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EXTRACTION OF PHENOLIC AND FLAVONOID COMPOUNDS FROM SOLID WASTES OF GRAPE SEED OIL PRODUCTION BY COLD PRESSING

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ABSTRACT

The objective of this investigation was to assess the advantages of phenolics removal from the grape seeds cold-pressing waste in comparison with their extraction from whole seeds. Series of batch solid-liquid experiments were carried out in order to determine: (a) at two temperature levels (25 and 60°C) the most convenient solvent from seven ethanol-water mixtures in the range 0 – 95 % tested (choosing flavonoid extraction capacity as criterion) and the extraction kinetics using the best solvent; (b) the effect of liquid to solid ratio in the range 2.5 - 50 ml g^{-1} on the total phenolic and flavonoid concentrations in the extracts from pressing waste; (c) the number of extractions necessary to attain a practically complete extraction from whole seeds and pressing waste. The data obtained from these experiments allowed to be evaluated the losses of phenolics and their flavonoid fraction in the course of the cold pressing process and their removal. The radical scavenging properties of the extracts from both sources were also investigated and correlated with the current total phenolic and flavonoid concentrations. The study has shown that the waste of grape seeds cold pressing can be classified as valuable phenol-rich resource. The fast one stage removal (within 20 min) at ambient temperature providing sufficiently high phenolic and flavonoid concentrations at reduced energy and solvent costs compensates for the losses and encourages the combination of seeds cold pressing with the liquid extraction of its solid wastes in industrial practice.

Keywords: *grape seeds, cold-pressing wastes, valorisation, phenolic antioxidants, extraction kinetics.*

INTRODUCTION

In recent years, there has been an increasing trend towards more efficient utilization of agro-industrial residues due to environmental and economic impacts. Among them, grape is one of the most economically important fruit crops and the processing industry transforms more than 20 % of the treated grapes into organic residues, part of which is used as compost, adsorbents, animal feed, or for biomass production [1]. Considering the growing demand for natural extracts and their derivatives, one of the most efficient options is the recovery of high value compounds suitable for use in various food, cosmetic and pharmaceutical products or other industrial applications.

Grape seed extracts are among the most studied and marketed grape-based byproducts because of their high and very specific content of phenolic compounds, which display a broad spectrum of biological, pharmacological and protective properties against free radicals and oxidative stress. Though quantitative and qualitative variabilities of phenolics and polyphenolics have been confirmed in seeds of numerous sorts at various climate conditions, the major individual phenolics identified are usually divided into two groups: flavonoid compounds, mainly monomeric flavan-3-ols (catechin, epicatechin),

their corresponding gallate compounds, cyclic oligomers and polymers (proanthocyanidins), and non-flavonoid phenolics occurring most often as phenolic acid esters or glycosides [2].

As a recyclable by-product, grape seeds represent a structurally complex material, containing some valuable substances, mainly phenolic compounds (5 - 8 %) and vegetable oil (7 - 16 %), along with natural fibers (40 %), proteins (10 %), carbohydrates, and other micro-components [3]. Roughly two-thirds of grape phenolics are located in the seed coats as expected from their protective function, providing mechanical strength and controlling permeability to water and oxidizing agents. It is also established that they are present in different forms, either as soluble phenolics (free and esterified) mainly localized in cell structures or as insoluble-bound phenolics, associated with other biopolymers in cell walls [4, 5]. Besides, phenolic acids are the main physically or covalently bonded compounds, forming insoluble molecular complexes with proteins, carbohydrates and lipids reinforcing the rigid cell wall matrix. Recent studies still show that regardless of the technological processing before extraction (juice or wine production), the insoluble-bound fraction may account for a large part (20 - 60 %) of the total phenolics in the different varieties, rendering them less extractable by these complex-forming mechanisms [6].

Due to inherent difficulties, the conventional solid-liquid extraction of grape seed phenolics requires long extraction times, lasting from several hours under moderate heating and stirring to few days under maceration. Hence various pretreatment techniques have been proposed with the aim to accelerate the extraction process at minimal reduction of extract quality [7 - 10]. The most practised techniques are the mechanical seed-size reduction, often performed under a controlled atmosphere (wet or cryogenic milling), thermal treatment or enzymatic hydrolysis, even though alternative technologies are currently being examined as a pretreatment step or for integration into the extraction process [10 - 12]. Other possibility explored is the separation of the oil fraction as a first step by extraction with nonpolar solvents (hexane, chloroform), supercritical CO_2 extraction or hydrodistillation, followed by extraction of phenolics with suitable polar solvents [13, 14].

The recovery of vegetable oil might be one primary target, and the oil-extracting technology is an essential element to assure further use of the beneficial ingredients, left in the residual cakes or meals. Advantageously, non-traditional oils from various seeds and oleaginous plants are now produced in small- and medium-sized pressing devices without heating and addition of organic solvent. The grape seeds are classified as a low oil containing material (< 20 %), but mechanical pressing at ambient temperature is highly suitable for them resulting in edible vegetable oil of superior quality, with high content of polyunsaturated fatty acids (85 - 90 %) and antioxidants as tocopherols, vitamin E and lipid-soluble phenolics, contributing to its nutritive values and oxidative stability. Despite the high phenolic content in grape seeds, it has been established that only a minor part of total phenolics is transferred to the pressed oil because of their low solubility in lipids at the temperature of processing [15, 16]. At the same time, due to the small yield (in the range of 100 g oil kg^{-1} seeds) after refining a crude oil a large quantity of treated seeds remains as waste.

Several studies have marked the wastes of seeds and oleaginous plants cold pressing as a source to prepare radical scavenging extracts [17, 18]. However, the kinetics of phenolic compounds extraction with various solvents has not been sufficiently tested.

The aim of this study was to evaluate the efficiency of phenolic fraction removal from the grape seeds cold pressing waste, investigating the kinetics of phenolic and flavonoid compounds extraction, as well as their contribution to the antioxidant activity of extracts. The removal from whole grape seeds was used as comparative base of evaluation.

EXPERIMENTAL

Materials

Grape by-products originated from red grape (Vitis vinifera L.) sort were procured from local producer of cold-pressed edible oils (Balcho Agro product Ltd, Bulgaria). Press cake (residual oil content < 5 %) was obtained in the form of dry brown powder with moisture content of 7.5 %. After classification using a vibrating sieve shaker, the following size fractions were collected: > 0.5 mm (15 %), 0.5 - 0.3 mm (80 %), < 0.3 mm (5 %), indicating quite uniform size distribution. Unclassified material was used for extraction. Whole non-defatted seeds with moisture content of 6.8 %, retained on the 4 mm-sized sieve, were used for comparative purposes.

The removal of the seed coat is difficult because the good adhesion with the lipid core, but the original core-shell structure is adequate for selective recovery of phenolic compounds from the seed coat, avoiding extraction of lipids from the core of the seeds.

The solvent used and other chemicals were of analytical grade. Aluminum chloride, anhydrous sodium carbonate, gallic acid, and (+)-catechin hydrate (> 96.0 %) were purchased from Sigma-Aldrich; Folin-Ciocalteu's phenolic reagent - from Merck and 2,2-diphenyl-1-picrylhydrazyl (free radical DPPH, 95 %) - from Alfa Aesar.

Solid-liquid extraction

Batch extraction experiments were carried out with laboratory scale reactor, at controlled temperature and protection from light to prevent oxidation of phenolic compounds. The mechanical stirring was maintained at 350 rpm proven as sufficient to eliminate the external mass transfer resistance. In order to make a proper choice of the extracting solvent and temperature, different ethanol-water mixtures (0 - 95 %, v/v) and liquid-solid ratios (2.5 to 50 ml g^{-1}) were tested in combination with two temperatures (25 and 60±2°C). For the kinetics study, the extractions were performed with a constant solvent to solid ratio (10 ml g^{-1}) for different extraction times (5, 10, 15, 20, 30, 60, and 120 min) with the chosen solvent for each material.

Additionally, the maximum extractable phenolic and flavonoid content were determined by successive extractions until completion, with addition of fresh solvent. All crude extracts were filtered through a 0.45 µm syringe before their spectrophotometric characterization (Boeco S-22 UV/Vis spectrophotometer) through standard colorimetric assays. The analysis were done in triplicate and the data were expressed as mean ± standard deviation.

Determination of total phenolics

The total phenolic concentration (TPC) in extracts was evaluated according to the Folin-Ciocalteu's assay [19]. Folin-Ciocalteu's reagent of 0.5 ml was added to 0.5 ml of the sample and 10 ml H_2O in a dark flask. After 5 min, 8 ml of 7.5 % aqueous Na_2CO_3 solution was added to the mixture. The mixtures were kept in dark for two hours and then the absorbance was measured at 765 nm. The TPC was expressed as mg ml^{-1} of gallic acid equivalents (GAE) (calibration curve equation: $A_{765nm} = 1.1353\ C_{GAE}$; $R^2 = 0.96$). The yield of total phenolics (TPY) was expressed as mg GAE g^{-1} of solid considering the TPC and the respective liquid to solid ratio.

Determination of total flavonoids

The total flavonoid concentration (TFC) was determined by the aluminum complexation assay [19]. One ml aliquot of grape seed extract (properly diluted) was added in a 10 ml volumetric flask containing 4 ml of distilled water, followed by the addition of 0.3 ml aqueous solution of $NaNO_2$ (5 % w/v). After 5 min, 0.3 ml of $AlCl_3$ solution (10 %) and 2 mL of NaOH (1 mol l^{-1}) was added to the mixture. The final volume was adjusted to 10 ml with distilled water, the sample was mixed and its absorbance was measured at 510 nm against water blank. The TFC was expressed as mg ml^{-1} of catechin equivalent (CE) (calibration curve equation: $A_{510nm} = 2.5018\ C_{CE} + 0.0545$; $R^2 = 0.98$). The yield of total flavonoids (TFY) was expressed as milligram of catechin equivalent per gram of solid (mg CE g^{-1}).

Determination of total phenolic content in crude grape seed oil

The phenolic content in lipidic product (crude oil) recovered after cold-pressing was also checked. After settling and decantation of the crude oil, two lipidic fractions were obtained (about 1/2 in volume) : clear reddish - yellow in color oil, and turbid sediments having a waxy paste-like consistency. Liquid-liquid extraction was performed to recover the phenolic compounds from both fractions. Oil aliquot of 15 ml was mixed with 20 ml of methanol-water solution (80 % v/v). After centrifugation at 3500 rpm for 15 min the methanolic fraction was separated and analyzed for total phenolic and flavonoid content as described above. The contents were expressed as mg GAE kg^{-1} oil or mg CE kg^{-1} oil , respectively .

Mass balance of oil cold-pressing process

In order to estimate the losses of phenolic compounds during oil cold-pressing, the mass balance of the phenolic and flavonoid contents in the grape seeds and in the fractions recovered is considered as follows:

$$G_s = G_w + G_0 \quad (1)$$

$$G_s Y_{Ps} = G_w Y_{Pw} + G_0 Y_{P0} + G_{LP} \quad (2)$$

$$G_s Y_{Fs} = G_w Y_{Fw} + G_0 Y_{F0} + G_{LF} \quad (3)$$

where G_s, G_w and G_0 are the amounts of raw material (whole seeds), press waste and lipid fraction, respectively; Y_{Ps}, Y_{Pw} and Y_{P0} - total extractable phenolics in the respective products (mg GAE g^{-1}); Y_{Fs}, Y_{Fw} and Y_{F0} - total extractable flavonoids in these products (mg CE g^{-1}), and G_{LP} and G_{LF} - losses of the compounds due to cold pressing.

Determination of antioxidant activity

Antioxidant activity of extracts was assessed by scavenging of the stable DPPH radical according to Brand-Williams et al. [20]. An aliquot of 0.1 ml from sample (properly diluted) was added to 3.9 ml of fresh DPPH in ethanol solution (6.10^{-5} M). The absorbance of tested extract solution (A_s) was measured at 515 nm after reaction time of 120 min to reach the plateau of colouration. The values of % DPPH scavenging were calculated by the following formula: $A_{AR}(\%) = (1-A_s/A_0)$ x100, where A_0 is absorption of control (0.1 ml ethanol and 3.9 ml ethanolic solution of DPPH).

RESULTS AND DISCUSSION

Effect of extraction conditions on phenolic extractability

Fig. 1 shows the variation of total phenolic (TPC) and total flavonoid (TFC) concentrations of extracts obtained in four series of experiments, performed with (a) whole seeds (reference), and (b) press wastes, at 25°C and 60°C, respectively, employing seven different mixtures of water and ethanol (0, 20, 40, 60, 70, 80, 95 %, v/v) in each series. All experiments were carried out at a liquid to solid ratio of 10 ml g^{-1} for 120 min and continuous stirring. The selected conditions are similar to those most often used for production of natural extracts from grape by-products, including their skins, stems, and seeds [6 - 9].

A detailed comparison of total phenolic and flavonoid extractability (yield at a given time) indicated that each solid-solvent system had a particular interaction mode and then the effects of the two basic extraction parameters appeared to be correlated with the physical structure of the vegetal material, e.g., unprocessed versus processed seeds. In the absence of pretreatment, the negligible TPC (< 0.65±0.01 mg GAE ml^{-1}) at 25°C confirmed the difficulty in extracting phenolics from intact seeds by any of the solvents used. When the temperature was increased to 60°C, the extractability from whole seeds was improved substantially, the highest TPC (6.68±0.17 mg GAE ml^{-1}) and TFC (2.82±0.06 mg CE ml^{-1}) were attained with 70 % ethanol in water and decreased sharply below and above this percent. Differently from the reference samples, the extraction from press waste was found to be comparatively less dependent on solvent composition, at least above 40 % ethanol and resulted in consistently higher phenolic con-

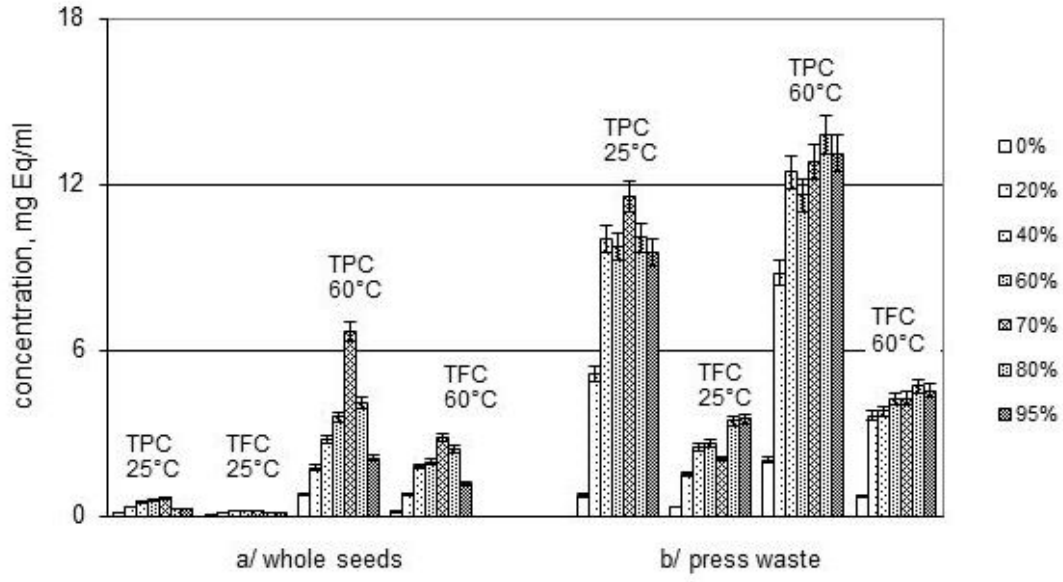

Fig. 1. Total phenolic concentrations (TPC) and total flavonoid concentrations (TFC) of extracts obtained with different ethanol-water solvents and temperatures.

centrations and colour intensity. This increase was particularly noticeable at 25°C, where again 70 % ethanol allowed to extract the highest TPC (11.54±0.28 mg GAE ml^{-1}) while the highest TFC (3.51±0.33 mg CE ml^{-1}) was observed for 80 % ethanol. The increase in temperature allowed to extract more total phenolics (13.77±0.34 mg GAE ml^{-1}) and total flavonoids (4.69±0.12 mg CE ml^{-1}) without any significant differences in the efficiency of solvents containing from 50 to 95 % ethanol. This is most likely due to the moderately polar nature of most the extractable phenolics in both grape by-products.

Such significant change in extractability by different aqueous solutions of ethanol (generally recognized as safe) has been reported in a number of comparative studies [1, 9, 12]. It has been essentially attributed to the diverse structures of natural phenolics even within a same chemical group that cause differences in their physicochemical properties such as chain length, polarity, and stability to external and solution conditions. However, their rate of extraction as well as their proportions in the extract could be controlled to some extent by using solvents of corresponding polarity: anthocyanins and polymerized flavonoids are well extracted by more polar solvents (≤ 50 % ethanol) whilst the predominating flavan-3-ols and smaller oligomers are extracted in higher amounts with solvents of medium polarity (≥70% ethanol). This fact is theoretically supported also by Hansen solubility parameters, calculated by a group contribution method [19]. According the results reported, there was no particular difference in the solubility of monomeric flavan-3-ols and oligomeric procyanidins and the solubility predicted was higher in ethanol than in ethanol-water mixtures. It is observed, however, that even minor increase in solvent polarity by using 95 % ethanol (relative polarity = 0.65) or 70 % aqueous ethanol (relative polarity = 0.758) provides higher TPC, most especially for intact seeds. The effect is not related to the modification of the relative polarity only but also to the specific interactions of solvents with the rest of the solid matrix, with its inert part or other soluble substances. Considering the high levels of fibers (cellulose and lignin) present in the seed coats (> 40 %), heating and addition of certain amount of water (up to 20 - 30 %) in ethanol hinder mass transfer because a more complete penetration of the solvent into the hydrophobic media, allowing the solvent to wet the seed matrix better. Rather, phenolic compounds usually occur in the form of glycosides or esters, which is the reason for their tendency to be highly water soluble, favoring an increase in the total soluble compounds which can be extracted (over the range of 1.4 - 22.5 g g^{-1} for different ethanol-water mixtures (0 - 95 %) [18]. In support to these considerations, Spigno et al. [8] have shown that overall phenols yield increased for water content in ethanol from 10 % to 30 % and remained constant from 30 % to 60 %, while phenols concentration of extracts decreased for water content above 50 %. In the case of press wastes, the substantial increase of the accessible surface area as a result of decreased seed-size and the removal of oil components from seed make the mass transfer of the extractable phenolics from the more open structure far easier and probably cause less matrix polarity limitations for the extracting agent. Thereby the flavonoids were more extracted at higher ethanol concentrations (> 80 % ethanol).

Fig. 2 illustrates that solvent effect alone or coupled with temperature can affect also the phenolic composition, characterized by the ratio of total flavonoids and total phenol concentration (TFC/TPC). It fluctuates between 0.2 and 0.6 (mg ml^{-1} flavonoids/mg ml^{-1} phenolics). On average this ratio is slightly lower (< 0.4) in the processed samples and this may be ascribed to the prevailing contribution of different kinds extractable phenolics over the flavonoids. The above results confirm that the flavonoids are important components of grape seeds by-products, and at the same time, are indirect evidence that the oil cold pressing induced both positive (suitable form of starting material and rapid access to extractable compounds) and negative effect (partial transfer of valuable compounds into the crude oil and their transformation/degradation). They are therefore not directly comparable, because on the one hand, the vegetal matrices are significantly different and on the other hand, the content and nature of extractable flavonoid and non-flavonoid phenolics before and after processing also differ. These differences are an explanation for the variations observed in the amount extracted from each material.

When interpreting the overall effect of processes on changes/losses in phenolic contents, it is difficult to distinguish the effect on the altered extractability (structural changes) and the effect on the extracted compounds (chemical modifications), which occur simultaneously and may be more or less beneficial on

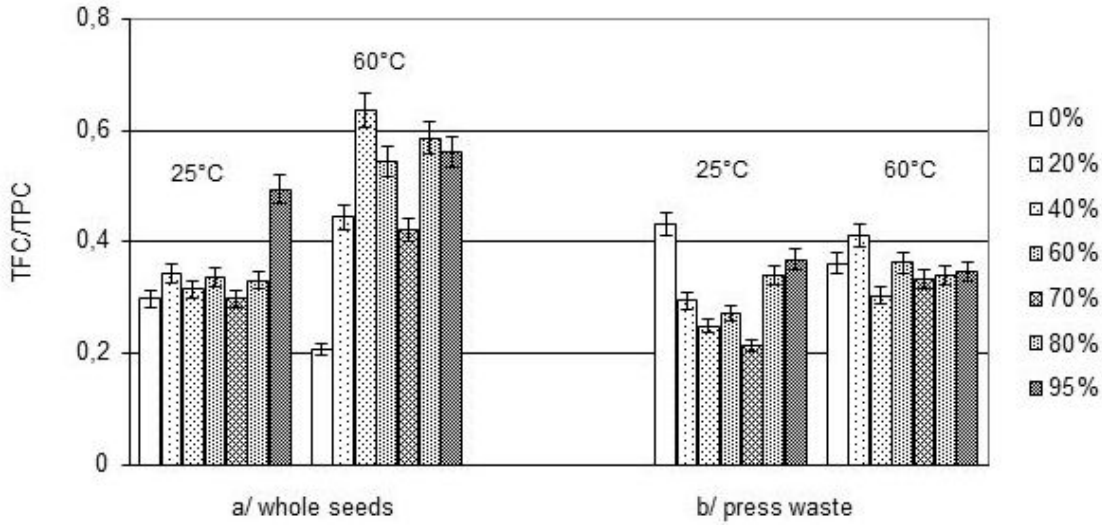

Fig. 2. Ratios of total flavonoid to the phenolic concentrations (TFC/TPC) of extracts obtained with different ethanol-water solvents and temperatures.

the final composition and quality of extracts. According to various articles related to the stability of phenolics under different heating conditions, the global tendency is to increase the extraction efficiency with the increase of temperature and processing time up to a certain combination of these parameters [3, 7, 10, 21]. Above it in many cases a variable and often non-negligible decrease of total or distinct groups of phenolic compounds have been registered, influenced by a number of factors such as intensity of heating, presence of oxygen, light, liquid environment, pH range, and vegetable characteristics. Shi et al. [3] state that the principal pathways for destroying phenolic compounds are the selective oxidation of easily-oxidized flavan-3-ols into less active and insoluble larger polymers or decomposition of more complex polyphenols even under moderate temperatures above 50 - 60°C, giving rise to new derivatives with different properties. On the other hand, the common mechanical operations for seed-size reduction as breaking or crushing are energy-consuming processes, associated with the physical resistance of the hard coat-protected seeds to disruption, which differentiates them from soft herbaceous plants. Next to energy requirement, depending on the mechanical process severity and degree of size reduction, this latter can cause loss and undesirable alterations of phenolic compounds by shear forces and local overheating through temperature gradients generated within the material. For these reasons, in an increasing number of studies the residues derived from pressure-assisted processing in the food industry are discussed as an opportunity to reduce costs and to retain more beneficial compounds [17, 18]. Peschel et al. [18] in their comparison of residual phenolic contents in several seed cakes (black currant, sesame, burdock, evening primrose) demonstrate that the net changes/losses of valuable compounds in the press cake are largely determined by the nature of the source material and press performances (pressure of hydraulic press, speed of rotation of screw press, time, temperature) and demonstrated that one technological processing that is effective with certain kinds of vegetables might not work for another. Therefore, a case-by-case approach is advisable. In fact, the exhaustive extraction (75 % ethanol, two extractions of 24 h each at room temperature) from black currant residues from mechanical juice extraction delivered far lower yields compared to the extracts from evening primrose seed cake, waste of oil production, amounting up to 76 mg GAE g^{-1} which was near to the commercial qualitative extracts from grape seeds (79 mg GAE g^{-1}).

In our present study, the results in Fig. 1 show that all extracts obtained from press waste with different aqueous ethanol solutions (10 - 95 %) have high levels of total phenolic compounds (51.35 - 100.59 mg GAE g^{-1}), comparable or even higher than those previously mentioned. These high levels classify the industrial waste of grape seed oil cold pressing as a valuable phenol-rich resource. Besides, the use of press waste enabled to

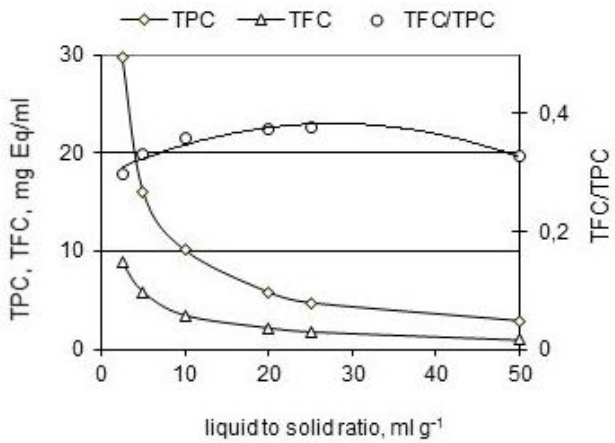

Fig. 3. Effect of liquid to solid ratio on phenolics extraction from press waste at 25°C in 95 % ethanol.

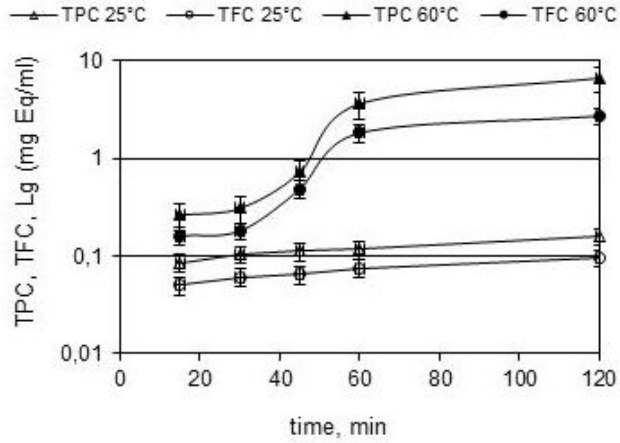

Fig. 4. Kinetics of extraction process from whole grape seeds with 70 % ethanol.

enhance significantly the initial extractability of different phenolic compounds and to reduce the temperature dependence of the extraction, and hence assuring mild conditions for the extraction. For example, the TPC and TFC values of extract obtained from press waste at 25˚C with 95 % ethanol were considerably higher (70 % and 25 %, respectively) than those attained at 60ºC with 70 % ethanol from whole grape seeds (see Figs. 1 and 2). The tendency to increase the relative contribution of the non-flavonoids fraction (phenolic acids) in press waste extracts indicates that transformation of some native phenolic compounds in seeds has occurred during processing and reflects possible chemical changes resulting in formation of new compounds. It would be important to determine the consequences of such changes in total phenolic and flavonoid contents on the antioxidant activity of extracts.

While the most favorable solvent and temperature for phenolic compounds extraction depend on the used vegetal matrices, the ratio of the liquid and solid phases (L/S) remains an important factor for each extracting system, affecting the mass transfer through regulation of concentration gradients. As illustration, in Fig. 3 the variation of the TPC and TFC together with TFC/TPC ratio is reported, carrying out the assays at 25°C for 120 min with L/S ratios ranging from 2.5 up to 50 ml g^{-1} at constant mass of solid. The rise of L/S decreased progressively the TPC and TFC and favoured an increase in extraction yield, since the relatively smaller sample amount can help to maintain favourable extraction equilibrium and higher concentration gradients between the solid and liquid phase, thus increasing the driving force and enhancing the mass transfer phenomena. The change in yields and composition was substantial up to approximately 20 ml g^{-1}, at which the extraction yield was about 1.5 times higher than that achieved at 5 ml g^{-1}, commonly used for large scale batch extractions from wastes of wine production [3]. As to avoid excessive consumption of organic solvents, a sufficiently large L/S value of 10 ml g^{-1} was chosen for further experiments, using 95 % ethanol for press wastes and 70 % ethanol for whole seeds for better extraction of flavonoids.

Solid-liquid extraction kinetics

The time evolution of phenolic and flavonoid concentrations extracting the whole seeds and press wastes with the chosen solvents can be seen in Figs. 4 and 5. Together with the character of the extracted material, they identify the effect of temperature increase from 25 to 60°C. These two factors can affect three major aspects of the process: liquid phase saturation (solid-liquid equilibrium), mass transfer kinetics (diffusivity), and stability of phenolic compounds (loss/conversion), taking a more complete account of the different mechanisms involved during extraction [8].

The complexity of interactions is clearly demonstrated by the changes in the kinetic trends for whole seeds (see Fig. 4). At ambient temperature, the solutes release was extremely retarded by a slow penetration of solvent into dry seeds. The higher temperature permitted to reduce the time necessary for solid impregnation to about 45 min, followed by a steep increase in the TPC

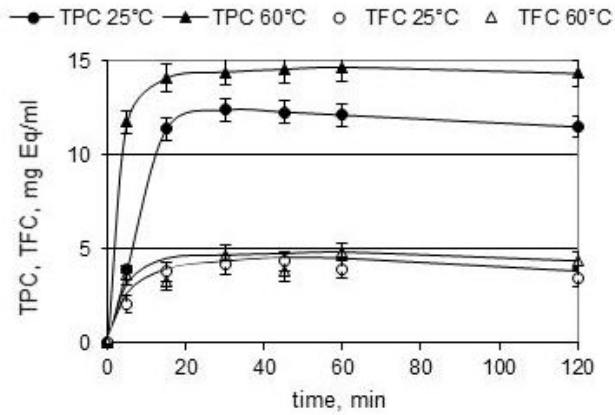

Fig. 5. Kinetics of extraction process from press waste with 95 % ethanol.

and TFC, but after 120 min the system remained still far from the saturation conditions. The extraction from press wastes, conversely, was a fast process, practically completed in 20 - 30 min (see Fig. 5). Moreover, the temperature changes had no appreciable influence on TFC obtained, while the extraction of the phenolic compounds appeared to be more temperature dependent with slightly higher (about 20 %) final TPC. The current extracts were composed of more or less equal TFC to TPC proportions which tended to decrease in the course of the process. Whole seed extracts had a higher TFC to TPC ratio of 0.61 at the beginning which then gradually declined down to 0.42 at 120 min. In the case of press wastes, at 25°C the ratio was reduced from 0.52 to 0.38 for the first 20 min while at 60°C maintained the lowest value of about 0.32 over the entire extraction time. From these changes it could be supposed that the intensive extraction conditions creating increased extraction rates may also result in higher content of other non-phenolic compounds or oxidation products possibly impairing the purity and quality of extracts.

For batch experiments, it is generally assumed that the particle characteristics such as size, shape and porosity play a relevant role in controlling both the rate and extent of extraction completion because they determine the area of contact with a solvent phase and the path of diffusion trough the solid particle [9, 10]. However, the very low (at 25°C) or weak (at 60°C) ability to release phenols from intact seeds, even if the seed coat is not too thick (< 0.2 mm), supports the premise that the internal structure complexity have a strong influence on it by the type of components present, their interactions one with other, and their relative spatial organization within the seed matrix. In this view, Cadot et al. [22] observed that the majority of monomeric flavan-3-ol units were located in the soft integument, between the restrictive cuticle and the medium integument whereas proantocyanidins were stored in the epidermis and inner cells of the outer integument and the inner cell layer of the inner integument surrounding the nutrient storage tissues of the germ. The hydrophobic cuticle, consisting of lipid and polysaccharide polymers impregnated with waxes and the highly lignified compact cell walls of medium integument present a sequence of physical and chemical resistances to diffusion of solvent and solutes inside the seed. The main implication is that the penetration of solvent into the interior of the intact grape seeds and the solubilization of the extractable phenolics, phenomena often considered fast and non-rate limiting in porous plant materials, in this case are decisive for the duration and extent of extraction. Typical example is the partial extraction from the solid parts of grapes during wine production: phenolics contained in skins are extracted during the first 4 - 5 days while those in seeds require over 10 days, with the help of increasing ethanol concentration and by decomposition of some cell wall compounds by macerating enzymes [21].

In the case of such hard-coated seeds, the enhancement of the overall extraction kinetics with increasing the temperature is basically attributed to the impact on the tissue permeability, which has been reported to increase suddenly in the range 50 - 55°C [4 - 6]. Some authors consider that the moderate heat energy supplied to the extraction system which may not be able to disrupt the cell walls and membranes, will be enough to weaken different intra or intermolecular non-covalent interactions which in turn results in an overall tissue softening and an increase in number and size of pores in the cell walls (intercellular spaces) allowing the target molecules to pass through by diffusion [23]. For whole seeds, temperature was an important determinant also because the decrease of solution viscosity and surface tension increasing the effective diffusivity rates of the solvent and targeted compounds, as evidenced by the shortening of the impregnation period (see Fig. 4). After saturating the porous matrix with solvent, the internal diffusion could be a controlling mechanism with corresponding limitations on the flux due particularly to the tortuous pore diffusion path through cell walls and the

insufficient external surface area of large seeds.

The fast and efficient extraction kinetics attained when using press wastes was primarily on account of the high degree of seed-size reduction with 80 % of particles having size smaller than 0.5 mm. At a reduction close to seed coat thickness the compressive forces most likely destroyed barrier tissues integrity and decreased the mass transfer resistances by minimizing the effect of unequal initial distribution of phenolics and rigid inert structures, irrespective of the extraction temperature. In addition, for smaller particles (< 0.3 mm), as revealed by Ghouila et al. [4] from SEM microphotographs, they can also lead to a breakage of cavities walls in the superficial layers, and thus making the diffusion from these areas faster than the one from the particle interiors containing cells with unbroken walls.

Based on Fig. 5, phenolic extraction curves consist of an initial fast rate part (washing stage) followed by a much slower one (diffusion-controlled stage) until the equilibrium is reached that is a common behavior in batch experiments performed with finely ground plant materials (< 1 mm) [24]. This transition occurs in the range of the first 5 - 10 min when more than 80 - 90 % of the extractable content at equilibrium is recovered, indicating that the washing of substantial amount of solutes on the particle surfaces represents the predominant mechanism for mass transfer. The observed weak solvent selectivity towards some compounds groups throughout the extraction can be explained by their different solubility and non-uniform effective diffusivity in the vegetal material. The free phenolic acids and monomeric flavan-3-ol units are likely to be representative of the easily extractable fraction, recovered during the process. Larger condensed tannins and their polymerization products, more or less weakly interacting with the matrix and located in the inner cell layers are increasingly difficult to dissolve, and their extraction is more influenced by the time and temperature. Moreover, a slight increment in the TPC and TFC at 60°C after the fast initial increase and a rapid approach to the equilibrium plateau may be related to the initiation of deteriorative reactions of phenolic compounds remained in the matrix. Consequently, milder extraction temperature below 60°C might be preferable not only because the reduced risk of adverse thermal effects and co-extraction of other inert components but also because the temperature effect on the extraction kinetics is not really significant if the final particle size is held less than 0.5 mm.

Several studies have reported results with similar issue performing experiments as to ensure reliable extraction by varying the size of grape seeds (whole, ground, or powdered) and the duration of the process. Our results obtained for whole seeds are consistent with those reported by Spigno et al. [8] where the extraction from coarsely ground seeds of 2 mm under comparable conditions (45 - 60°C, 80 % ethanol) needed approximately 8 h to approach equilibrium plateau. In another study, Bucić-Kojić et al. [25] have reported that the temperature within the range 25 - 80°C influences stronger the initial rate and extent of extraction with the bigger of the three fractions (> 0.63, 0.63 - 0.4, 0.4 - 0.16 mm) where equilibrium is nearly reached after 40 min. This behavior is explained by the change in activation energy required for the desorption process, decreasing from 8.01 to 0.71 kJ mol^{-1} with the particle size decrease. Kim et al. [23] have shown that powdered seeds need less intense thermal treatment (at 100°C for 10 min) before extraction compared to whole seeds (at 150°C for 40 min) as to obtain maximum TPC and antioxidant activity but provide less resistance to thermal damage. In terms of extraction duration, the short extraction time found matches the one reported for the more expensive physical methods of cell disruption, like pulsed electric field treatment [11].

Total extractable phenolic content

The yields of total phenolics (TPY) and flavonoids (TFY) according to the number of treatments depict further the impact of the oil cold-pressing on the extraction efficiency (Fig. 6). For press wastes, three successive extractions (95 % ethanol, 10 ml g^{-1} solid, 25°C, 30 min) appeared sufficient for almost complete extraction and gave 153.92±4.17 mg GAE g^{-1} and 46.98±1.42 mg CE g^{-1}. These values were comparable to those attained by a single extraction with an excessive L/S ratio of 50 ml g^{-1} (Fig. 3). A higher phenolic content in the whole seeds was evidenced after four extractions (70 % ethanol, 10 ml g^{-1} solid, 60°C, 120 min), yielding 197.61±5.33 mg GAE g^{-1} and 74.61±2.24 mg CE g^{-1}. However, the first extracts contain more than 90 % of initial phenolic content in press wastes but only 30 - 40 % of that in whole seeds. Therefore, a second extraction of the wet seeds is unavoidable as to attain satisfactory phenolic recovery, i.e. the use of press wastes instead of whole seeds is saving at least the cost of one extraction stage.

The evaluation of the total phenolic and flavonoid

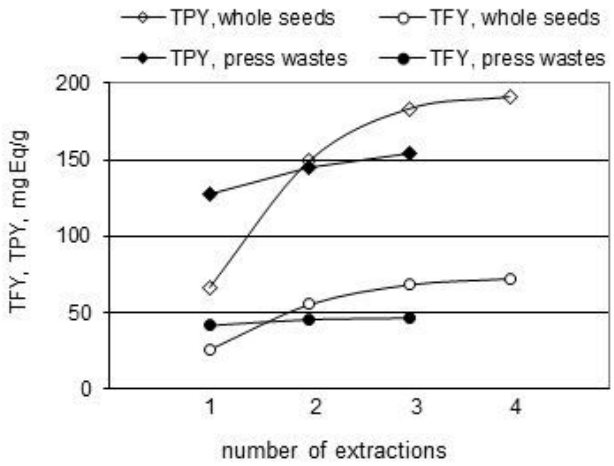

Fig. 6. Comparison of total phenolic and flavonoid yields from whole seeds and press waste increasing the number of extractions.

content in the cold press grape seed oil tested showed about 60 mg GAE kg^{-1} of total phenolics and 25 mg CE kg^{-1} of total flavonoids in the clear oil fraction and amounted to 630 mg GAE kg^{-1} and 310 mg CE kg^{-1} - in the turbid sediment.

Assuming the ratio of the amount of crude press-oil fraction (G_0) to grape seeds (G_s) is within a range from 15 % to 25 % in the industrial processing, the mass balance has been calculated according to Eqs.1 - 3 and presented in Table 1. Practically, a very low part of phenolic compounds initially present in the grape seeds has been transferred to the press oil (0.44 % phenolics and 0.57 % flavonoids). Their contents are in order of magnitude lower than those with grape seeds and so they do not contribute greatly to the overall mass balance. The major part of phenolic and flavonoid initial amounts has been retained in the press wastes (66 % and 56 %, respectively). The losses have reached up to 37 % of total phenolic content and 48 % of total flavonoid content with respect to the amount of these compounds in the

Table 1. Distribution of total phenolics and flavonoids in the process of grape seed oil cold pressing.

	Total phenolics, g GAE kg^{-1}	Total flavonoids, g CE kg^{-1}
Grape seeds	197.61±.9.81	74.61±3.72
Press waste	123.14±3.25	37.59±1.87
Press oil	0.87±0.25	0.43±0.26
Losses	73.59±3.68	36.59±1.83

untreated grape seeds. These significant losses might be attributed to the degradation of certain unstable components, moistly flavonoids and to the cake lost during processing. However, the press wastes still remain an important source of phenolics because the noticed losses can be compensated for by the accelerated extraction and completeness of recovery, including probably some bonded polyphenols that could not be extracted from the whole seeds.

Comparable degree of losses in original phenol contents was observed by Maier et al. [17] concerning grape seed oil production by a pilot-plant scale screw press. Total phenolic yields in the grape seed extracts from seven red and white grape cultivars varied between 107.4 and 226 mg GAE g^{-1} and decreased to 57 - 119 mg GAE g^{-1} in press wastes extracts, depending of grape variety. The relative phenolic losses amounted to 35.8 % on average, however, without a significant alteration of the individual phenolic profiles detected by HPLC-DAD. Accordingly, the studied grape seeds were less variable in terms to their contents before and after oil recovery as compared to the typical strong variations in the contents of total phenols and flavonoids found in the different grape sorts.

The potential of press wastes valorization is supported also by the fact that, when are used in excessive amount (> 200 ppm) in lipid systems, some phenolics may act as pro-oxidants regenerating peroxyl radicals [16]. Consequently, the amount of losses of grape seed phenolics in the pressed oil must not be high if the operating conditions, especially temperature, are well selected. Even so, the press wastes are usually underappreciated because of the expected reduction of the valuable fraction that could be discarded, distorted or destroyed during the different handling and processing operations (seed preparation, pressing, separation of the oil from the seed cake) that must be completed under optimized and strictly regulated conditions as to keep the maximum possible level of bioactive compounds in the processed material and in the end products.

Antioxidant activity

In order to assess the impact of the extraction conditions and vegetal materials used on the quality of extracts, the overall scavenging activity toward the DPPH radical (A_{AR}) was traced in the course of the extraction and correlated with the current total phenolic and flavonoid concentrations.

Two sets of experiments were carried out to this end.

In the first one whole seeds were subjected to extraction with 70 % ethanol lasting 120 min at 60°C. In the second - press wastes were used at 25°C and 95 % ethanol with a duration of 30 min. In the both cases samples were taken and analysed for phenolic and flavonoid content as well as for scavenging activity. The results obtained are illustrated in Figs. 7 and 8 where A_{AR} is plotted against TPC and TFC, respectively. The advantage of press waste as a source of antioxidant extract is well demonstrated. While with whole seeds the scavenging activity increases progressively to maximum of about 70 % after 120 min, with press wastes at lower temperature and shorter time it becomes over 90 % which is close to the saturation limit of DPPH radical in test systems. It can be also observed that each set of data follows a non-linear trend analogous to that of the kinetic curves. Such a trend has been observed in other studies, wherein the results have been correlated by equations of various kinds [26]. In our case a single second degree polynomial function different for TPC and TFC has given a satisfactory accuracy, for both sets of data ($R^2 = 0.97$ for total phenols, and $R^2 = 0.98$ for total flavonoids, respectively). This is somewhat surprising accounting for their distinguishable experimental conditions.

Numerous studies concerning the relationship structure-activity in model systems of individual phenolic compounds (groups of compounds) have discovered that the number and position of hydrogen-donating hydroxyl groups, the substituents present on the rings of flavonoids and the degree of polymerization may affect their reactivity toward DPPH radical [1, 2, 9]. According to Rice-Evans et al. [20], the structural requirement considered essential for better scavenging capacity include the presence of (i) the 3-OH groups on C ring; (ii) 2,3-double bond with the 3-OH group in conjunction with a 4-ketone in the C ring; or (iii) an ortho-OH substitution in the B ring with not glycated -OH groups. Accordingly, the relative antioxidant ability of the main groups of grape seed phenolics decreases typically in the order: procyanidin dimmers > flavanol > flavonol > simple phenolic acids. Flavan-3-ol monomers and oligomers that fulfill the first and third criteria are among the most powerful natural antioxidants but due to the same structural features have proven to be more prone to degradation compared to structurally simpler phenolic acids that are relatively stable and potentially important for the antioxidant properties of grape seed by-products, the most potent being gallic acid (trihydroxylated) that is recognized as the strongest antioxidant on mass basis [17, 20]. However, the presentation of total phenolic and total flavonoid concentrations in GAE and CE probably decreases the effect of the compounds individuality.

It is obvious from Figs. 7 and 8, that extracts from press wastes present almost the same antiradical capacity as those from whole seeds, when the concentration required to lower the initial absorbance of DPPH solution by 50 % (approximately 4 - 4.5 mg GAE ml^{-1}) is concerned. However, this concentration was attained for 10 min at 25°C with the press wastes against 80 min at 60°C with the whole seeds. A first conclusion can be, that the recovery of all the phenolic classes (TPC) makes a contribution to A_{AR}, a phenomenon, princi-

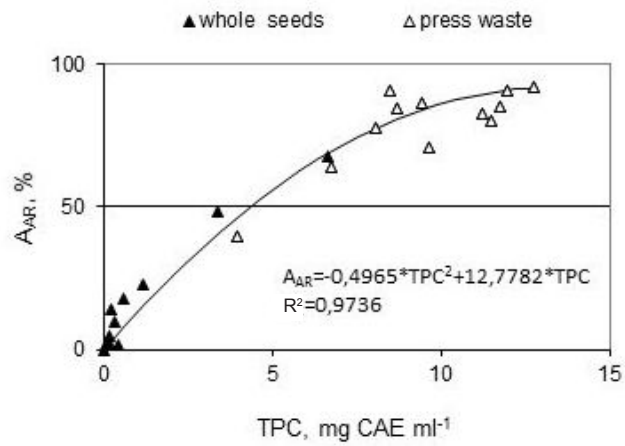

Fig. 7. Correlation between radical scavenging activity (A_{AR}) and total phenolic concentration (TPC).

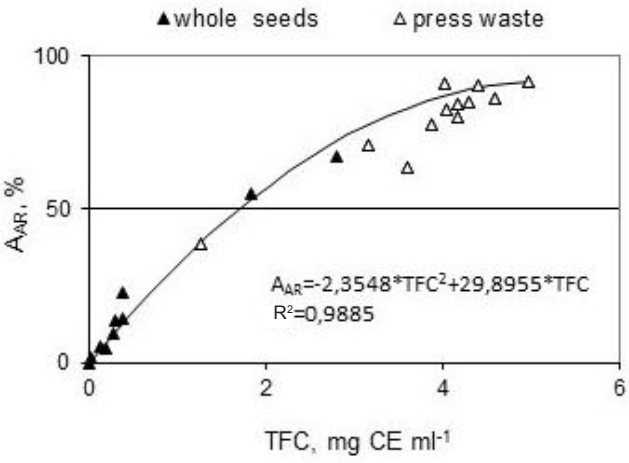

Fig. 8. Correlation between radical scavenging activity (A_{AR}) and total flavonoid concentration (TFC).

pally associated to the synergistic combination of these phenolic compounds and other soluble compounds in plant-derived extracts [2, 9]. At the same time, the high values of TFC to TPC ratio (close to 0.5) in both systems at this stage of extraction attest also the fact that the relative content of the more active flavonoids can be more important factor for activity level, as reported in studies on various phenol-containing vegetables [17]. These results provide also some confirmations to the equivalence of time-temperature combination relevant to particular extraction process, suggesting the possibility to provide extracts with similar TPC and A_{AR} by prolonged extraction at moderately increased temperatures from intact seeds or by rapid extraction at room temperature from processed residues, supporting previous works of Rajha et al. in this context [9].

The current kinetic study does not prove any apparent decline in the values of TPC and A_{AR} after the extraction period of 120 min at 60°C even if the mass transfer rate and the TFC/TPC ratios show a declining trend with a diminutive increment in the A_{AR} values in the later stages of the process. Thus, the raw material influenced mainly the kinetics of valuable compounds removal, and the kinetic curves of total phenolics and flavonoids could be used to get a compromise between an acceptable degree of extraction and safeguarding of their bioactivity. For whole grape seeds, phenolics were extracted to a limited extent (up to 30 %), leaving behind the more active phenolic oligomers, naturally protected in the innermost layers of the seed coat, and in such case the resulting extracts could not be representative of the entire bioactive content in the seeds. Thus, the reduced oligomers extractability resulted in incomplete recovery with a consequence of reduced antioxidant capacity. It is interesting to analyse the A_{AR} when the extraction conditions are optimized aiming to remove the majority of highly antioxidant molecules (with multiple extraction and better solvent). Regarding the press wastes, the above discussed phenomena remain away, and an extraction time of about 10 - 15 min at ambient temperature was sufficient to recover the extractable antioxidant content almost entirely (Fig. 6). These results and other literature reports [9, 17, 18] clearly reveal that industrial cold-pressing of grape seeds is not as deleterious as expected, and good radical scavenging properties of the extracts might be maintained by the unaffected antioxidants easily available for extraction or even by different chemical structures formed from the degradation reactions that might still be active as antioxidants.

The altered extractability, however, can confound the estimation of antioxidant capacity of different crude extracts, as described by other authors [1, 2]. Although each one of the analytical procedures for phenolics UV detection can be used to compare samples with similar compositions, it exhibits comparatively less precision in multicomponent mixtures of various classes of chemical compounds because of their different responses to the assay reagents and potential interferences with other nonphenolic reducing molecules. These uncertainties may partly explain the much higher TPC for press wastes extracts and their somewhat weaker correlation with the A_{AR} values (see Fig. 7). Further investigations are needed as to characterize the major individual compounds, as well as the extent of structural changes caused by cold-pressing and to establish a more reliable correlation between the operating conditions, structure modifications and yield of the active ingredients.

CONCLUSIONS

The study confirms that the solid wastes after the oil cold-pressing of grape seeds are a cost-effective and readily-exploitive source of phenolic acids and flavonoids. Results from the kinetic study show that the use of press wastes increases significantly the yield and reduces considerably the processing time of almost complete recovery of extractable phenolic content that may compensate for the losses of some unstable phenolics and flavonoids during the oil production. These losses have been evaluated on the base of material balance and found to be in order of 37 % and 48 % of the phenolic and flavonoid compounds initial contents. In terms of products quality, the combination of cold pressing with antioxidants recovery as compared to extraction from whole seeds will be preventing harms of oil and phenolic compounds, in addition to reduced solvent and energy consumption. From a practical perspective, such combination will increase the economic viability of the linked processes and may assist in a more efficient valorisation of industrial byproducts.

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