 ± 2.1mg GAE/g DW), followed by almost comparable TCRC values of CSE and MHG extracts (Table 13). Interestingly, the VMHG and MHG extracts afforded lower concentration of total flavonol content than CSE, even though the CSE extract contained a good percentage of free quercetin MHG proved itself comparable and VMHG slightly more efficient than CSE for extracting the overall pool of reducing compounds. The TCRC values reported in this work are consistent with the literature [21, 25]. TCRC values generally correlate with the DPPH-scavenging capacity with lower IC_{50} value 4.39 mg/ml (means more efficient) for VMHG extract followed by CSE and MHG with almost comparable IC_{50} values (Table 14).

Table 14: DPPH reducing capacity of different extracts, their inhibition action against linoleic acid peroxidation in SDS micelles expressed as IC_{50} values

Samples	DPPH test			Inhibition of lipid peroxidation		
	[A]IC_{50}	r^2	[B]IC_{50}	[A]IC_{50}	r^2	[B]IC_{50}
MHG	7.4	0.9981	4.9	7.7	0.9997	5.1
VMHG	4.39	0.9909	3.0	6.2	0.9985	4.3
CSE	6.27	0.9943	4.2	7.4	0.9969	5.0

[A] g of onion extract per L; $IC_{50,}$ concentration for 50% inhibition
[B] mmol of gallic acid equivalent per L

Almost the similar pattern we have got with a more reliable antioxidant activity test, *Inhibition of the AAPH-induced peroxidation of linoleic acid in SDS micelles*. The VMHG extract have comparatively low IC_{50} value in comparison to CSE and MHG extracts (Table 14). In the inhibition of lipid peroxidation, the hydrophilic onion antioxidants are expected to act in the aqueous phase and/or at the interface by reducing the initiating peroxyl radicals and not in the lipid phase where the propagating lipid peroxyl radicals are located [19]. Consistently, no lag phase (typical of chain-breaking antioxidants) is observed and the peroxidation is simply retarded (Figure 22).

It can be assumed from above discussed results that the flavonol contents are not totally responsible for the overall antioxidant activity of onion extracts. As CSE extract is 1.3 and 3.1 times more concentrated in flavonol content than VMHG and MHG extracts, even though it posses higher concentration of free quercetin, respectively but it has revealed less antioxidant competence in comparison to VMHG extract as demonstrated by the DPPH and lipid peroxidation tests. It's surprising that even though the CSE extract have enough amount of free quercetin, which is considered as a potent antioxidant, only make a marginal increase in antioxidant activity when compared with MHG extract which posses much less concentration of quercetin aglycone but exhibited less antioxidant competence in comparison to VMHG extract. On the other hand, when the antioxidant tests are expressed by reference of the total contents of reducing compounds, only weak differences emerge between extracts. These results strongly confirm that flavonols (even free quercetin) are not responsible for over all antioxidant activity of extracts.

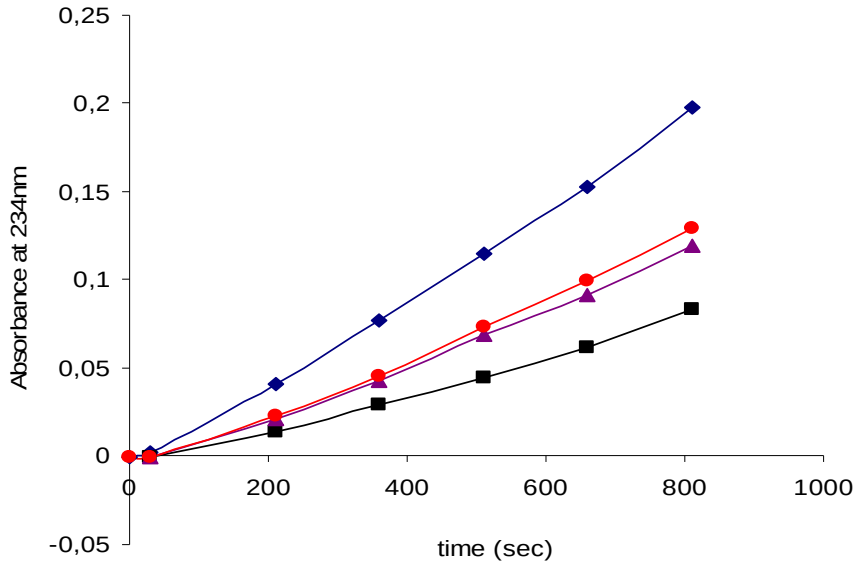

Figure 22: Relative accumulation of hydroperoxides at 234nm generated from peroxidation of linoleic acid (2.55 mM) in SDS micelles (0.1 M) initiated by AAPH (1 mM). Samples conc. (5mg/ml). = Blank, = MHG, = CSE, = VMHG

The overall pool of reducing compounds is responsible for the antioxidant and radical scavenging activities, which may include phenols, reducing sugars and sulphur-containing compounds (S-alk(en)yl-L-cysteine and sulfoxides, cysteine-containing peptides) [26 – 28]. These results strongly indicate the remarkable influence of microwaves in extraction, which played a vital role in increasing the antioxidant activity by extracting more antioxidant components, especially with application of vacuum in MHG system it's possible to extract more components without the onset of burning until 26 min of extraction.

III.4. CONCLUSION

VMHG, an extraction technique which is the combination of MHG with vacuum system, was designed and applied for extraction of onion by-products as model in the absence of any solvent or water. The results revealed the efficiency of this new process by extracting more moisture and dry contents yield at less temperature. VMHG has shown its predominance for efficient and increase recovery of onion flavonol contents (57%) in comparison to MHG and thus more antioxidant not only in contrast to MHG but also CSE extracts. This novel extraction method offers an opportunity to develop efficient procedure for the extraction of valuable heat sensitive plant components with maximum recovery of *in situ* moisture content. We proposed this system as a green and environment friendly for extraction of components which have wide application especially in the field of food, pharmaceutical and cosmetic.

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Chapter IV

A comparison technique for flavonoids extraction: Ultrasound assisted extraction

IV.1. INTRODUCTION

IV.1.1 Green extraction techniques

Shortcomings of existing extraction technologies, like increase consumption of energy (more than 70% of total process require energy), high rejection of CO_2 and more consumption of harmful chemicals, have forced the food and chemical industries to find new separation "green" techniques which typically use less solvent and energy, such as microwave extraction, supercritical fluid extraction, ultrasound extraction, ultrafiltration, flash distillation, the controlled pressure drop process and subcritical water extraction. Separation under extreme or non-classical conditions is currently a dynamically developing area in applied research and industry.

IV.1.1.1. Microwave-assisted extraction (MAE)

Using microwaves, extraction and distillation can now be completed in minutes instead of hours with high reproducibility, reducing the consumption of solvent, simplifying manipulation and work-up, giving higher purity of the final product, eliminating post-treatment of waste water and consuming only a fraction of the energy normally needed for a conventional separation method such as distillation or solvent extraction. Several classes of compounds such as essential oils, aromas, pigments, antioxidants, and other organic compounds have been extracted efficiently from a variety of matrices (mainly animal tissues, food and plant materials). The advantages of using microwave energy, which is a non contact heat source, includes: more effective heating, faster energy transfer, reduced thermal gradients, selective heating, reduced equipment size, faster response to process heating control, faster start-up, increased production, and elimination of process steps [1-2].

IV.1.1.2. Supercritical fluid extraction (SFE)

SFE is an innovative method with green theme behind its development for the extraction of solid materials using a supercritical fluid [5-6]. Carbon dioxide is the most widely used solvent in SFE because it is non-toxic, non-flammable, cheap, easily eliminated after extraction and endowed with a high solvating capacity for nonpolar molecules. Other possible solvents are Freon, ammonia and some organic solvents [7]. In a typical SFE procedure, the supercritical fluid continuously enters the solid matrix where it dissolves the material of interest. The extraction can be achieved with a remarkably high selectivity by

adjusting the solvating capacity of the supercritical fluid by changing the pressure and/or temperature. Major advantages of SFE include preconcentration effects, cleanliness, safety and simplicity [8]. The drawbacks of SFE are the need of more expensive equipment with the difficulty of extracting polar molecules without adding modifiers to CO_2.

IV.1.1.3. Pulsed electric field extraction (PEF)

Application of pulsed electric field (PEF) either concurrently or before pressure application is an emerging extraction method, which has improved non thermal pressing efficiency by enhancing extraction of valuable components with adequate yield and purity of extracted juice in very short time with very less energy consumption [9]. PEF actually influence textural properties of plant material and improve tissue softness by electroporation of cell membrane resulted with rapid and enhance mass transfer. These short duration pulses of high intensity are not only applicable for extraction of juice at large scale but also permit extraction of sensitive coloring pigment at room temperature [10].

IV.1.1.4. Instantaneous controlled pressure drop (DIC, Détente Instanttanée Contrôlée)

‘‘Instant Controlled Pressure-Drop” process (DIC: Détente Instantanée Contrôlée), is actually a swelling operation which was developed few years ago by Allaf and Louka [11]. The aim behind the development of this process was to improve the hydration capacity and dissolution rate of dry fruits and vegetables, which normally oppose resistance to penetration of solvent due to their natural structure. The matrix is subjected to an abrupt transition from high steam pressure to close to vacuum, resulted with plant product with more expended structure. Initially it was used for food industries especially for drying purpose but laterally it was also used for extraction of volatile components and essential oil from different plant parts including fruits and seeds. Recently the effect of this process was also studied for anthocyanin extraction from Roselle calyces which resulted with increase yield due to higher overall diffusivity [12].

IV.1.1.5. Ultrasound-assisted extraction (UAE*)*

Ultrasound assisted extraction is an emerging potential technology that can accelerate heat and mass transfer and has been successively used in extraction field. Ultrasound waves after interaction with subjected plant material alter its physical and chemical properties and their cavitational effect facilitates the release of extractable compounds and enhances the

mass transport by disrupting the plant cell walls. UAE is a clean method that avoids the use of large quantity of solvent along with cutting down in the working time. Ultrasounds are successively employed in plant extraction field [3-4].

IV.1.2. Applications of ultrasound in food technology

In pre-historic ages, the competition of food urged the processing of food to preserve it for longer times. Nowadays, the processed foods that are flourishing in supermarkets are modern processed foods and traditional foods, but their manufacturing, processing and packaging technologies have been advanced and rationalized to an incomparable extent. The principle aims of these technologies are to reduce the processing time, save energy and improve the shelf life and quality of food products. Thermal technologies (radio frequency and microwave heating), vacuum cooling technology, high pressure processing and pulsed electric field technology are those novel technologies who have potential for producing high-quality and safe food products but current limitations related with high investment costs, full control of variables associated with the process operation, lack of regulatory approval and importantly consumer acceptance have been delaying a wider implementation of these technologies at the industrial scale. In recent years, ultrasound (US) in the food industry has been the subject of research and development. There is a great interest in ultrasound due to the fact that industries can be provided with practical and reliable ultrasound equipment. Nowadays, its emergence as green novel technology has also attracted the attention to its role in the environment sustainability. Ultrasound applications are based on three different methods:

- Direct application to the product
- Coupling with the device
- Submergence in an ultrasonic bath

There are a large number of potential applications of high intensity ultrasound in food processing e.g filtration [13], defoaming [14], degassing [15], depolymerisation, emulsification, cleaning and cutting; others are in use but still under development e.g. drying, freezing and sterilisation and several are of great potential interest but await full realisation e.g. extraction, fermentation and meat tenderisation. A key aspect of the application of ultrasonic methods is that they are often used in conjunction with existing technologies to provide more efficient treatments e.g. thermosonication and drying.

IV.1.3. Ultrasounds theory

Ultrasounds are mechanic waves that necessitate an elastic medium to spread over. The difference between sound and ultrasounds is the frequency of the wave (figure 23): sounds are at human earring frequencies (from16 Hz to 16-20 kHz) while ultrasounds have frequencies above human earring but below microwaves frequencies (from 20 kHz to 10 MHz).

As a sound wave passes through an elastic medium, it induces a longitudinal displacement of particles. In fact, the source of the sound wave acts as a piston on the surface of the medium [16] (figure 23). This results in a succession of compression and rarefaction phases into the medium. When the piston is in its opened position it induces a compression into the medium and when the piston is in its contracted (pull) position it creates a rarefaction phase.

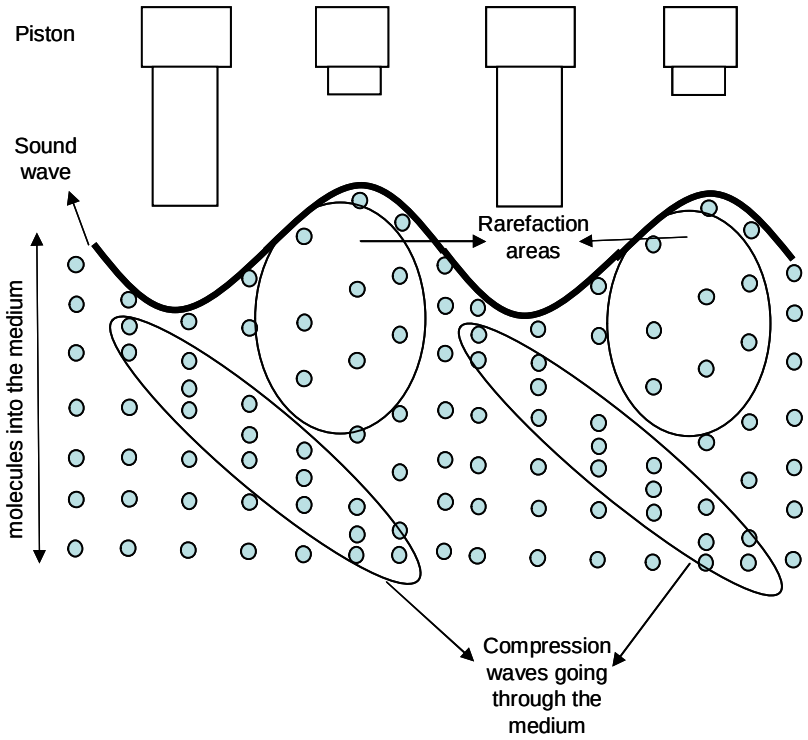

Figure 23: Compression and rarefaction cycles induced by a sound wave

The series of compression and rarefaction regions generated as the sound wave passes through the medium creates an acoustic pressure in this medium. The acoustic pressure into the medium is positive during the compression cycle and is negative during the rarefaction cycle. These variations of pressure during compression and rarefaction phases induce molecular movements involving constriction and distension between groups of molecules. During the rarefaction phase, the distance between contiguous molecules (d) increases (figure 24).

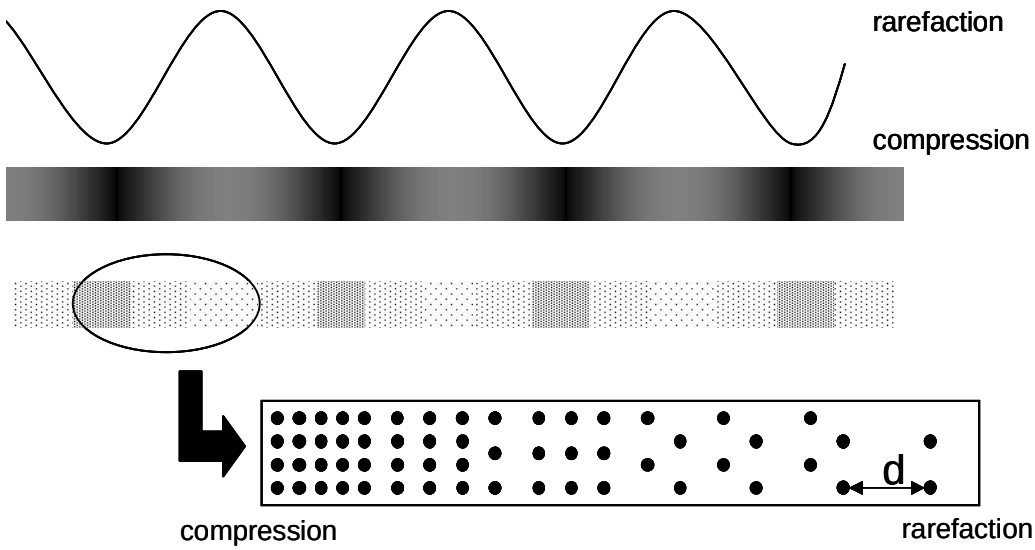

Figure 24: Critical distance between contiguous particles

Every medium has a critical molecular distance: below this critical value, the liquid remains intact, but above this distance, the liquid would break down and voids can be generated into the liquid. In the case of ultrasounds, if the rarefaction cycle is strong enough, the distance (d) between contiguous molecules can reach or even exceed the critical molecular distance of the liquid. The voids created into the medium are the cavitation bubbles which are responsive of ultrasonic effect. In fact theses cavitation bubbles are able to grow during rarefaction phases and decrease in size during compression cycles. When the size of these bubbles reach a critical point they collapse during a compression cycle and release large amounts of energy. The temperature and the pressure at the moment of collapse have been estimated to be up to 5000 K and 2000 atmospheres in an ultrasonic bath at room temperature. This creates hotspots that are able to accelerate dramatically the chemical reactivity into the

medium. When these bubbles collapse onto the surface of a solid material, the high pressure and temperature released generate microjets directed towards the solid surface. These microjets are responsible for the degreasing effect of ultrasounds on metallic surfaces which is widely used for cleaning materials. Another application of microjets in food industry is the extraction of vegetal compounds. As shown in figure 25, a cavitation bubble can be generated close to the plant material surface (a), then during a compression cycle, this bubble collapse (b) and a microjet directed toward the plant matrix is created (b and c). The high pressure and temperature involved in this process will destroy the cell walls of the plant matrix and its content can be released into the medium (d). This is a very interesting tool for ingredient extraction from natural products.

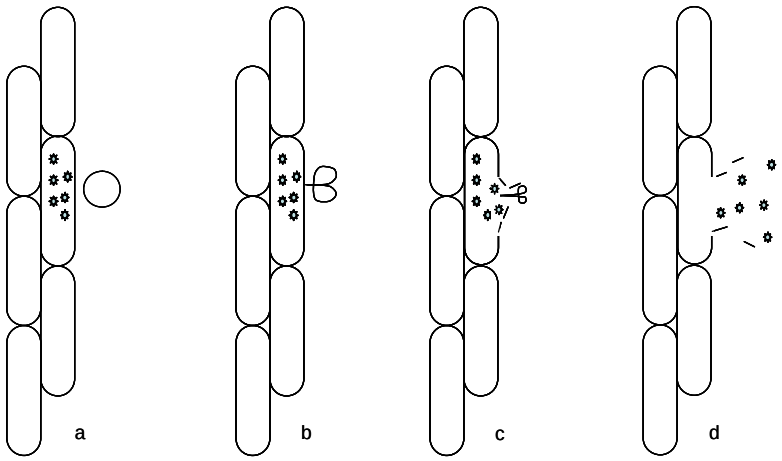

Figure 25: Cavitation bubble collapse and plant material releasing

IV.1.4. Application of US in extraction field

The use of ultrasound can enhance the extraction process by increasing the mass transfer between the solvent and plant material. The collapse of cavitation bubbles leads to better cell disruption through the formation of microjets due to assymetrical bubble collapse near a solid surface. This allows for improved solvent penetration into the plant body itself and can also break down cell walls [17]. As a consequence, employing ultrasound in the use of plant extraction has benefits in increased mass transfer, better solvent penetration, less dependence on solvent used, extraction at lower temperatures, faster extraction rates and greater yields of product [18]. These features make sonication an attractive proposition for many extractions and for scale-up it should be used in the extraction unit itself where the plant material is in direct contact with the solvent. Flavour and fragrances are complex mixtures of

volatile compounds usually present in low concentrations. They are present in varieties of aromatic plants, either in the roots, stems, seeds, leaves, flowers or fruits. The molecules responsive for the flavour of the aromatic plant can be extracted and used in the food industry, medicine or perfumeries. The first aroma extractions are difficult to date precisely. Old testimonies allow concluding that the Hindus controlled fermentation and obtained odorant oils starting from rudimentary distilling apparatuses. Now a days, this distillation process has been improved by its coupling with ultrasound or even the development of new ultrasound-assisted extraction (UAE). Literature reported the extraction of different aroma components from aged brandies [19], tea [20] and wine [21] by ultrasound baths. UAE allowed better yields and a shorter extraction time compared to conventional methods.

UAE has also been developed for essential oil from aromatic plants such as peppermint leaves [22], artemisia [23] and lavender [24], or from other vegetal matrix such as garlic [25] and citrus flowers [26]. Increased yields of essential oil were found for peppermint leaves (up to 12%) and for artemisia when using UAE, and increase by 2 to 3 fold of the main compounds of lavandula essential oil when comparing UAE to conventional distillation. Moreover, UAE not only improved yields but as the method is fast and run at low temperature, the final product usually showed less thermal degradation than traditional methods.

Several studies have been run out on extraction of the main aroma compounds from spices. For example the vanillin was extracted from vanilla pods [27] carvone from caraway seeds [28] and safranal from Greek saffron [29]. Yields of vanillin were comparables after one hour by UAE vs 8 h in conventional extraction. Another publication showed that 80% of the pure vanillin was obtained after only 120 sec of ultrasounds (ultrasonic probe) whilst it took 24 hours with the conventional method to obtain 100% [30]. For carvone extraction, the UAE method was compared to the Soxhlet. Unwanted fatty materials were extracted by Soxhlet extraction while the extract with UAE was of better quality and richer in carvone than in limonene

Wide varieties of fruits and vegetables have been studied by UAE because antioxidants are present in different amounts in different varieties of plants and these antioxidants come from different families. One of the most common antioxidant is the lycopene extracted from tomatoes [31]. In this particular work, authors not only worked on UAE but also on coupling ultrasounds with microwaves which gives high lycopene extraction

in only 6 min. Herrera et al. [32] showed that similar amounts of phenolic compounds could be extracted from strawberries in 2 minutes while it took 20 h with conventional method and 3 h using supercritical fluid extraction. Another example is the extraction of anthocyanins from raspberries developed by Chen et al. [33]. A fast UAE of capsaicinoids from pepper as also been set up by Barbero et al. [34] who developed a reproducible ultrasonic extraction method using methanol as solvent. Citrus by-products are not only used for aromatic compounds contained in the essential oils, but phenolic compounds are also extractable from citrus peels [35] by using ulrtrasound. Other phenolic compounds have been extracted from coconut shell [36] and from wheat bran [37].

Furthermore, it has a number of applications for extraction of polyphenols from different plant matrix but the literature search did not yield any reference about earlier reports on the UAE of phenolic compounds from onions just by using water as a solvent. The objective of this work is to use ultrasound as a novel comparative extraction technique for onion flavonols extraction.

IV.2. MATERIALS AND METHODS

IV.2.1. Raw material

Yellow onion (*Allium cepa* L.) by-products which were rejected by local supermarket in Avignon province (South France) were purchased. These onion by-products were used completely with their outer dry layer for their following processing after cutting in small slices.

IV.2.2. Chemicals

All solvents used for chromatographic purposes were HPLC grade. Methanol and Formic acid were from Merck (Darmstadt, Germany) and Acetonitrile was from Fisher Scientific Ltd. (Bishop Meadow Road, Loughborough, UK). The HPLC grade flavonol standards quercetin-3,4′-diglucoside and quercetin-4′-glucoside (spiraeoside) were purchased from Extrasynthese (Lyon, France). Quercetin and quercetin-3-glucoside were purchased from Sigma Chemicals Chimie (Fallavier, France). DPPH (2,2-diphenyl-1-picrylhydrazyl) was from Sigma-Aldrich (Steinheim, Germany).

IV.2.3. Moisture content determination

Moisture content determination of onion by-products (whole bulb of onion including outer dry layer) was carried out firstly by conventional Dean-Stark distillation apparatus according to the official method of the American Oil Chemical Society (AOCS) [38] and also by dehydration in an electric oven at 80°C. The average moisture content measured by both processes was 84.5 ± 0.5%.

IV.2.4. Extraction procedures

Ultrasound-assisted extraction (UAE) was performed with a PEX 3 Sonifier (R.E.U.S., Contes, France) composed of an inox jug having 23 _ 13.7 cm internal dimensions with a maximal capacity of 3 L, and a transducer, in the base of jug, operating at a frequency of 25 kHz with maximum input power of 250 W. The double layered mantle allowed us to control the temperature of the medium by cooling/heating systems. The detailed diagram of the apparatus has shown in the Fig. 26. Conventional maceration, made for comparison, was carried out in the exactly same conditions without ultrasound. Extraction experiments were performed in triplicate.

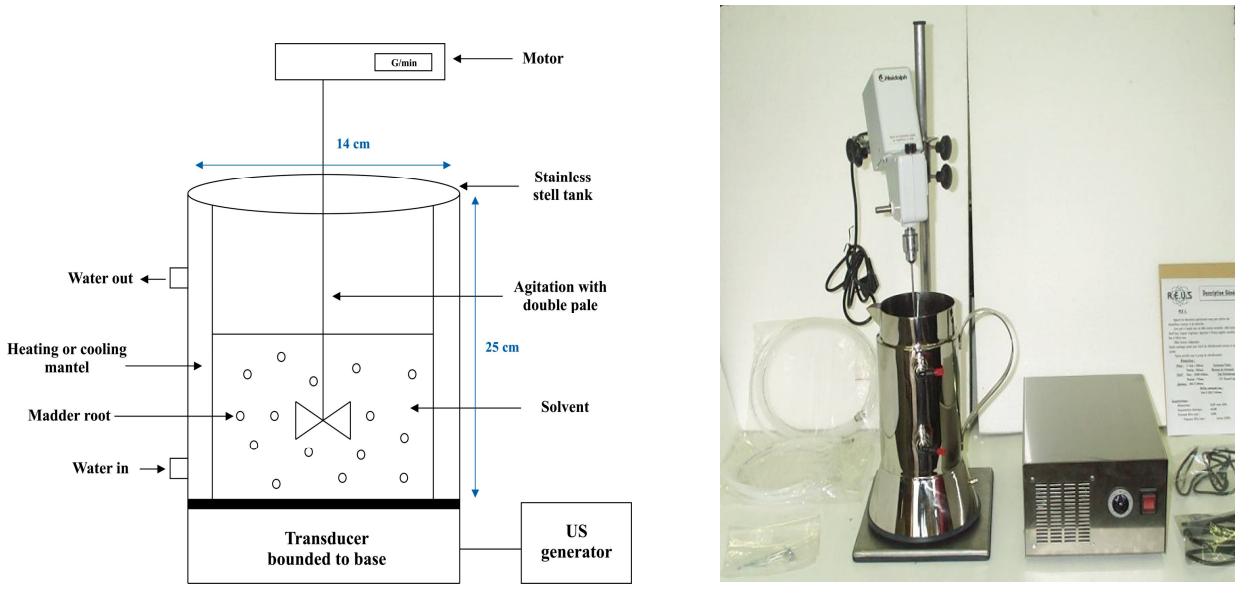

Figure 26: Laboratory sono-extraction reactor (3 liter system)

IV.2.5. HPLC analysis

HPLC analyses were performed using a Waters (Milford, MA) HPLC system consisting of a Waters 600E pump, a Waters 717 manual injector rheodyn, a Waters 2996 photodiode array detector. The HPLC pumps, manual injector rheodyn, column temperature,

and diode array system were monitored and controlled by using Waters Empower 2 Chromatography Data software program. The wavelength used for the quantification of the onion flavonoids with the diode detector was 360 nm. The chromatographic separation was carried out on a Purospher Star RP-18 end-capped column (250 mm × 4 mm I.D.; 5 µm particle size from VWR), with a RP18 guard column (4 mm×4mm I.D.; 5µm particle size also from VWR). The end-capped column and guard column were held at 37°C and the flow rate was set at 1mL/min. The mobile phase consisted of two solvents: (A) acidified water (0.5% formic acid) and (B) 100% acetonitrile. The solvent gradient used was the following: 0 min, (A) 95% and (B) 5%; 20min, (A) 60% and (B) 40%; 30min, (A) 0% and (B) 100%; 45min, (A) 95% and (B) 5%. The injection volume was 20 µL and all analyses were performed at least three times and only mean values were reported. Identification of flavonoids was done by comparing the elution order and UV-visible spectra. Quantification was carried out by using the external standards of known concentration. Peak areas were used to quantify the compounds in the sample. A linear regression analysis was carried out on the data of the peak area versus concentration. Linear calibration curves of the standards ranging from 10 to 100 mg/L were obtained with good linearity and R2 values which were more than 99.5% accurate for all the standards. Final concentrations of different flavonoids were calculated in mg/100g DW.

IV.2.6. UV-Visible Spectroscopy

All absorbances of total phenolic contents (TPC) and DPPH assay were performed on a Hewlett-Packard 8453 diode array UV-vis spectrometer equipped with a magnetically stirred quartz cell (optical path length = 1 cm). The temperature in the cell was controlled by means of a water thermostated bath [39].

IV.2.6.1. Determination of the total content of reducing compounds (TCRC)

TPC was estimated colorimetrically using the Folin-Ciocalteu method [40], with a kit (SEPPAL (Isitec-lab), France) especially suitable for TPC measurement of food products. This kit includes reagent A (modified Folin-Ciocalteu reagent), reagent B (alkaline buffer) and a gallic acid solution (3 g/L). A small volume (20 µL) of H_2O (blank), gallic acid solution (standard) and 200 µL the extract (sample) was mixed with reagent A (2 mL). After 1 min, 1 mL of reagent B was added in both water and gallic acid standard and 850 µL in sample. The mixtures were allowed to stand for 30 min in the dark at room temperature. Then, their

absorbance was measured at 760 nm. TPC were calculated by using the following formula: TPC = 3 × (sample absorbance – blank absorbance) / (standard absorbance – blank absorbance). TPC measurements were performed thrice and mean values, expressed as mg gallic acid/g of dry weight (mg GAE/g DW), were reported.

IV.2.6.2. DPPH assay

DPPH is a stable highly coloured free radical that can abstract labile hydrogen atoms from phenolic antioxidants with simultaneous formation of a colourless hydrazine (DPPH-H) [41]. The radical scavenging activity of extracts was evaluated according to the method described by Monzocco [42], with some modifications. About 200 µL of a solution of onion extract in water were added to 2 mL of a DPPH solution ($6x10^{-5}$ M) in MeOH and the mixture was left in the dark at room temperature for 30 min. The decrease in absorbance was measured at 517 nm. The percentage of DPPH consumed was calculated from the following equation: I % = 100 x (A_0 – A_1) / A_0, where A_0 was the initial absorbance (no antioxidant) and A_1 the absorbance in the presence of the extract or standard at different concentrations. The IC_{50} of each extract or standard (concentration giving I % = 50) was calculated by plotting I % as a function of the extract concentrations. All experiments were performed in triplicate.

IV.3. RESULTS AND DISCUSSION

IV.3.1. Preliminary study

Various experiments aim to optimize and evaluate phenolic compounds extraction in a convenient time, with appropriate solid/liquid was carried out in the preliminary study without ultrasonic assistance. Solvent we have used for extraction was just water at pH 7 at ambient temperature 25°C with theme of solvent free cold extraction.

IV.3.1.1. Solid/liquid ratio

The solid/liquid ratio was optimized, to get maximum amounts of polyphenols, 5 to 30g of onion slices were extracted by conventional maceration method in 100mL of water. TPC concentrations were found to increase with the solid/liquid ratio (Fig. 27) upto 25g/100mL, further increase of solid contents doesn't affect on more recovery of phenolic contents.

IV.3.1.2. Power optimization

After selection of solid/liquid ratio we have performed experiments at maximum ultrasound power 250W to determine the time limit to get the maximum phenolic contents. We have got maximum phenolic contents in 30mins of extraction, with further passage of time there was no remarkable increase of phenolic contents was detected. To optimize the power for extraction of onion flavonoids, we have analyzed various powers including 30W, 60W, 90W, 150 and 250W for total dry extract yield and phenolic contents and we have got maximum recovery of yield 1.95% at power of 250W. This obtained yield was clearly more than we have got with conventional maceration method (0.98%) at same conditions but in absence of ultrasound waves.

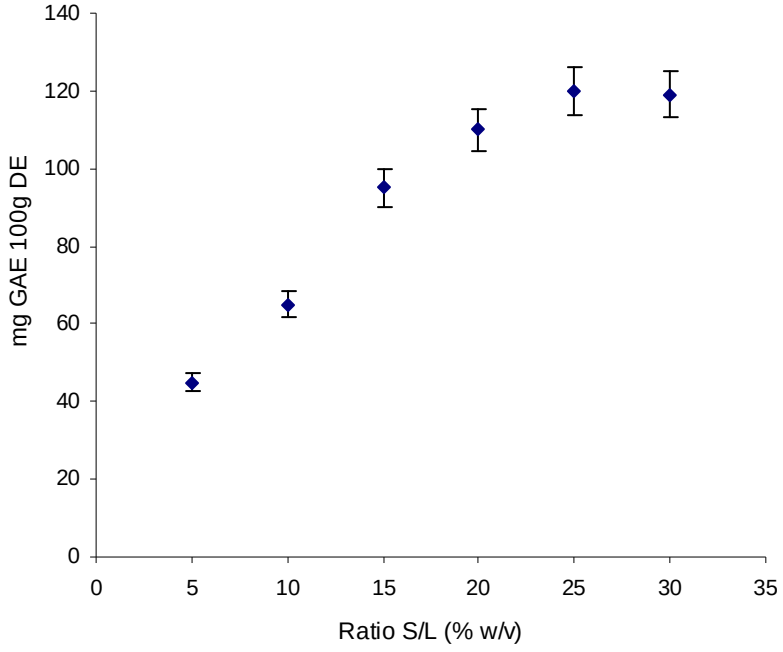

Figure 27: Effect of different solid liquid ratios on phenolic contents

IV.3.2. Comparison of US vs Conventional method

Moving towards green extraction, we have tried to minimise the loss of nutritionally important components from onion by-products by extracting them with ultrasound waves at ambient temperature (25°C) by using water having pH 7. We have analysed the extracts obtained by UAE and conv. method for quantification of flavonols by HPLC, the data

Table 15: Flavonol contents obtained by UAE and conventional maceration extraction process

Extraction method	QA* (mg/100gDW) 23.81 min	QDG* (mg/100gDW) 16.18 min	Q4G* (mg/100gDW) 19.85 min	Q3G* (mg/100gDW) 17.88 min	Total quercetin (mg QE/100g DW)
UAE	71.52 ± 3.8	449.29 ± 17.1	230.5 ± 7.8	18.9 ± 0.59	450.5
Conv	22.40 ± 1.1	401.3 ± 24.5	163.2 ± 5.6	13.8 ± 0.83	345.6

revealed the presence of quercetin-3,4′-diglucoside and quercetin 4′-glucoside as predominant form along with possessing higher concentration of quercetin aglycone (Figure 28) in both of extracts (Table 15).

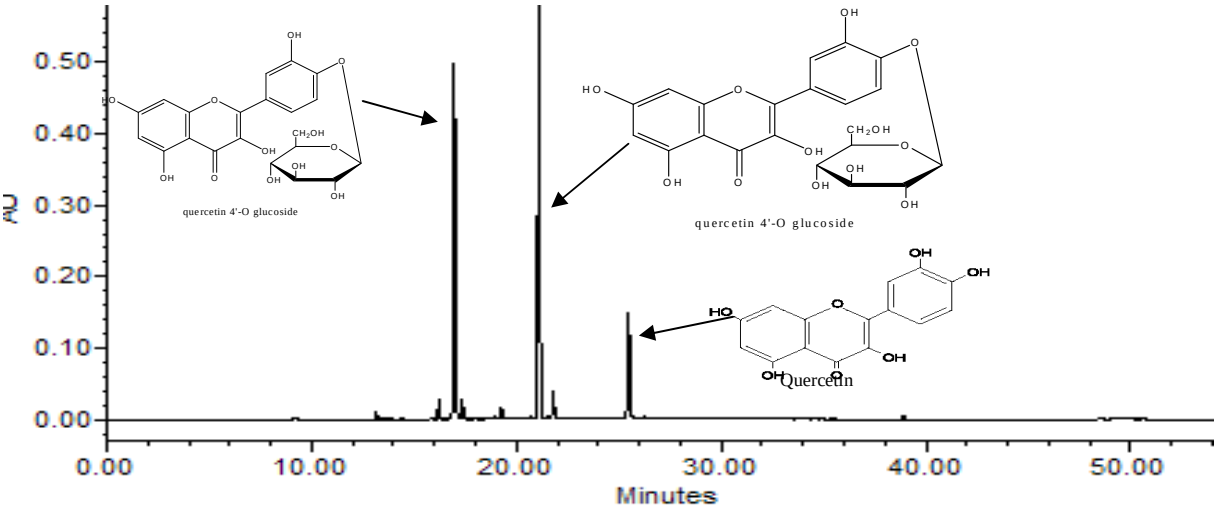

Figure 28: HPLC Chromatogram of ultrasound extracted onion byproducts

The application of ultrasound waves resulted with increased yields of these flavonols from 401.3 to 449.29 for quercetin-3,4′-diglucoside , from 163.2 to 230.5 for quercetin 4′-glucoside and from 22.40 mg to 71.52 mg per 100 g dry extract for quercetin aglycone. Higher concentration of these flavonols in UAE extracts in comparison with their content obtained by traditional extraction process at same conditions reveals clearly the effectiveness of this process in the field of antioxidants extraction. To found the specific impact of ultrasound, extracts (ultrasound and conventional) (figure 29) were compared for their total phenolic contents and antioxidant activity by DPPH assay. Ultrasound-assisted extraction lead

to a yield of gallic acid equivalents (TPC) (121 ± 3.8 mg GAE/g DW) increased by more than 20% than the yield obtained by conventional maceration method (89.6 ± 2.3mg GAE/g DW) in 30mins of extraction. The kinetic of extraction was clearly improved, which could be attributed to ultrasonic cavitation while it is the only variable added between both experiments. Chemat et al. showed, by-using electronic microscopy, physical effects of ultrasound on cells when applied on plant material [43].

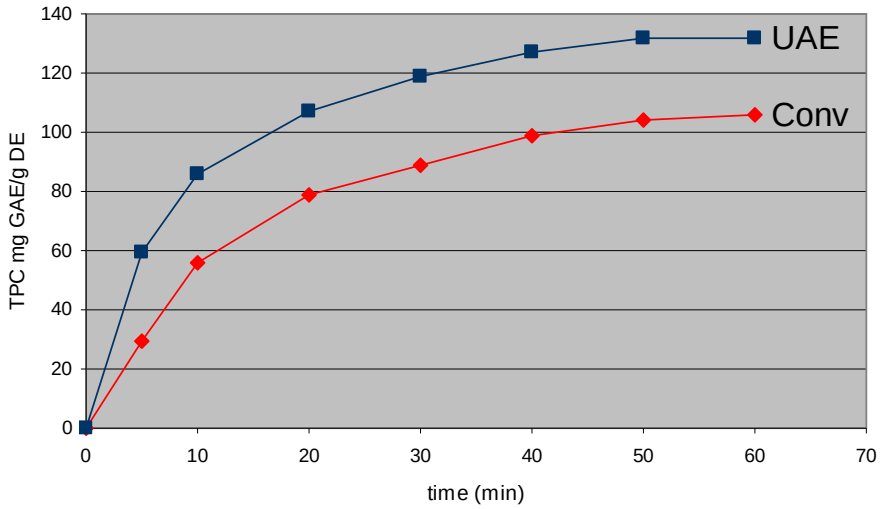

Figure 29: Comparison of UAE vs CSE on phenolic contents extraction

Total phenolics generally correlate with antioxidant capacities displayed by the DPPH assay. Radical scavenging activities of both extracts in terms of their IC_{50} values are calculated. The DPPH method is one of the oldest and most frequently used methods as it provides a convenient and rapid way to evaluate antiradical activities of antioxidants. For measuring the antioxidant activity we have chosen the concentration of samples providing 50% inhibition (IC_{50}) of DPPH solution. Both extracts have given linear response above then R^2=0.9900 in the concentration range 1-200mg/ml. Similar to the total phenolic content, ultrasound extracted sample was more effective with IC_{50} value 10.01mg/ml against IC_{50} value of conventional extract (13.21mg/ml).

Among newer techniques used in extraction technology, ultrasound assisted extraction (UAE) of food components has been employed as a new tool to improve the yield and quality of extraction products and to reduce the duration of procedures. UAE is a clean method that avoids the use of large quantity of solvent; this reduced environmental impact of UAE is clearly advantageous in terms of energy and time. UAE makes use of physical and chemical phenomena that are fundamentally different from those applied in conventional extraction

Table 16: Advantages and drawbacks of traditional and recent extraction techniques

	MHG	UAE	MAE	SFE	ASE
Name	Microwave hydrodiffusion and gravity	Ultrasound-Assisted Extraction	Microwave Assisted Extraction	Supercritical Fluid Extraction	Accelerated Solvent Extraction
Brief description	Sample is directly put in the reactor and subjected to microwave irradiations	Sample is immersed in solvent and submitted to ultrasound using a US probe or US bath.	Sample is immersed in solvent and submitted to microwave energy.	Sample is placed in a high pressure vessel and crossed continuously by the supercritical fluid.	Sample is heated by a conventional oven and crossed by the extraction solvent under pressure.
Extraction time	15-30 min	10-60 min	3-30 min	10-60 min	10-20 min
Sample size	150g-1kg	1-30 g	1-10 g	1-5 g	1-30 g
Solvent use	No solvent	50-200 ml	10-40 ml	2-5 ml (solid trap)30-60 ml (liquid trap)	15-60 ml
Investment	Moderate	Low	Moderate	High	High
Advantages	Rapid Easy to handle No filtration required No solvent consumption	Easy to use	Rapid Easy to handle Moderate solvent consumption	Rapid Low solvent consumption Concentration of the extract No filtration necessary Possible high selectivity	Rapid No filtration necessary Low solvent consumption
Drawbacks	Appropriate selection of extraction parameters is its critical step, less dry extract yield	Large amount of solvent consumption Filtration step required	Extraction solvent must absorb microwave energy Filtration step required	Many parameters to optimize	Possible degradation of thermolabile analytes

techniques. The present study reports on the extraction of polyphenols especially flavonols from onion (*Allium cepa* L.) by-products range by using water having pH 7 as solvent. The high total phenolic content and extraction yield obtained from optimised UAE proved its efficiency when compared with the conventional method. Furthermore, the antioxidant activity determined by the DPPH test confirmed the suitability of UAE for the preparation of antioxidant-rich plant extracts.

Finally we have tried to make the comparison by comparing advantages and disadvantages of MHG with other recent and traditional extraction methods like UAE, Supercritical Fluid Extraction (SFE), Accelerated Solvent Extraction (ASE) and Microwave-Assisted Extraction (MAE) in Table 16.

IV.4. CONCLUSION

Due to the use of ambient temperature (25°C) and water having pH 7 during extraction step, ultrasounds have been found to be an interesting alternative to conventional methods for antoxidants extraction. Extracts obtained in presence of ultrasound waves have shown clearly their impact in increase of total dry extract yield along with increase extraction of flavonol contents. Another advantage of using ultrasounds is the rapidity of the extraction. This extraction step require several hours in conventional processes, but it only required few minutes in ultrasounds assisted systems which induced better recovery of flavonol contents.

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General Conclusion

The aim of this study was to develop, optimized and implements a new microwave extraction technology for the extraction of antioxidants from different plant matrix. The main features of this recent developed extraction technique, which proved it to be advantageous over traditional methods, is its extraction conditions (absence of solvent and short extraction time). An important observation of this study which was done initially during optimization of this device for onion flavonoids extraction was the detailed prediction of temperature distributions in different parts of plant matrix and also in the reactor.

This microwave heating profile shows a fundamental difference between microwave and conventional heating: in conventional heating heat transfers occur from the heating device to the medium, while in microwave heating heat is dissipated inside the irradiated medium. In our microwave heated reactor, the average temperature of the onion is significantly higher than the surrounding temperature. This is due to the fact that the microwave power is dissipated over the whole volume of the onion (which contains 88% water). In conventional solvent extraction, mass transfer occurs from the inside to the outside while heat transfer occurs from the outside to the inside. For solvent free microwave, the two transport phenomena (heat and mass transfer) are in the same direction from the inside of the extracted material to the bulk reactor.

At optimized extraction conditions it was also possible to extract a good yield of onion juice with retention of fresh organoleptic qualities and also by retaining increase content of valuable phenolic components. The MHG efficiency was also evaluated by detemining the flavonol contents (total quercetin (TQ), quercetin aglycon (QA) quercetin-3,4′-diglucoside (QDG), quercetin-4′-monoglucoside (Q4G), quercetin-3-monoglucoside (Q3G) in dry extracts along with measuring the antioxidant activity of extracts by different tests. The results obtained revealed the ability and effectiveness of this method in improving the overall antioxidant activity of extracts in comparison to conventional solvent extracts with higher recoveries of flavonols in initial 15mins of extraction.

These findings were also confirmed by extracting and analysing a byproduct (sea buckthorn) for flavonol contents and antioxidant activity. Juice production of sea buckthorn results in generation of large amount of by-products, which are suggested to contain

substantial amounts of valuable natural antioxidants. We have applied MHG technique for extraction of flavonoids along with remaining moisture content from the by-products of sea buckthorn and then analysed the extracts for quantification of flavonoids along with evaluating their phenolic contents and reducing power. The short extraction time and no consumption of solvents were the main advantage of this technique.

However, the less dry extract and flavonol content yield was a drawback, due to rapid onset of burning process it was not possible to extract completely maximum contents of different compounds along with insitu water of lant material. Introduction of vacuum in this system was an attempt to improve the process by suppressing these above discussed drawbacks which worked by reducing the extraction temperature both in the matrix (from 100°C to 87°C) and also in the reactor (from 100°C to 81°C). The results revealed the efficiency of this new process by extracting more moisture and dry contents yield at less temperature. VMHG has shown its predominance for efficient and increase recovery of onion flavonol contents (57%) in comparison to MHG and thus more antioxidant not only in contrast to MHG but also solvent extracts.

Finally this sytem also proved to be more compatible in comparison to another recent extraction technique (UAE), which definitely requires a medium to work. Moreover, the development of this method is a step towards growth of green chemistry and environment by reducing energy consumtion and its rejection, by eliminating the usage of toxic hazardous solvents and avoiding the generation of wastes. This attractive novel technology is clearly offering opportunities for food, pharmaceutical and cosmetic industries to meet the growing demand of consumers for healthier food products. MHG could also be used to produce larger quantities of extracts by using existing large-scale microwave extraction reactors (figure 30) (www.archimex.com). These microwave reactors which are mostly continuous microwave reactors are suitable for the extraction of 10, 20, 100 or 1000 kg of plant material per hour in a batch. However, in the application of microwaves for heating of food materials, the occurance of uneven temperature profiles within the extraction plant matrix is a problem and challenging in the future, more attention needs to be paid to solve this problem and making it more user friendly.

Figure 30: Batch and continuous microwave oven in industry

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Abstract

Microwave hydrodiffusion and gravity (MHG) technique is an attempt towards development of green extraction, as this environment friendly technique has completely eliminated the use of organic solvents. After describing the effectiveness of microwave radiations in extraction field in the first part of this manuscript, we have optimized this noval extraction method to get antioxidants rich extract. Along with studying the temperature distributions in different parts of plant material under the effect of microwave irradiations, we have analyzed the influence of microwaves in enhancing the antioxidant activity of extracts by using different tests. We have got the promising results concerning about the antioxidant rich extracts of different onion varieties and sea buckthorn in generalization step against the conventional solvent extracts. The application of vacuum system in this extraction system helped in restraining the limitations like dry extract yield and flavonol contents. In comparison to traditional and recent extraction systems, the MHG extracts doesn't require any filtration and purification steps as it works in absence of any solvent and water and are highly recommended for direct application in industrial products.

Keywords: microwave, solvent free, onion, flavonols, antioxidant activity, vacuum

RESUME

L'hydrodiffusion générée par micro-ondes est une nouvelle technique d'extraction mise au point au sein de l'Université d'Avignon et des pays de Vaucluse. Ce procédé est une combinaison entre une technique traditionnelle et une technologie innovante. En effet, le chauffage par micro-ondes a permis d'initier et de générer le transfert de matière et de chaleur de l'intérieur des matrices végétales (oignons) vers l'extérieur et de réduire de facon considérable les temps d'extraction des antioxydants sans aucune intervention de solvant.

A titre de comparaison, les polyphenols de différentes variétés d'oignons ont été extraits par l'hydrodiffusion générée par micro-ondes et par la technique conventionnelle, l'extraction par solvant. Les rendements obtenus par micro-ondes sont presque identiques à ceux obtenus à l'aide d'un solvant alors que les temps d'extraction sont réduits. La capacité antioxydante des extraits micro-ondes est supérieure à celle obtenue par technique conventionel. Ce qui présage des potentialités d'application dans le domaine agroalimentaire en particulier pour la valorisation des co-produits.

Une étude cinétique de l'extraction, ainsi qu'une observation au microscope optique (cytologie) des matrices traités soumises aux micro-ondes et au solvant ont mis en évidence la spécificité de l'extraction sans solvant assistée par micro-ondes au niveau des mécanismes de libération et d'extraction des molécules antioxydantes au sein du végétal. L'effet des micro-ondes a pour conséquence une libération plus rapide des principes actifs contenue dans la plante grâce à l'ouverture quasi instantanée des glandes et l'explosion des cellules. L'explication de la différence de composition chimique entre les procédés d'extraction par solvant et par micro-ondes pourrait être basée principalement sur des phénomènes de solubilité, de polarisation diélectrique ainsi qu'un transfert de matière et de chaleur inversé.

Mots clés : extraction, micro-ondes, oignons, cinétique, microscopie optique, antioxydants.