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Review

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Grape Pomace as a Sustainable Source of Bioactive Compounds: Extraction, Characterization, and Biotechnological Applications of Phenolics

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ABSTRACT: Grape pomaces (GPs) are characterized by high contents of phenolics due to an incomplete extraction during the winemaking process. These phenolics are secondary plant metabolites with potential beneficial effects on human health because of their antioxidant activity and antimicrobial, antiviral, and anti-inflammatory properties. Therefore, GP constitutes an inexpensive source for the extraction of phytochemicals that can be used in the pharmaceutical, cosmetic, and food industries. As a result of the increased attention to sustainability of agricultural practices, efforts have been made to use GP in different fields of industry. Thus, it is necessary to have efficient extraction techniques to achieve good recoveries of compounds. In this respect, sensitive and selective analytical methods have been tried for the characterization of phenolic extracts. This review summarizes the most recent developments in the extraction of polyphenols from GPs. Furthermore, the techniques used for characterization of extracts are explained, with emphasis on sample preparation, separation, and analysis of phenolics. Finally, the possible applications of GP extracts in diverse biotechnological fields are also discussed.

KEYWORDS: *grape pomace, polyphenols, extraction, purification, characterization, liquid chromatography−mass spectrometry*

INTRODUCTION

Grape is the world's largest fruit crop with an annual production of more than 67 million tons.[1] Grapes and products obtained therefrom, such as wine, grape juice, jams, and raisins, have then an obvious economic importance. Eighty percent of the worldwide grape production is used in winemaking.[1,2] The wine industry produces millions of tons of residues (that is, grape pomace, GP) after fermentation,[3] which represents a waste management issue both ecologically and economically.

Actually, the industrial recovery of GP is performed by its partial use for tartaric acid extraction or ethanol production,[4] and the final solid residue is sometimes cast off as fertilizer, although the high levels of phenolics constitute a problem because they inhibit seed germination.[3] As well, GP has been utilized as an additive in animal feeding, but the presence of polymeric polyphenols (lignin) reduces digestibility because they inhibit cellulolytic and proteolytic enzymes as well as the growth of rumen bacteria.[5] Additionally, the high content of dietary fiber, especially glycans, cellulose, and pectins, emphasizes the possible nutritive value of GP with a wide range of applications as food ingredients.[6,7]

GP as a Source of Bioactive Compounds. With the increase of consumers' awareness of the use of additives in foods and the attention that functional foods have acquired in recent years, there is a need for the identification of alternative natural and (purportedly) safer sources of food antioxidants. In the same way, modern industries are focused on diminishing the environmental impact of industrial byproducts. Therefore, most attention has been paid to the recovery of bioactive phenolics from grape byproducts from the winemaking industry.[8] When grape berries are processed for red winemaking, the skins and seeds are usually in contact with the fermenting broth for several days. Thus, grapes are subjected to a slight but prolonged extraction with a hydroethanol mixture that provides the red wine with a variable content of polyphenols. However, the residue remaining after fermentation (i.e., GP), which mainly consists of skins and seeds, still contains high levels of polyphenols. These polyphenols have known health-promoting effects and other properties in different biological and food systems. These features are related to the antioxidant characteristics as reducing agents (i.e., by donating hydrogen-quenching free radicals such as singlet oxygen), so inhibiting and delaying lipid oxidation in diverse food systems.[9−11] As a consequence, GP is considered a valuable source of phytochemicals that may be recovered as functional compounds for the pharmaceutical, cosmetic, and food industries as well as used as biopesticides. In this way, the recovery of phenolics from grape byproducts from the winemaking industry has attracted increasing attention in the past years, and industries are finding a high value and sustainable alternative to their residues. Recently, Yu et al.[11] reviewed the functional components of GP focusing on its composition and biological properties. They discussed the GP phenolics profile and their biological, antioxidant, and antimicrobial activities. Besides, the interactions of GP phenolics with other food ingredients as well as the functionalities of grape seed oil and GP fiber were covered. In the same way, Peralbo-Molina et al.[12] recently reviewed the potential of residues from the Mediterranean agriculture and related industries, focusing on residues with presently minor application such as vine shoots and leaves. They explain the

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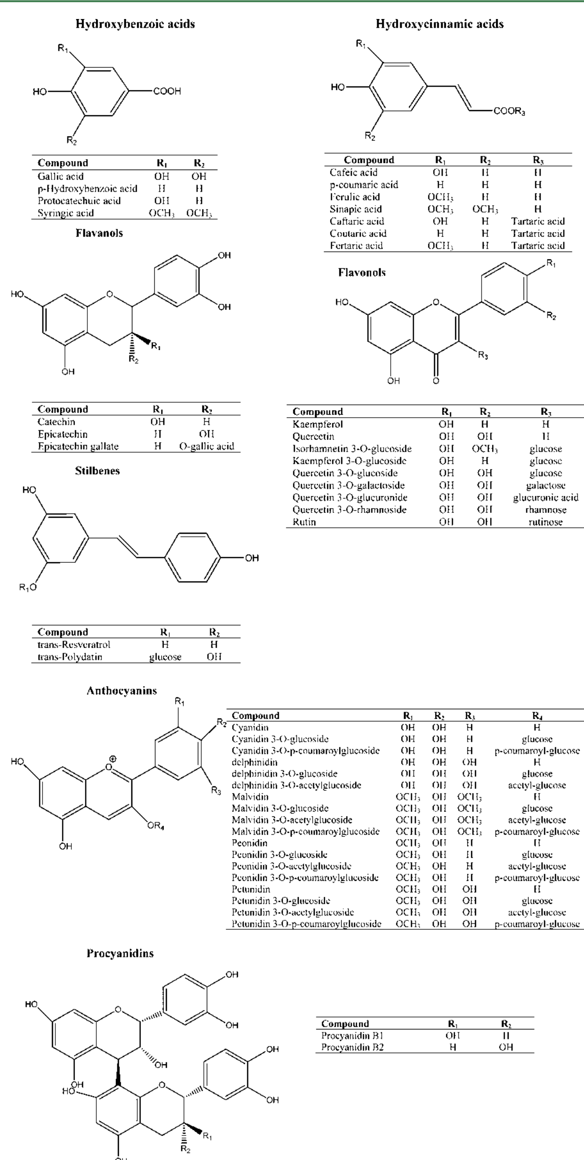
composition of these residues and their possibility of application in the nutraceutical field.

Phenolics (polyphenols) constitute one of the most numerous and widely distributed groups of natural products in the plant kingdom. Phenolics include not only an ample variety of molecules with a polyphenol structure (i.e., several hydroxyl groups on aromatic rings) but also molecules with one phenol ring, such as phenolic acids and phenolic alcohols. Phenolics contained in grapes and wine can in general be classified into three main groups: (1) phenolic acids (mainly benzoic and hydroxycinnamic acids), (2) simple flavonoids (catechins, flavonols, and anthocyanins), and (3) tannins and proanthocyanidins. Many phenolics have been identified in GP, where the most abundant are anthocyanins, hydroxybenzoic and hydroxycinnamic acids, flavan-3-ols, flavonols, and stilbenes.[3,13] Figure 1 summarizes the chemical structures of the phenolics identified in GP. As can be observed, an ample variety of compounds have been reported, justifying the investigation in this field related to sustainable uses of natural resources.



Compound	R_1	R_2
Gallic acid	OH	OH
p-Hydroxybenzoic acid	H	H
Protocatechuic acid	OH	H
Syringic acid	OCH_3	OCH_3

Compound	R_1	R_2	R_3
Cafeic acid	OH	H	H
p-coumaric acid	H	H	H
Ferulic acid	OCH_3	H	H
Sinapic acid	OCH_3	OCH_3	H
Caftaric acid	OH	H	Tartaric acid
Coutaric acid	H	H	Tartaric acid
Fertaric acid	OCH_3	H	Tartaric acid

Compound	R_1	R_2
Catechin	OH	H
Epicatechin	H	OH
Epicatechin gallate	H	O-gallic acid

Compound	R_1	R_2	R_3
Kaempferol	OH	H	H
Quercetin	OH	OH	H
Isorhamnetin 3-O-glucoside	OH	OCH_3	glucose
Kaempferol 3-O-glucoside	OH	H	glucose
Quercetin 3-O-glucoside	OH	OH	glucose
Quercetin 3-O-galactoside	OH	OH	galactose
Quercetin 3-O-glucuronide	OH	OH	glucuronic acid
Quercetin 3-O-rhamnoside	OH	OH	rhamnose
Rutin	OH	OH	rutinose

Compound	R_1	R_2
trans-Resveratrol	H	H
trans-Polydatin	glucose	OH

Compound	R_1	R_2	R_3	R_4
Cyanidin	OH	OH	H	H
Cyanidin 3-O-glucoside	OH	OH	H	glucose
Cyanidin 3-O-p-coumaroylglucoside	OH	OH	H	p-coumaroyl-glucose
delphinidin	OH	OH	OH	H
delphinidin 3-O-glucoside	OH	OH	OH	glucose
delphinidin 3-O-acetylglucoside	OH	OH	OH	acetyl-glucose
Malvidin	OCH_3	OH	OCH_3	H
Malvidin 3-O-glucoside	OCH_3	OH	OCH_3	glucose
Malvidin 3-O-acetylglucoside	OCH_3	OH	OCH_3	acetyl-glucose
Malvidin 3-O-p-coumaroylglucoside	OCH_3	OH	OCH_3	p-coumaroyl-glucose
Peonidin	OCH_3	OH	H	H
Peonidin 3-O-glucoside	OCH_3	OH	H	glucose
Peonidin 3-O-acetylglucoside	OCH_3	OH	H	acetyl-glucose
Peonidin 3-O-p-coumaroylglucoside	OCH_3	OH	H	p-coumaroyl-glucose
Petunidin	OCH_3	OH	OH	H
Petunidin 3-O-glucoside	OCH_3	OH	OH	glucose
Petunidin 3-O-acetylglucoside	OCH_3	OH	OH	acetyl-glucose
Petunidin 3-O-p-coumaroylglucoside	OCH_3	OH	OH	p-coumaroyl-glucose

Compound	R_1	R_2
Procyanidin B1	OH	H
Procyanidin B2	H	OH

Figure 1. Chemical structures of the common classes of phenolics identified in GP.

Analytical Methods for Characterization. Because of sample complexity, which includes high quantity of target analytes with different chemical natures, highly selective and sensitive analytical methods are necessary for the characterization of GP extracts. Because of the complexity of the problem, extraction techniques have been widely investigated to obtain high recoveries of valuable natural compounds for the commercialization of GP. Traditional extraction techniques have been gradually switched to novel extraction methods with reduced extraction times and low consumption of organic solvents, which increase the sustainability of the process. The chemical characteristics of GP extracts are related to the content of phytochemicals with bioactive properties, and so it is of utmost importance to determine their composition. Although the reported studies are mainly focused on quantitative data as total phenolic contents and antioxidant activity, the determination of individual compounds is of particular interest to recognize possible interactions between the content of phenolics and the antioxidant properties of GP extracts. These data may provide valuable information for the characterization of samples and also increase the economic value of the product. In this sense, it is necessary to rely on highly sensitive and selective instrumental techniques for unequivocal identification. Chromatographic techniques, especially high-performance liquid chromatography (HPLC), have been the choice for the analysis of phenolics in GP extracts. Nowadays, novel separation techniques with ultrahigh-pressure systems can achieve rapid, versatile, and high-throughput methods, particularly suitable for the analysis of complex samples such as those from plant extracts. As a resumé of the process to achieve the characterization of GP, Figure 2 shows a scheme of the steps associated with the chemical identification of phenolics in this matrix. The common steps in this flow diagram will be considered in detail below, with attention to both routine procedures and recent analytical developments.

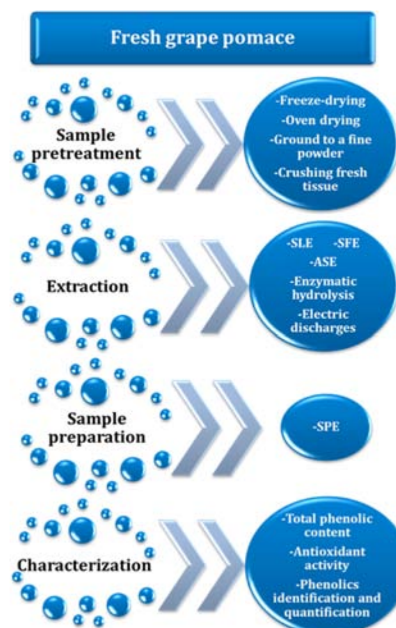


Figure 2. Steps associated with the characterization of GP, starting from fresh product discarded by wineries.

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Table 1. Reported Solid–Liquid Extraction Techniques for Phenolics in GP

sample pretreatment	solvent/solvent mixture	extraction mode, time (h)	temperature (°C)	TPC (mg GAE/g GPE)[a]	ref
freeze-dried and ground to a fine powder	80% ethanol (ratio 10:1)	overnight shaking	ni[b]	475.4	92
freeze-dried	50% acidified ethanol (pH 1 HCl)	2	60	139	25
oven-dried	50 or 75% acetone (ratio 10:1)	sonication, 0.25	ni	53	19
		shaking, 0.5			
chopped fresh tissue	methanol/acetone/water (6:3:1) (acidified 0.1% HCl)	stirring, 0.25	ni	54	94
chopped fresh tissue	methanol/acetone/water (6:3:1) (acidified 0.1% HCl) (ratio 10:1)	maceration, 0.25	ni	48–54	95
		stirring, 0.5			
air-dried at room temperature and ground to a fine powder	water (ratio 10:1)	continuous refluxing, 1.7	50	12.0–15.3	96
freeze-dried and ground to a fine powder	methanol (0.1% HCl) (ratio 20:1)	stirring, 2	ni	ni	3
freeze-dried	methanol/ethanol (8:2)	ultrasound, 0.25	ni	ni	97
		shaking, 12			
ni	methanol (ratio 10:1)	shaking, 0.5	ni	2.5–4.8 (fresh weight)	63
freeze-dried and ground to a fine powder	methanol (1% 1 N HCl) (ratio 6:1)	continuous stirring, 4	25	6.91–49.33	98
vacuum-dried at 50 °C	50% acetone (ratio 20:1)	ni	60	16.7	99
oven-dried at 60 °C	60% ethanol (ratio 4:1)	continuous stirring, 1	60	261.1	55
oven-dried at 50 °C and crushed	80% ethanol (0.5% 0.1 N HCl) (ratio 30:1)	ni	ni	41.9	52
oven-dried at 60 °C and ground to a fine powder	ethanol (ratio 4:1)	5	60	ni	100
air-dried at room temperature	methanol (ratio 5:1)	shaking, 1.5	50	10.9	24
freeze-dried and powdered in liquid nitrogen	methanol (0.1% HCl) (ratio 50:1)	shaking, 1	4	32.62–74.75	101
hot air-dried at 60 °C	50% methanol and 70% acetone	1	room	ni	7
freeze-dried and ground to a fine powder	methanol (1% 1 N HCl) (ratio 4:1)	stirring, 12	ni	ni	102
oven-dried at 60 °C and ground to a fine powder	50% acetone (ratio 5:1)	shaking, 0.7	25	77.5–148.3	18
freeze-dried and ground to a fine powder	70% ethanol (ratio 4:1)	shaking, 5	60	≈20	103
ni	ethyl acetate (ratio 10:1)	0.3	ni	ni	51
oven-dried at 50 °C and ground to a fine powder	53% ethanol (ratio 50:1)	ultrasound, 0.5	56	5.44 (100 mL extract)	27
oven-dried at 50 °C and ground to a fine powder	53% ethanol (ratio 50:1)	ultrasound, 0.5	56	5.44 (100 mL extract)	27
freeze-dried	methanol/ethanol/water (2:1:1) (ratio 10:1)	ultrasound, 0.3	ni	344–618	74
freeze-dried	methanol (ratio 10:1)	ultrasound, 0.25	ni	1.9–4.5 (100 mL extract)	104
freeze-dried	74% methanol	ultrasound, 0.25	ni	ni	67

[a]TPC, total phenolic content; GAE, gallic acid equivalent; GPE, grape pomace extract. [b]ni, no information.

This review presents an overview of the different techniques reported for bioactive phenolics in GP published in recent years, focusing on sample extraction strategies as well as sample preparation, separation, and analysis of these compounds by instrumental techniques. Also, we critically discuss the limitations and potential of methods with the aim to improve the determination of phenolics in complex matrixes. Finally, the applications of GP extracts in diverse fields of biotechnological industries are also presented.

EXTRACTION TECHNIQUES

Extraction is an important step in the recovery, isolation, and identification of compounds prior to the use of phenolic extracts, and there is no standard extraction method. Traditional techniques such as solid–liquid or Soxhlet extractions have been used for many decades, but they are time-consuming and require relatively large quantities of solvents.[14] Also, due to the common extractive steps used by these techniques, including heating, boiling, or refluxing, a loss of polyphenols due to ionization, hydrolysis, and oxidation occurs during the procedure. Besides, factors such as extraction solvent and sample/solvent ratio are relevant to achieving good recoveries, especially considering the polar nature of compounds. In recent years with the development of miniaturization in chemistry, some new techniques were successfully proposed for extraction of phenolics from GP. These techniques are primarily focused on shortening the extraction time, reducing organic solvent consumption, and increasing sustainability while maintaining (or even improving) recovery of compounds of interest.

Solid–Liquid Extraction (SLE). The most common technique reported for extraction of polyphenols from GP is SLE. SLE can be defined as a phenomenon of mass transport in which the analytes contained in a solid matrix migrate into a solvent phase that is in contact with the matrix. Mass transport phenomena, and thus the extraction efficiency, can be improved by changes in concentration gradients, diffusion coefficients, or boundary layer, all of these being affected by the extraction method, solvent type, particle size, temperature, and extraction time as well as the presence of interfering substances in the matrix.[15,16] Solvent type is one of the main factors affecting the

extraction efficiency of the process. Due to the polar nature of polyphenols, they are easily solubilized in polar protic media such as hydroalcoholic solutions. Phenolic fractions could be then obtained by varying the alcohol concentration in mixtures with increasing concentration of low-polar solvents such as ethyl acetate.[17] Table 1 summarizes the reported techniques based on SLE for phenolics in GP. As can be observed, solvents such as methanol, ethanol, acetone, and water have been the most used for the recovery of polyphenols from GP with variable extraction efficiencies.[18] As well, different degrees of acidification with HCl have been reported (see Table 1). Among these solvents, methanol exhibits the highest capacity to extract phenolics. When alcohols are utilized as extraction solvents, a progressive release of polyphenols from GP as a function of the extraction time is observed, whereas contact time is not as significant when water is used.[8] However, if the aim is to select the best extraction solvent system among the mentioned alternatives, it could be a really difficult task. The published results are not conclusive about an ideal solvent, and different mixtures have been proposed (see Table 1). The mixtures based on acetone/water have been suggested to give better results for the extraction of procyanidins from grape seeds.[19] Other researchers have found that the extraction of catechins and procyanidins was more efficient with the use of an ethanol/water mixture.[20] In contrast, methanol was reported to be the best solvent for extracting catechins, epicatechins, and epigallocatechins from grape seeds.[21] Instead of individual extraction of compound families, the most developed methods are aimed to obtain extracts with high phenolic content prior to their fractionation and characterization. As can be seen from Table 1, several researchers have used organic/alcoholic solvent/water mixtures for the extraction of grape byproducts because the presence of water increases the permeability of cell tissue, enabling a better mass transfer by molecular diffusion as well as the recovery of water-soluble compounds.[18,22] In terms of the extraction of total phenolic content (TPC), ethanol/water mixtures showed relatively better results as compared to acetone or methanol/water mixtures. Additionally, ethanol is cheaper and has GRAS (generally recognized as safe according to U.S. Food and Drug Administration definition) status. As well, this solvent may be preferable in the case of later food utilization. However, it is necessary to have in mind that extraction of TPC from GP also depends on waste fraction, grape variety, extraction time, temperature, acidification of solvents, and interactions among all of these variables. As can be observed from Table 1, increasing solvent-to-solid ratio has also been found to work positively to enhance yields. However, equilibrium between the uses of high and low solvent-to-solid ratios, involving a balance between cost and solvent waste and avoiding saturation effects, needs to be found for specific situations.

Extraction time and temperature are important parameters to be optimized to minimize the energy cost of the process and to achieve high recoveries of compounds. According to results summarized in Table 1, many authors agree that an increase in the working temperature favors extraction efficiency by enhancing both the solubility of solutes and the diffusion coefficient.[23,24] Regardless of the positive effects of high temperatures on the extraction yields, this factor cannot be increased indefinitely because at temperatures >50 °C the stability of phenolics decreases and denaturation of membranes may occur.[24] Some works reported that temperatures of 60 °C increase the extraction of phenolics from GP. Spigno et al.[23] performed an interesting comparison between the simultaneous effect of extraction temperature and time. The authors demonstrated that both variables exert a significant effect, and they may be regulated after an economical appraisal of the energy cost for the whole procedure.

The pH of the extraction solution also affects the recovery of phenolics, and there are many works that reported increased TPC by using an acidified extraction solvent mixture (see Table 1). The added concentrations ranged from 0.1 to 1% HCl, achieving variable increases in extraction efficiencies. As an example, Vatai et al.[25] stated that the addition of HCl to the solvent resulted in an increase of 3-fold TPC in the extract. However, there was no significant effect on total anthocyanins. In this way, the addition of acid to the extraction system will be a convenient alternative to achieve higher recoveries of selected compounds that are affected by this variable. Different organic solvent/water ratios with and without acid addition have been evaluated to optimize the extraction. Most literature reported that the majority of polyphenols are extracted after one or two extraction steps, whereas the application of three extraction steps produces only a slight increase in recovery.[18] Besides, extraction of compounds of interest may be increased with one or two steps combined with the use of alternative energies. The application of ultrasound radiation (US) is a convenient alternative to conventional SLE by shaking or stirring because it may increase the extraction efficiencies of compounds in a single extraction. It is simple and inexpensive, being only a slight modification of the conventional SLE approach. The effects of US are primarily related with the cavitation phenomenon, which involves the implosion of bubbles formed in the liquid medium during US. The bubble implosion generates rapid adiabatic compression of gases and vapors within the bubbles or cavities and, as a consequence, high temperature and pressure are produced.[26] The increased pressure favors penetration of the extraction solvent into the sample matrix and also improves transport between the solid matrix and the liquid phase. This leads to an increment in the solubility of the analytes and their diffusivity from the sample matrix to the solvent phase, which is the limiting step of mass transfer.[26] Ghafoor et al.[27] reported that US was as effective as any other extraction process such as high temperature or long shaking time. The main virtue of US is that it greatly reduces the extraction time without necessity of high temperatures that may affect phenol stability. The efficiency of US extraction could be explained by the fact that sonication simultaneously enhances the hydration and fragmentation process while facilitating the mass transfer of solutes to the extraction solvent.[27] Liu et al.[28] performed a comparative study of US assisted and conventionally stirred to facilitate dead-end microfiltration (MF) of GP extracts. They compared the energy consumption and liquid flux enhancement of the two filtration systems, achieving much flux enhancement for the US-assisted MF. The authors suggested that ultrasonic power and stirring speed did not influence markedly the permeate quality and, as well, that no modification of intrinsic permeability of the membrane by US irradiation was observed. However, further precise analysis is needed to ensure the structural integrity of the membranes after US application.

In summary, the SLE of phenolics from GP depends on several variables that could be optimized. The utilization of solvent mixtures containing ethanol/water is preferred in terms of their similar extraction efficiencies and lower cost and the sustainability of the process. With the aim to achieve better

recoveries in a one-step extraction, the application of US results in a reliable option to conventional long-term shaking and high-temperature procedures. Besides the extraction techniques used, other factors involved in winemaking processes, as well as the genetic and environmental characteristics of grapes, may affect the phenolic composition of the final byproduct and thereafter the final TPC extraction yield of the technique. Thus, it is important to take into account other parameters affecting the phenolic composition of GP to have a better appraisal of the efficiency of extraction techniques.

Supercritical Fluid Extraction (SFE). SFE is still a fairly novel technique to extract target analytes from solid matrices. SFE employs the unique properties of supercritical fluids to facilitate the extraction of organic analytes from solid samples.[29] A supercritical fluid is a substance above its critical temperature and pressure that has a good solvating power (like liquid), high diffusivity, low viscosity, and marginal surface tension.[29] Due to these characteristics, SFE permits a rapid mass transfer in the supercritical phase and an improved ability to penetrate the pores in the sample matrix, achieving a fast and efficient extraction. SFE may be an environmentally sustainable alternative to the conventional organic solvent extraction because it avoids the use of large amounts of toxic solvents, being also rapid, automatable, and selective. Likewise, the absence of light and air during the extraction reduces the degradation processes that may occur during the traditional organic solvent extraction.[16]

SFE basically consists of two major steps: (1) extraction of the soluble substances from the solid matrix by the supercritical fluid solvent and (2) separation of extracted compounds from the supercritical solvent after the expansion. The extraction phenomenon can be resumed as follows: At first, the solid matrix absorbs the supercritical solvent, which favors the dilatation of the cellular structures. This step facilitates solvent flow through the sample by decreasing mass transfer resistance. At the same time, soluble compounds are dissolved by the solvent and transferred by diffusion to the solid surface. Finally, the compounds are transported by the solvent and then removed from the extractor.[30]

To develop an effective SFE, several factors must be taken into consideration. These factors include selection of supercritical fluids, sample preparation, use of modifiers, and extraction conditions. In the case of the extraction of phenolics from GP, the most used solvent is supercritical carbon dioxide (SC-CO_2). The addition of modifiers to a supercritical fluid (SCF) (such as methanol) can change its polarity, obtaining a more selective extraction power. Therefore, SC-CO_2 methods are a convenient alternative for the extraction of natural products from plant materials. As well, they are principally recommended for the extraction of thermolabile compounds for which low temperatures are required.[16] In this sense, the SC-CO_2 extraction uses a moderate extraction temperature as low as 30 °C, avoiding the degradation of these susceptible analytes.[14] Additionally, SC-CO_2 has the advantage of being environmentally friendly to achieve extracts free of residual toxic solvents. Exclusion of organic solvents is desirable for extraction of natural products to be used as functional foods and nutraceuticals.

De Campos et al.[31] studied the effect of various extraction methods in relation to the antioxidant potential present in GPE. Other features such as phenolics yields and lipophilic composition of extracts were also evaluated. The authors compared the performance of conventional SLE, Soxhlet, and SFE with SC-CO_2 and with SC-CO_2 plus a cosolvent. The results showed that the addition of a cosolvent such as 15% ethanol enhances the yield and antioxidant activity due to proportional changes in the solvent mixture characteristics. However, the antioxidant activity and TPC of the obtained extracts were considerably lower than those achieved by using other extraction methods. SFE gave better results for the extraction of nonpolar compounds such as fatty acids and was able to extract important compounds not detected in the conventional extracts.

Palenzuela et al.[32] used methanol instead of ethanol as a modifier with better results; 3% methanol added to the extraction system increased the efficiency of SFE in terms of yield.

The possible industrial application of SFE for the extraction of grape seed oil was exploited by Fiori.[33] In this work the author proposed an integrated system producing grape seed oil and related byproducts from exhausted GP, achieving recoveries of 86%. The results appear interesting because the spread between production cost and retailing price, as analyzed by the author, is enough to make the supercritical process economically convenient.

As well, it is worth mentioning that SFE has the ability to extract in a high extent nonpolar compounds. Future work may expand this technique to the extraction of a wider spectrum of compounds, giving a more versatile technique for phenolics.

Accelerated Solvent Extraction (ASE). ASE is also known as pressurized fluid extraction or pressurized liquid extraction. It uses conventional solvents at elevated temperatures (100−180 °C) and high pressures (1500−2000 psi) to enhance the extraction of organic analytes from solid samples. The elevated pressure and temperature used in ASE affects solvent, sample, and the interactions between them. The solvent boiling point increases under high pressure; thus the extraction can be conducted at higher temperatures. The high pressure also allows the solvent to penetrate deeper into the sample matrix, increasing the extraction of analytes confined in matrix pores. At elevated temperatures, the solubility of analytes increases and mass transfer become faster. The high temperature also weakens the solute−matrix interaction due to van der Waals forces, hydrogen bonding, and dipole attractions. In addition, solvent viscosity and surface tension are reduced at high temperature, enhancing solvent penetration into the matrix. All of these factors lead to faster extraction and higher recoveries of analytes.

Taking into consideration the mentioned features, many works applied ASE for the extraction of phenolics from GP. Monrad et al.[34,35] developed two alternative methods for the extraction of anthocyanins and procyanidins from GP and studied the variables influencing the extraction by ASE. The authors reported that a mixture of 50:50 ethanol/water (v/v) extracted more procyanidins from red GP than other ethanol/water solvents mixtures studied, working at an optimal ASE temperature range of 80−140 °C. Although ethanol/water mixtures were less effective than individual solvents in extracting high molecular weight polymers, they were especially effective in extracting monomers, dimers, trimers, tetramers, and pentamers. Similar conditions gave the best results for the extraction of anthocyanins. In this sense, the authors proposed ASE as a convenient extraction technique that can be applied in wine and juice industries to extract bioactive compounds, because it is environmentally friendly (use of a GRAS solvent) and inexpensive. Rockenbach et al.[36] also reported the use of

ASE using a mixture of acetone/water (70:30) at 25 °C, reporting the extraction of several galloylated and non-galloylated flavan-3-ol compounds and condensed products of catechin with acetaldehyde. As well, they identified the elemental compositions of 251 different flavan-3-ol compounds in the Cabernet Sauvignon variety, including isomers of 28 different molecular classes, proving the ability of ASE for the extraction of these compounds. Because the stability of phenolics under high temperature decreases, the method reported by Rockenbach et al.[36] appears to be more suitable in comparison to others using temperatures >80 °C. In this way, this aspect should be considered during the development of ASE methods for phenolics to avoid high extraction temperatures. However, it needs to be evaluated according to the phenolics that we want to extract from the sample and the relative content of each one.

Recently, Aliakbarian et al.[37] proposed a novel ASE approach using subcritical water as extraction solvent. They reported recoveries of phenolics from GP in subcritical water at 140 °C similar as those obtained by using traditional organic solvents. This was explained as the increasing temperature also raised the solute vapor pressure, promoting the mass transfer of phenolics by enhancing the diffusivity and decreasing the viscosity.[38] Nonsignificant differences between the total flavonoids contents (TFC) of extracts obtained by ASE and SLE with ethanol were observed. Extracts with higher TPC (although with lower antioxidant power) were achieved with water as ASE solvent instead of ethanol.

Vergara-Salinas et al.[39] optimized a pressurized hot water extraction method for antioxidants in GP. The presented results showed that elevating the extraction temperature increased total antioxidant extraction and antioxidant activity. The maximum anthocyanin extraction yields were achieved at 100 °C and at 150 °C for tannins and tannin−anthocyanin adducts. The application of higher temperatures and longer extraction times resulted in a sharp decrease of polyphenol extraction yield. In the case of relevant proanthocyanidins, they were extracted only at 50 and 100 °C. The authors applied the optimized methods to compare the extraction of antioxidants from fermented and unfermented GP, achieving a greater recovery of phenolics and antioxidant activity equivalent to fermented GP.

In the last extraction methods, the authors stated that the technique has the advantage of employing water as the unique solvent for the extraction of phenolics, achieving similar extraction efficiencies as conventional organic solvent extraction, with the additional advantage that the processing time was remarkably shorter.

Other Emerging Sample Extraction Techniques. Enzymatic release is another useful technique for extraction of phenolics from GP. The reduction of particle size in GP has a positive effect on the recovery of phenols from the matrix, promoting an increase in surface contact between solids of the GP and the solvent and, so, an increase in the extraction efficiency. The application of various mixed pectinolytic and cell-wall polysaccharide degrading enzyme preparations catalyzes polysaccharide hydrolysis. Kammerer et al.[40] optimized the enzymatic hydrolysis of GP extracts by using a combination of pectinolytic and cellulolytic enzymes during extraction with water to increase yields of phenolics. The authors performed a pre-extraction of the GP followed by enzymatic treatment with a combination of the mentioned enzymes. After pre-extraction, 70.1% of phenolic acids, 75.2% of nonanthocyanin flavonoids, and 1.7% of anthocyanins were recovered. After enzymatic treatment, total contents of phenolics obtained in this two-stage extraction process amounted to 98.1% (phenolic acids), 96.8% (nonanthocyanin flavonoids and stilbenes), and 2.9% (anthocyanins). The success was explained by a reduced inhibitory effect on enzymatic digestion because phenolics were partly extracted during the first step. However, from the presented data, it can be seen that even pre-extraction did not improve anthocyanin recovery considerably. The proposed two-step (pre-extraction and digestion) process may be a good strategy to obtain highly concentrated extracts to be used as food additives or functional food ingredients. Lately, Chamorro et al.[41] examined the release of phenolics from GP extracts after treatment with tannase, cellulase, and pectinase, establishing their relationship with the antioxidant capacity. The results demonstrated that the extracts obtained after these enzymatic reactions showed higher antioxidant capacity than the untreated byproducts. The use of tannase in grape seed and GP and pectinase in GP changed the galloylated catechin to its free form, releasing gallic acid. Thus, the enzyme-assisted polyphenol extraction process may be used to enhance the release of bioactive compounds from these matrices. Nonetheless, further studies are needed to improve the knowledge about the identity of the cell-wall polysaccharides and how the phenols are inserted and/or bound in the cell wall of grape berries. Such knowledge will allow the use of more specific enzymatic preparations to accomplish the release of grape skin phenolics.

Another extraction technology recently introduced is high-voltage electric discharge (HVED). This technique is based on the application of a high voltage between two electrodes, so the electrons are accelerated and reach sufficient energy to excite water molecules. Then, a flood of electrons called streamer is created. If the applied electric field is intense enough, the streamer propagates from the positive to the negative electrode. When one of the streamers attains the negative electrode, electrical breakdown occurs. During such electrical breakdown, high-amplitude pressure shock waves, bubble cavitation, and liquid turbulence are created. These phenomena result in particle fragmentation and cell structure damage that accelerate the extraction of intracellular compounds.[42] With the positive effects of HVED taken into account, the optimum conditions for the recovery of phenols and the possibility of applying these results at a pilot scale have been established.[42−45] The initial development was focused on a method to accelerate the extraction of total soluble matter and polyphenols from GP into distilled water. These authors also reported a synergistic effect on polyphenol extraction when HVED was combined with freezing or elevated temperatures. Such an effect may be explained because an impact on cell membranes like any freezing process as well as changes in cell membranes by heating increases the cell permeability.[42] The results showed that higher energy is required to obtain equivalent extraction rates at the pilot scale when compared with laboratory scale.[45] Thus, with the objective of obtaining a reliable and sustainable alternative to traditional extraction techniques, further studies are required to determine the energy costs of HVED related to other reported extraction techniques.

Pulsed ohmic heating (POH) combining electrical and thermal treatments can be an effective method to extract valuable cell compounds using moderate electric field and temperatures. El Darra et al.[46] proposed POH to produce cell membrane damage and increase phenolics extraction from red

GP. They studied the effects of electric field strength (100−800 V/cm) and the percentage of ethanol in water (0−50%) on phenolics extraction. The achieved results showed that POH treatment results in cell membrane denaturation, and this effect increases with the elevation of electric field strength. A synergy was observed with respect to phenolics extraction when POH was combined with moderate diffusion temperature (50 °C) and 30% ethanol. POH pretreatment accelerates the extraction kinetics of total phenolics from GP and is not require to work at elevated temperatures. The authors assumed that the POH-accelerated extraction is promising for future application in the valorization of pomace from fruits and vegetables without hydroalcoholic solvent use.

Critical Comparison of Extraction Techniques. On the basis of the available data about extraction of bioactive phenolics from GP, some comparative remarks may be stated. The application of conventional methods (SLE, US-SLE) has the advantage of being accessible for most laboratories, with satisfactory recoveries when extraction parameters have been successfully optimized. However, they are often restricted by several difficulties such as overheating of the matrix, which may produce a loss of activity and poor stability of the final product, and high energy consumption and general cost, as well as the utilization of organic solvents that could be a problem for later application. Extraction techniques such as SFE and ASE have demonstrated their suitability for the extraction of bioactive compounds, achieving recoveries similar to or better than conventional ones. Because of advantages such as lower solvent consumption and relatively lower operative necessity, they appear to be convenient alternatives. However, although the yield of compounds using the aforementioned technologies is important for the industries in the recovery processing, other factors such as product safety and general cost govern the final decision for the selected methodology. These aspects are critical in the case of emerging technologies, as they could be too sophisticated in comparison to the yield improvement they are promising. Besides, because most of the reported techniques have been tested only in laboratory-scale experiments, pilot-scale studies are necessary to select the optimal technique after evaluation of the costs to produce commercial reliable extracts. Novel extraction processes are usually complex thermodynamic systems with high costs. The modeling of these extraction processes can provide a better understanding of the extraction mechanisms, and they may be used to optimize the extraction conditions and scale up any design. Moreover, it is necessary to establish safety considerations related to the unknown impact of innovative technologies and the possible negative effects on the consumers.

SAMPLE PREPARATION AND FRACTIONATION BEFORE INSTRUMENTAL ANALYSIS

The production of concentrated extracts of specific bioactive components from GP is relevant from both industrial and analytical points of view. The extract obtained from recovery processes includes many bioactive phytochemicals, so pre-concentration of specific chemical groups sometimes is required because it has been reported that individual polyphenols exhibit different functionalities and chemical activities.[9] Thus, the complexity of the extracts makes it necessary to apply some sample preparation step such as preconcentration and purification to isolate different groups of phenolics prior to instrumental analysis. There are different reviews reporting sample preparation strategies for extracting and cleaning up extracts of plant and, more particularly, grape phenolics.[16,17,47−50] In this sense, we focus in this section on discussing the developments used for GP characterization. The application of solid-phase extraction (SPE) to increase the selectivity of the chromatographic techniques for the identification of phenolics was reported by different authors. Diverse sorbent materials have been used for the fractionation between phenolic acids, flavonoids, and anthocyanins present in GP extracts. The most commonly used sorbent is reverse-phase octadecylsilane (C_{18}), which has a good affinity for phenolic compounds. Kammerer et al.[3] developed a method for the fractionation of phenolics based on the adsorption of such compounds onto C_{18} minicolumns followed by sequential elution with acidified water (hydroxybenzoic and hydroxycinnamic acids), ethyl acetate (flavanols, flavonols, and stilbenes), and acidified methanol (anthocyanins). Anthocyanins were analyzed directly in the acidified extract of GP extracts prior to SPE. Also before the SPE step, the authors applied an extraction of nonanthocyanin phenolics from crude GP extracts with ethyl acetate. The purpose of the former procedure was to reduce the quantitative abundance of anthocyanins, increasing the retention capacity of C_{18} cartridges.[5] The method reported by Kammerer et al.[3] was quite efficient in terms of the number of compounds reported in GP extracts that was applied to many different samples. Other authors used different variations of the SPE technique (with C_{18} as sorbent) for the profiling of anthocyanins or anthocyanidins in GP etracts with reasonable results in terms of purification and selectivity prior to the analysis by HPLC.[51−53] Yi et al.[54] proposed the use of hydrophilic−lipophilic-balanced (HLB) reversed-phase sorbent for the initial separation of two fractions: (1) phenolic acids and (2) anthocyanins and other flavonoids. Then, the suspended extract was loaded onto a Sephadex LH20 column to elute first the anthocyanins and flavonols using methanol acidified with formic acid, and then the column was washed with 70% acetone for the elution of tannins and proanthocyanidins. After freeze-drying, the anthocyanin and flavonol fractions were solubilized in 5% formic acid in water and applied to a second HLB cartridge. The cartridge was washed with 5% formic acid, followed by ethyl acetate and then 10% formic acid in methanol. The ethyl acetate eluted the flavonols and the acidified methanol eluted the anthocyanins. The authors utilized this method for the isolation of different fractions prior to evaluation of these phenolics against viability and apoptosis in cancer cells. This procedure has several steps that augment sample manipulation and therefore may increase the loss in reproducibility of the analytical method. Also, it could be time-consuming if we compare it with the method reported by Kammerer et al.[3]

ANALYTICAL CHARACTERIZATION

There is an increasing demand for highly sensitive and selective analytical methods for the determination of phenolics. Despite a great number of investigations, the separation and quantification of different phenolics remain challenging, especially the simultaneous determination of diverse groups of them. Different spectrophotometric methods for quantification of phenolics have been developed. These procedures are based on different principles and are used to determine various structural groups present in phenolic molecules. Several authors have studied the properties of GP extracts by determining the antioxidant power using different techniques as well as the TFC of extracts or different isolated fractions. Because synergistic

and antagonistic effects have been observed in in vitro tests, similar interactions are also expected in vivo. In this way, the mentioned methods that give broad-spectrum information about the extract might be complemented by using chromatographic techniques for the identification and quantification of individual phenolics present in each fraction. This section will discuss the analytical methods used for GP extracts with the aim of establishing the correlation between properties of extracts or their fractions with the results about profiling of each individual phenolic. Taking into account that there are several reviews describing the principles of techniques used for the analysis of plant and more particularly grape phenolics, the section is focused on applications to GP.

Spectrophotometric Methods for Quantification of Total Phenolics. The determination of TPC is the starting point for the characterization of GPE because it gives useful information about the relative composition of the sample. There are several spectrophotometric methods based on different principles for the quantification of phenolics in plant samples which are used to determine diverse structural groups. These techniques have been properly reviewed and explained by different authors.[48] The simplest method for a quick estimation of TPC is the measurement of absorption at 280 nm (in a sample properly diluted). The second method most commonly used for TPC assessment is the Folin–Ciocalteu assay. Amendola et al.[55] suggested that direct reading of absorbance at 280 nm is preferable to the Folin–Ciocalteu method. They stated that yields of total phenols, based on gallic acid equivalents (GAE-280), were lower but better correlated to those based on GAE obtained from Folin–Ciocalteu method. However, most papers about GPE used either method with apparently satisfactory results. The obtained data give an estimation of TPC that in combination with antioxidant activity is the starting point for the chemical characterization of GP extracts. In this regard, most papers correlate the GP extract antioxidant activity with TPC to explain their biological and nutritional properties.

Determination of Antioxidant Activity. As stated above, the determination of GP antioxidant activity is a useful strategy to evaluate extraction methods as well as for the preliminary characterization of samples before chromatographic analysis. Hence, the assessment of the antioxidant activity of GP by an adequate assay is critical. There are two general types of assays widely used for antioxidant studies: assays associated with lipid peroxidation and techniques related with electron or radical scavenging. Several authors have applied different in vivo and in vitro assays, the latter being preferred when chemical characterization of GP extracts or evaluation of the extraction methods is needed. There are different reviews reporting techniques for the assessment of in vitro and in vivo antioxidant activity. López-Alarcón et al.[56] presented the chemical-based methodologies employed for screening antioxidant activity of natural products, discussing the classical and nonclassical mechanisms of action. As well, Frankel et al.[57] reported an interesting perspective about standardization of different methods for evaluating antioxidant activity in foods. They give definitions related to the convenience of each technique, as well as drawbacks of each one. Taking into account the mentioned reviews, we focused this section on those reports related to the application of a particular technique for the analysis of GP extracts and encourage the reading of these previous works to explain each technique.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent has been used for characterization of the GPE antioxidant power[31] because flavonols are able to scavenge electrons from superoxide and hydroxyl radicals and DPPH by single transference. This spectrophotometric technique consists of measuring the decrease in absorbance of the colored radical ($DPPH^•$) provoked by the presence of antioxidants. $DPPH^•$ accepts a hydrogen from an antioxidant, and the antioxidant activity is proportional to the disappearance of $DPPH^•$. The color change from purple to yellow after the formation of $DPPH_2$ occurs when the radical $DPPH^•$ takes hydrogen from the antioxidant. This reaction is stoichiometric with respect to the number of absorbed hydrogen atoms. Therefore, the antioxidant effect can be easily evaluated by following the decrease of absorption at 517 nm. With the aim of normalizing the results from different studies, the Trolox equivalent (TE) unit has been the most used.[58] This method is simple and highly sensitive, so it has been commonly used for the determination of antioxidant activity in GP extracts with acceptable results in terms of antioxidant activity evaluation.[7,18,20,31,37,53,59−63]

Another method increasingly used is oxygen radical antioxidant capacity (ORAC). The ORAC method is based on the inhibition of the peroxyl radical induced oxidation initiated by thermal decomposition of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). The presence of antioxidants in the medium protects a fluorescent probe (fluorescein or pycoerithrin) from oxidation by the peroxyl radical, so the time of fluorescence emission is prolonged. Then, the antioxidant activity of foods is quantified by the areas under the curves of relative fluorescence intensity.[64] The ORAC assay utilizes a biologically relevant radical source and is the only technique that combines both time and degree of inhibition into a single magnitude.

Monagas et al.[65] used ORAC to relate the antioxidant activity of commercial dietary grape seed extracts (GSE) with their phenolic composition. They observed product-to-product and batch-to-batch variations in ORAC values and flavan-3-ol composition among the products, demonstrating poor standardization of commercial extracts.

Yilmaz et al.[19,64] reported the antioxidant activity of GSE and skin extracts obtained from winemaking and juice industries of grape cv. Chardonay, Merlot, and Muscadine by the ORAC method. They also described the contribution of the major monomeric flavonols and phenolic acids to the total antioxidant activity of these extracts. The results showed that GSE had higher ORAC values as compared with skin extracts. They also proposed that the high antioxidant activities of GSE would most likely be due to the presence of polymeric procyanidins, in addition to the monomers.

Rodriguez-Rodriguez et al.[66] used DPPH and ORAC methods to assess the antioxidant activity of GP extracts obtained from aqueous–enzymatic extraction and to evaluate in vivo the effects of GP extracts in aorta tissue of rats. They found that GP extracts induce endothelium-dependent vasodilatation and attenuate vascular contraction through a NO-dependent mechanism.

Although DPPH and ORAC are the most common assays, there are other methods reported for antioxidant activity determination in GP extracts. González Paramás et al.[67] used the Trolox equivalent antioxidant capacity (TEAC) assay. It measures the capacity of compounds to scavenge the relatively stable blue/green chromosphere radical cation 2,2′-azinobis(3-

ethylbenzothiazoline-6-sulfonate) (ABTS), changing it into a colorless product when an antioxidant is applied.

The thiobarbituric acid (TBA) assay has been used to quantify the antioxidant activity in lipid phase. It is based on lipid peroxidation, and the antioxidant activity is spectrophotometrically determined by production of thiobarbituric acid reactive substances (TBARS).[58] Caillet et al.[68] applied the *N*,*N*-diethyl-*p*-phenylenediamine (DPD) method to evaluate the antioxidant activity of hydrophobic and hydrophilic fractions of GPE and GSE. It consists of the production of reactive oxygen species (ROS) in an electrochemical way. The ROS oxidize the DPD colored reactive, and the capacity of grape polyphenols to inhibit the accumulation of ROS is spectrophotometrically quantified.

As can be seen from the research discussed above, there is disparity in the types of tests with different bases that are applied to evaluate the antioxidant activity of extracts. Also, differences in the expression of results and use of reference antioxidants are found. The DPPH method appears as the most suitable alternative, although it is generally recognized that the use of two different methods to investigate antioxidant activities will give more reliable results. A combination of assays is recommended for scavenging electrons or radicals and also for lipid peroxidation.

Identification and Quantification of Phenolics from GPE. Due to the polar nature of phenolics present in GPE, the most commonly used analytical technique for separation of these compounds is HPLC. As can be appreciated from Table 2, the chosen columns for phenolics are almost exclusively of the reverse phase type, with C_{18} as stationary phase, an internal diameter between 2 and 4.6 mm, and a particle size ranging from 3 to 10 μm. Narrow-bore columns with small internal diameter packed with very small particles for ultrahigh-performance liquid chromatography (UHPLC) have the advantage of better resolution and sensitivity, being quite versatile and fast and so augmenting sample throughput of the methodology.[69] However, UHPLC has not been used for GP extract analysis, being an alternative to conventional HPLC that deserves to be explored. The solvent system consists of an aqueous phase and an organic phase (mainly methanol or acetonitrile) that are mixed isocratically or in a gradient. For the separation of procyanidins, mixtures of $CH_2Cl_2/CH_3OH/H_2O$ have been preferred.[35,70] Usually, an acid is added to the solvents in concentrations ranging from 0.1 to 10%, to get good peak shape but also to improve ionization during electrospray ionization (ESI) of compounds if the system is coupled to a mass spectrometry (MS) detector. Acetic and formic acid are the most commonly used, although phosphoric and trifluoroacetic acids have also been employed. The mentioned combination of columns, solvent systems, and conditions has been successfully applied for the separation of families of phenolics such as anthocyanins, procyanidins, flavanols, isoflavones, flavonols, phenolic acids, flavanones, and stilbenes (see Table 2). Irrespective of the chromatographic program used, different runs are required for separation of compounds from diverse families because it is practically impossible to achieve good separation of phenolics present in GP extracts with a single HPLC run. Kammerer et al.[3] reported a successful HPLC approach for the separation of phenolics in white and red GP by using three different chromatographic systems: system I for anthocyanins, system II for phenolic acids, and system III for flavanols, flavonols, procyanidins, and stilbenes (see Table 2). By using this method the authors identified and quantified 40 compounds in different GP samples. Chromatographic methods performed by other authors have the drawback that only a few compounds of each family, namely, flavanols, flavonols, etc., were evaluated. This fact affects negatively the interpretation of the results because it does not allow knowing the qualitative and quantitative importance of each individual compound in the extract. This information is relevant to justify possible technological applications with added value to the wine industry.

Another method recently reported for the determination of the main anthocyans in pomace refers to planar chromatography. Krüger et al.[71] developed an efficient method for quantification of 11 anthocyans using silica gel with a mixture of ethyl acetate/2-butanone/formic acid/water for anthocyanins and ethyl acetate/toluene/formic acid/water for anthocyanidins. They performed the identification of unknown anthocyanin sample components by MS, eluting the zones of interest with the TLC-MS interface, which was helpful for further characterization of unknowns. The analytical figures of merit showed LOQs ranged between 7 and 90 ng/zone, repeatabilities were ≤1.8%, the intermediate precisions within a laboratory over several months were ≤6.7%, and the robustness of the method was ≤5.5%. The proposed method appears to be a suitable strategy for the identification and quantification of the main anthocyans because minor compounds are unlikely to be present in sufficient concentration to be detected by this method.

Classically, the routine detection in HPLC is based on measurement of UV−vis absorption, often using diode array detection (DAD). Different reviews reported that a match between UV−vis spectrum and retention time can lead to strongly positive identification of the analytes separated by HPLC as well as document the merits and drawbacks of DAD for the identification of different groups of polyphenols present in one sample.[49,50] Table 2 shows the wavelengths used for the identification of different families of phenolics. Although HPLC methods combined with multiwavelength UV−vis or DAD have been widely used and have proven to be highly effective in phenolics research, the use of these techniques is often limited by sample complexity such as those containing chemically similar phenolics. The weakness of these detection methods is the lack of structural information that leads to the possibility of sample matrix interference and misattribution of peaks.[49]

Over the past few years the coupling of MS detectors to HPLC systems has increased, improving outstandingly the identification and structural characterization of phenolics. In comparison with other detection tools, MS allows the elucidation of chemical structures of unidentified compounds, increasing the selectivity and sensitivity of the method. In combination with UV−vis or DAD, HPLC-MS can provide structural information for each individual peak in a chromatogram, allowing a rapid identification.[50,69] HPLC-MS is the best analytical approach to study phenolics and the most effective tool in the elucidation of anthocyanin structures, especially by permitting anthocyanin aglycone and sugar moiety characterization.[3,16] Furthermore, HPLC-MS allows the characterization of complex structures such as procyanidins, proanthocyanidins, prodelphinidins, and tannins, providing experimental evidence of several structures that had been previously conjectured.[16] In this sense, several authors have reviewed the application of MS detectors for the qualitative and quantitative analysis of phenolics in food samples.[48,49,69]

Table 2. Reported HPLC Methods for the Characterization of Phenolic Compounds in GP Extracts

analyte	detection	stationary phase	mobile phase A	mobile phase B	flow rate (mL min^{-1})	λ (nm)	ref
anthocyanins	UV−vis and MS	C_{18} Waters Symmetry 250 × 4.6 mm, 5 μm	H_2O/formic acid (95:5)	MeOH	1	510	34
procyanidins	UV−vis	silica Phenomenex 250 × 4.6 mm, 5 μm	DCM/MeOH/H_2O/ acetic acid (82:14:2:2)	MeOH/H_2O/acetic acid (96:2:2)	1	280	70
procyanidins	UV−vis and MS/MS	silica Phenomenex Luna 150 × 4.6 mm	DCM/MeOH/H_2O/ acetic acid (82:14:2:2)	MeOH/H_2O/acetic acid (96:2:2)	1	276 and 316	35
phenolic acids, flavanols and stilbenes	UV−vis	ODS Waters Spherisorb 250 × 4.6 mm, 5 μm	25% aqueous methanol in 1% acetic acid	75% aqueous methanol 1% acetic acid	0.75	280 and 360	64
flavonols and anthocyanins	DAD	C_{18} Phenomenex 250 × 4.6 mm, 5 μm	H_2O/formic acid (90:10)	AcN/formic acid (90:10)	ni[a]	350 and 530	60
flavanols	MS	C_{18} Phenomenex Gemini 250 × 4.6 mm, 5 μm	H_2O/formic acid (0.1%)	AcN (formic acid 0.1%)	1		41
phenolic acids, flavanols, and stilbenes	DAD	C_{18} Agilent Eclipse 250 × 4.6 mm, 5 μm	H_2O/ acetic acid (98:2)	MeOH	0.8	278	61
phenolic acids, flavonols, flavanols, and stilbenes	UV−vis	C_{18}	H_2O/ acetic acid (99:1)	MeOH/AcN (50:50)	1	280	105
flavanols, flavonols, and anthocyanins	DAD and MS	C_{18} Phenomenex Luna 250 × 4.6 mm, 5 μm	H_2O/formic acid (90:10)	AcN/formic acid (90:10)	1	280, 350, 480, and 530	102
phenolic acids, flavanols, and flavonols	UV−vis	C_{18} X-Terra	methanol	H_2O/acetic acid (95:5)	1	280 and 350	103
anthocyanins and procyanidins	MS	C_{18} Waters symmetry 250 × 4.6 mm	H_2O/formic acid (95:5) and DCM/MeOH/H_2O/acetic acid (82:14:2:2)	MeOH and MeOH/H_2O/acetic acid (96:2:2)	1	276, 316, and 510	106
phenolic acids, flavanols, flavonols, and anthocyanins	DAD and MS	C_{18} Alltech Altima 250 × 2.1 mm, 5 μm	H_2O/acetic acid (98:2)	MeOH/acetic acid (98:2)	1.5	250, 700, and 520	18
phenolic acids, flavanols, flavonols, stilbenes, and anthocyanins	MS	C_{18} Phenomenex Luna 150 × 2.1 mm, 4 μm	H_2O/formic acid (99:1)	AcN/formic acid (99:1)	0.2		92
phenolic acids, flavanols, and procyanidins	DAD	C_{18} AlphaBond 250 × 4.6 mm, 5 μm	H_2O/ acetic acid (98:2)	AcN	1	280 and 530	63
flavanols, flavonols, procyanidins, and anthocyanins	DAD and MS	C_{18} Waters Nova-Pak 150 × 3.9 mm, 4 μm	H_2O/formic acid (90:10)	H_2O/MeOH/formic acid (45:45:10)	0.8	280, 340, and 530	65
flavonols and anthocyanins	DAD and MS	C_{18} Phenomenex Luna 250 × 4.6 mm, 5 μm	H_2O/formic acid (90:10)	AcN/formic acid (90:10)	1	350, 480, and 530	98
anthocyanins	DAD and MS	C_{18} Phenomenex Aqua 250 × 4.6 mm, 5 μm	H_2O/formic acid/AcN (87:10:3)	H_2O/formic acid/AcN (40:10:50)	0.8	520	2
stilbenes and flavonols	DAD and MS	C_{18} Phenomenex Luna 250 × 4.6 mm, 5 μm	H_2O (1% formic acid)/AcN/2-propanol (70:22:8)		0.2	306 and 370	97
flavonols, flavanols, and stilbenes	DAD	C_{18} Waters symmetry 250 × 4.6 mm, 5 μm	20 mM H_2KPO_4 buffer pH 3 with 6 M HCl or H_2O (5% acetic acid)/AcN (91:9)	MeOH and AcN	0.8 or 1	367, 280, 315, 324, and 370	25
stilbenes	UV−vis	C_{18} Supelco Hypersil 250 × 4.6 mm, 5 μm	H_2O/MeOH/acetic acid (75:20:5)		1.5	306	107
phenolic acids, flavanols, and flavonols	DAD	C_{18} Hypersil Gold Aqua 150 × 4.6 mm, 5 μm	H_2O/acetic acid (98:2)	AcN/H_2O (98:2)	1	280 and 370	44
phenolic acids, flavonols, and anthocyanins	UV−vis	C_{18} Beckman Ultrasphere 250 × 4.6 mm	H_2O/MeOH/acetic acid (88:10:2) or H_2O/MeOH/phosphoric acid (5:10:85)	AcN	1 or 0.5	260, 313, 360, and 520	54
phenolic acids, flavanols, and flavonols	DAD	C_{18} Hypersil Gold Aqua 150 × 4.6 mm, 5 μm	H_2O/acetic acid (98:2)	AcN:H_2O (98:2)	1	280 and 370	44
phenolic acids, flavanols, flavonols, stilbenes, and anthocyanins	DAD and MS	C_{18} Phenomenex Luna 250 × 4.6 mm, 5 μm	H_2O/MeOH/acetic acid (89.8:10:0.2) or H_2O/AcN/acetic acid/phosphoric acid (84:5:10:1)	AcN	0.6 or 1	275, 340, and 520	53

Table 2. continued

analyte	detection	stationary phase	mobile phase A	mobile phase B	flow rate (mL min^{-1})	λ (nm)	ref
flavanols	DAD and MS	C_{18} Phenomenex Aqua 150 × 2 mm, 3 μm	H_2O/acetic acid (99:1)	AcN/H_2O (99:1)	0.3	280	36
phenolic acids, flavanols, flavonols, stilbenes, and anthocyanins	DAD	C_{18} Phenomenex Prodigy 250 × 4.6 mm, 5 μm	H_2O/tetrahydrofuran: trifluoroacetic acid (98:2:0.1)	MeOH/tetrahydrofuran/trifluoroacetic acid (98:2:0.1)	ni	270, 328, 370. and 525	101
phenolic acids, flavanols, and procyanidins	DAD and MS	C_{18} Phenomenex Luna 250 × 4.6 mm, 10 μm	H_2O/acetic acid (99.8:0.2)	AcN/MeOH (50:50)	1	280 and 320	108
phenolic acids, flavanols, flavonols, procyanidins, stilbenes, and anthocyanins	DAD and MS/MS	C_{18} Phenomenex Aqua 250 × 4.6 mm, 5 μm	H_2O/formic acid/AcN (87:10:3) or H_2O/acetic acid (98:2)	H_2O/AcN/formic acid (40:10:50) or H_2O (0.5% acetic acid)/AcN (50:50)	0.8 or 1	280, 320, 370, and 520	3
flavanols and proanthocyanidins	DAD	C_{18} Waters Spherisorb 150 × 4.6 mm, 3 μm	H_2O/ acetic acid (97.5:2.5)	H_2O (2% acetic acid)/AcN (90:10)	0.5	280	67

[a] ni, no information.

Taking into account the mentioned advantages of MS detectors in the structural elucidation of compounds, Rockenbach et al.[36] applied a LC-ESI-FTICR-MS method to explain the presence of isomers and other flavan-3-ol compounds in seeds from different GPs. A variety of flavan-3-ols, including both galloylated and nongalloylated compounds with oligomers up to seven monomeric units, were detected, and also epicatechin−ethyl trimers and tetramers as well as their respective dimers were described for the first time. The authors pointed out that FTICR provides the highest mass resolution and most accurate mass determination, making it theoretically possible to assign unambiguously elemental composition for most detected compounds in a complex sample such as GSE. Different authors have exploited the use of tandem mass spectrometry (MS/MS) for the identification of substitution pattern of anthocyanins and procyanidins with satisfactory results in terms of selectivity and sensitivity.[3,34,35] The use of MS/MS avoids ambiguous identification of compounds. Nevertheless, the majority of reported methods use single MS detection. The latter has the disadvantage of being quite nonspecific for the determination of pattern substitution and does not allow the identification of new compounds in GP extracts. Despite these, its relatively low costs of purchasing and maintenance, combined with its relatively good sensitivity (and selectivity if is combined with UV detection), are the primary reasons for its selection.

The determination of phenolics by gas chromatography−mass spectrometry (GC-MS) has been scarcely explored because of the chemical nature of the compounds. GC-MS analysis reveals that low-volatile polar compounds, such as phenolics, exhibit low sensitivity and peak tailing. Therefore, the determination of these compounds by GC-MS requires a derivatization step, which sometimes is tedious and time-consuming and can lead to analyte losses. However, the GC-MS technique is highly selective and sensitive and can be used for nonpolar compounds present in GP such as terpenes. Furthermore, there are a lot of spectral libraries to achieve a successful identification of analytes that could be explored in the future.

■ BIOTECHNOLOGICAL APPLICATIONS

Phenolics from the agro-food industry have received considerable attention in recent years because of the wide range of possible applications. One of the most valuable options is the recovery of bioactive plant constituents that have antioxidant properties and could be used in pharmaceutical, cosmetic, and food industries. GP extracts have been researched as a source of natural antioxidants due to the content of large quantities of monomeric phenolics such as (+)-catechin, (−)-epicatechin, and (−)-epicatechin-3-*O*-gallate, as well as dimeric, trimeric, and tetrameric procyanidins, among others. In this sense, this section discusses the reported applications of GPE in different fields of industry.

Antimicrobial Effects. Because of the environmental and health concerns related to the use of antimicrobial compounds of synthetic origin, interest in obtaining these compounds from natural sources such as fruits and vegetables has recently increased. As mentioned above, a particularly important field is the utilization of industry residues to obtain these compounds in a cheaper way. Baydar et al.[72] studied the antibacterial effects of GSE and bagasse. They did not find antibacterial activities against 15 pathogenic bacteria in extracts obtained from grape cv. Narince bagasse, but they did discover antibacterial activities

in the GSE (both from nonvinified grapes). Jayaprakasha et al.[70] applied GSE collected from juice-processing industries to study the minimum inhibitory concentration (MIC) with antibacterial capacity (that is, the lowest concentration capable of inhibiting the complete growth of the bacterium tested). Their results indicated that the GSE exhibited antibacterial effect against all bacteria tested (*Bacillus cereus*, *Bacillus coagulans*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*), and the extracts were more effective against Gram-positive as compared to Gram-negative bacteria. A similar study was performed by Anastasiadi et al.[73] in evaluating the MIC of GP extract against *Listeria monocytogenes*, and the results justified its incorporation in food systems to prevent the growth of these bacteria. Thimothe et al.[74] studied the chemical composition and the biological activity of phenolics obtained from several red wine grape varieties and their GP extracts on the virulence of *Streptococcus mutans*. They observed that the biological activity of GP was as effective as (or significantly better) than those of whole fruit grape extracts. For example, some of the studied GP extracts produced a higher degree of inhibition of glucosyltransferase (GTF) activities (>60% of inhibition) and acidogenicity of *Str. mutans* (≥48%) than whole grape fruit extracts. These enzymes synthesize the glucans, which are the principal components of bacterial biofilms. As well, GTFs are specific and proven virulence traits of *Str. mutans* associated with the pathogenesis of dental caries and the structural integrity of dental biofilm (plaque). Therefore, one of the strategies to control biofilm formation and dental caries is to inhibit the activity of GTFs. The higher percentage of inhibition of GTF by GP extracts could be due to the higher content of phenolics in GP as compared to whole grape extracts. The results also showed that grape phenolics, especially from GP extracts, are highly effective against virulence of *S. mutans*, although bacterial viability was not affected. That is, the extracts acted as bacteriostatic but not as bactericide. In this sense, GP is a promising and feasible (low cost and largely available) source for the extraction and isolation of compounds for the prevention of oral diseases, such as dental caries.

Food Systems. Antioxidants are used in the food industry to prevent lipid oxidation, and they are added to fresh or further processed meats to prevent oxidative rancidity, to retard development of off-flavors, and to improve color stability. The use of synthetic antioxidants is restricted in some countries because of their toxic or carcinogenic effects. In view of these negative health effects, in the past decade a considerable interest has emerged for the use of antioxidants of natural origin. Maestre et al.[75] highlight that the use of natural antioxidants in the food industry is a common practice, the use of phenolics from agricultural and forestry byproducts being an excellent option that fulfills this objective. The effect of GP extracts or GSE in different lipid food systems has been analyzed by several authors and is commented on below.

Recent data suggest that the effects of GSE components over free radicals, including the monomers catechin and epicatechin, as well the procyanidin B dimer, in a lipid bilayer model and in fish muscle may be due to inhibition of the propagation of free radicals in the lipid bilayer. This effect can be explained by the interaction between phenolics and lipid bilayers, so forming rigid structures. The capacity of these compounds to chelate metals, to donate electrons, and to scavenge free radicals may explain the higher antioxidant capacity found in fish muscle.[75]

Pazos et al.[76] studied the antioxidant activity of flavonoids from GP extracts in food systems containing fish oils and frozen fatty fish. They extracted the phenolic fraction of fresh GP obtained from the wine industry. Then, the phenolic extract was separated into a set of fractions differing in composition and procyanidin structure. Flavanol monomers showed higher antioxidant activity than flavonol monomers, and the efficiency detected in fish lipids did not show a direct relationship with the number of phenolic residues and the galloylation. The same group studied the influence of the grade of polymerization (number of monomeric units) and galloylation of polyphenols. The authors found that proanthocyanidins with medium size (2−3 monomeric units) and low galloylation degree (0.15−0.25 gallate group/molecule) are more suitable than highly polymerized and galloylated compounds to inhibit lipid oxidation in pelagic fish muscle.[77]

Some studies have shown that the use of low-sulfite vegetable extract combinations (GSE or green tea extracts) preserve raw meat products. The use of extracts improved the preservative effects of low sulfite doses and had good sensory properties.[78] The authors observed positive effects on the major causes of raw meat deterioration such as microbial spoilage, loss of redness, and lipid oxidation of low-sulfite cooked beef patties. Selani et al.[79] studied the addition of GP extracts (from grape cv. Isabel and Niagara) on lipid oxidation, color, pH, and sensory properties of raw and cooked chicken meat. Like other authors, they also found a preventive lipid oxidation in raw and cooked chicken meat without changes in pH or color of raw products. However, they observed changes of color in cooked meat. A similar study was performed by Garrido et al.,[80] who evaluated two different red GP extracts on meat quality of pork burgers. They found that the addition of GP produced color stability and decreased lipid oxidation, and both changes correlated with a potent antioxidant effect of these extracts. At the same time, the extracts did not affect the pH value or the microbial spoilage in raw pork burgers at the 0.06 g GPE/100 g concentration.

Lau et al.[81] applied GSE in dark- poultry meat to inhibit the development of malonaldehyde (MDA, an indicator of peroxidation); they observed that 1.0% GSE has lower values of TBARS than 2.0% GSE, and both were nearly 10-fold lower than the control. The authors suggested that the observed effect of lowering the level of TBARS at relatively low contents of GSE (behavior found with other antioxidants such as *R*-tocopherol) may be due to high concentrations of compounds acting as pro-oxidants. Furthermore, the patties formulated with GSE were not rated sensorially objectionable as compared to the control, despite the evaluators noticing a wine odor that masked the mild chicken flavor and a slightly bitter aftertaste in GSE patties. Also, GSE patties were crumbly and lacked cohesion. In this sense, further research is needed to avoid the negative organoleptic effects produced by the addition of GSE and increase the applicability of this promising source of antioxidants.

The application of phenolics from extracts in cheese has been reported by Han et al.[82] Their results evidenced that cheese products formulated with some phenolics improved the antioxidant properties. The addition of polyphenols at 0.5 mg mL^{-1} milk did not affect physical attributes such as the texture or firmness of the final cheese. However, increased gel strength along with decreased curd moisture content would eventually occur following an increase in the concentration of added polyphenols. The rennet-induced milk gels formulated with

bioactive compounds showed slightly altered characteristics of cheese curds. On the basis of these results, the authors proposed the possibility of producing milk gels with varied textural properties. However, additional studies are needed to examine the consequences on cheesemaking and the possibility of application of this technology to other dairy products. In another study, these authors found that the antiradical–antioxidant properties of extracts from whole grapes were comparatively better than those of green tea and cranberry,[83] suggesting a promising utilization for GP.

Another interesting application was recently reported by Tseng et al.,[84] who applied GP (from grape cv. Pinot noir) as antioxidant dietary fiber for enhancing nutritional value and improving storability of yogurt and salad dressing. They demonstrated that Pinot noir GP may be utilized as an alternative source of antioxidant dietary fiber to fortify yogurt and salad dressing with the aim of increasing the dietary fiber and TPC as well as delaying lipid oxidation of samples during refrigerated storage. As a negative point, the authors observed a reduction in the TPC and DPPH radical scavenging activity (RSA) of fortified samples during the storage time. Consequently, further research is necessary to investigate the mechanisms and methods of retention of TPC and RSA in the mentioned products for a long time.

Mildner-Szkudlarz et al.[62] proposed the utilization of white GP as an additive to wheat flour for the evaluation of its effect on physical and nutraceutical characteristics of wheat biscuits. The results showed that white GP enriched biscuits (up to 10% addition to flour) showed considerably higher dietary fiber contents than control samples and were characterized by significantly higher antioxidant activities associated with their phenolic contents. Biscuits with 20 and 30% white GP-added flour were characterized by high scores for fruity–acidic as well as brown notes, which was also evident in the color of those samples. In this sense, it is necessary to find a compromise between positive nutritive effects and negative organoleptic alterations after the addition of GP that could represent the rejection of the product by the consumer.

Other authors determined the antimicrobial effects of crude and powdered GP extracts against *E. coli* and *S. aureus* in vegetable soup. In soup samples, *S. aureus* was more sensitive than *E. coli* when treated with crude or powdered GP.[85] The authors also reported that antibacterial effects of crude GP were more effective than those of the powdered GP, where bacterial counts decreased with the increase in extract concentration. This study showed that crude and powdered GP could be used as antimicrobial agents in a model food system, although a scaling up to pilot plants and to different foods is necessary to justify a suitable application.

Other Emerging Applications. Because of the low exploited applicability of GP extracts, in recent years scientists focused on evaluating different alternative uses to these natural sources of protective compounds. In this sense, the effect of administering GP or GSE in animals' feeds has been studied with the aim to establish antioxidant properties in both food quality and health effects.

Brenes et al.[86] studied the effect of a commercial GSE included in the diet of chickens. They found that the antioxidant activity in GSE diets and excreta exhibited higher scavenging free radical capacity than the control diet. The oxidative stability in breast meat with GSE diets (equivalent to vitamin E diet) suggests that GSE could be a new source of antioxidants in animal nutrition that could improve oxidant stability in chicken meat. In other research, the inclusion of a concentrate of GP extract in the diet of chickens evidenced lower TBARS values during storage of raw and cooked breast chicken patties in comparison to breast samples obtained from animals fed the control diet.[87]

Another recently reported application refers to the use of GP extracts as inducers or elicitors of tobacco plant defense properties. These options for plant protection have been described by Goupil et al.[88] as attractive alternatives for sustainable agriculture and environmentally friendly practices to sustain pest management.

A novel nanoemulsion-based delivery system formulated with natural ingredients has been proposed by Sessa et al.[89] Phenolics were extracted from GP by using high pressure and temperature. To increase their dispersion in the aqueous phase, the phenolic extracts were encapsulated using either a liquid (sunflower oil) or a solid (palm oil) lipid phase, as well as the combination of a hydrophilic and hydrophobic emulsifier. The nanoemulsions were produced by high-pressure homogenization. The authors observed that the cellular antioxidant activity was significantly higher for the encapsulated GP polyphenols than for the nonencapsulated ones, suggesting a fundamental role of the nanoemulsions in favoring delivery through the biological membranes.

As protective effects, modifications in enzyme activities as well as modifications in lipid peroxidation in rats have been reported.[90] These indicated that the GP is capable of protecting the activities of hepatic enzymes, which play important roles in combating the ROS. However, different activities of GP extracts can be ascribed to their different phenolic compositions. Therefore, further studies with individual or families of phenolics should be performed to clarify the mechanisms involved in the enhancement of enzyme activity and protection provided to the liver and also to explore the possible synergism that may potentiate the protective effects against ROS. Hogan et al.[91] reported that GP extracts suppress postprandial hyperglycemia in diabetic mice by specifically inhibiting α-glucosidase, a strategic enzyme for oligosaccharide digestion and further glucose absorption. The achieved results suggest a potential application of GP-derived bioactive compounds in the management of diabetes. In other research, the same authors have found that supplementation with GP produced anti-inflammatory activity but not a reduction of oxidative stress in mice with an obesity-induced diet.[92] Terra et al.[93] observed that commercial GSE prevented low-grade inflammation in rats fed a high-fat diet by adjusting adipose tissue cytokine imbalance, enhancing anti-inflammatory molecules, and diminishing pro-inflammatory ones.

As can be seen, there are several applications in different fields. The principal drawback is the need for more studies in the same group of foods to achieve representative results that argue for industrial application and the scaling up of proposed GP extract uses.

■ REMARKS AND FUTURE TRENDS

The recovery of phenolic compounds from GP is an important challenge for the field-related scientists and also the industry, although the commercial implementation is a complex approach depending on several parameters that should be considered. Besides the described developments and the criteria that must be involved, researchers should be able to succeed in scaling up the process without affecting the functional properties of the target compounds, developing a product

that meets the high quality standards for safety and organoleptic characteristics that consumers need.

One of the most significant facts that needs further research is in the field of physiological activities of phenolics. There are many studies that justify positive health effects, basically associated with the known antioxidative properties of GP. However, much work is necessary to screen in vitro potential bioactivities of plant-obtained extracts prior to transferring the results to in vivo conditions.

The sustainable exploitation of GP will be a useful strategy for wineries with the aim of reducing environmental contamination and as an alternative to reduce the carbon footprint in the whole production process. In this sense, simplified processes (with few extraction and purification steps) will be the choice with the aim of an easier scale-up as well as achieving a cheaper production. Besides, experience focused on investigating and establishing definite food-targeted applications of the final product will be strategically evaluated.

The characterization of achieved GP extracts is a relevant point in terms of increasing the economic value of the obtained product. Knowledge of the identity and individual concentrations of recovered phenolics after extraction is a significant fact during the application of extracts. This information will provide tools to support the technological application of extracted bioactive compounds in diverse industries. Also, the application of modern detectors will be a useful way to identify unknown compounds that could be affecting the antioxidant properties of extracts.

Although the reviewed techniques are well established and can be successfully applied for the extraction and characterization of phenolics in GP, researchers should focus on the prospect of applying emerging technologies, particularly by using simplified extraction techniques and nontoxic solvents that meet the GRAS status. These will give more sustainable processes for the recovery of bioactive phenolics, increasing the safety of recovered products from food wastes and justifying the recycling process from both technological and environmental points of view.

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■ ABBREVIATIONS USED

GP grape pomace
GSE grape seed extract
HPLC high-performance liquid chromatography
SLE solid−liquid extraction
TPC total phenolic content
GRAS generally recognized as safe
US ultrasound radiation
MF microfiltration
SFE supercritical fluid extraction
SC-CO_2 supercritical carbon dioxide
SCF supercritical fluid
ASE accelerated solvent extraction
TFC total flavonoids contents
HVED high-voltage electric discharge
POH pulsed ohmic heating
SPE solid-phase extraction
HLB hydrophilic−lipophilic-balanced reverse phase
GAE gallic acid equivalent
DPPH 2,2-diphenyl-1-picrylhydrazyl
TE trolox equivalent
ORAC oxygen radical antioxidant capacity
AAPH 2,2′-azobis(2-amidinopropane) dihydrochloride
TEAC trolox equivalent antioxidant capacity
ABTS 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate)
TBA thiobarbituric acid
DPD *N*,*N*-diethyl-*p*-phenylenediamine
ROS reactive oxygen species
UHPLC ultrahigh-performance liquid chromatography
ESI electrospray ionization
MS mass spectrometry
DAD diode array detection
FTICR Fourier-transform ion cyclotron resonance
MS/MS tandem mass spectrometry
GC gas chromatography
MIC minimum inhibitory concentration
GTF glucosyltransferases
MDA malonaldehyde
TBARS thiobarbituric acid reactive substances
RSA radical scavenging activity

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