

Characterization and recovery of phenolic compounds from grape pomace - A review

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Summary

Recent findings have indicated potential health-beneficial effects of plant secondary metabolites, such as polyphenolics and carotenoids. Due to their widespread occurrence in plant foods, a diet rich in fruits and vegetables has been propagated by health authorities, as can be seen from the '5 a day campaign'. On the other hand sources are being searched for the recovery of phenolic compounds which may be used for the production of functional or enriched foods. Therefore, in a comprehensive screening the contents of individual phenolic compounds were determined in the skins and seeds of 14 grape pomace samples originating from red and white winemaking. The results indicate by-products of winemaking to be an inexpensive source for a wide range of different polyphenolics. With those pomace samples exhibiting the highest polyphenol levels a novel process for their extraction was developed on a pilot-plant scale, employing cell wall degrading enzymes for an enhanced release of phenolics. Finally, the crude extracts were purified using the adsorber technology to obtain a highly concentrated purified polyphenol extract.

Introduction

Phenolic compounds are aromatic secondary plant metabolites which are found ubiquitously in the plant kingdom. Modern analytical techniques have revealed a vast range of highly diverse structures, the number of which is supposed to be above 8,000, with molecular weights ranging from < 100 Da for simple phenolic compounds to > 30,000 Da for highly polymerized structures. Solely among the flavonoids more than 5,000 compounds have been described (HARBORNE, 1994).

According to this great diversity of polyphenolics in plants, they perform a wide range of different functions. By producing appealing colors and hues, anthocyanins attract animals both for pollination and seed dispersal, but they may also act as antifeedants (STINTZING and CARLE, 2004). Furthermore, they may function as light screens against damaging radiation and as transport vehicles for monosaccharides or as osmoregulators during periods of drought and low temperatures (CHALKER-SCOTT, 1999). In general, most phenolic compounds exhibit antioxidant activity and are therefore assumed to increase the response of plants to oxidative damage in order to maintain the regular physiological status in tissues which are directly or indirectly affected by biotic or abiotic stress factors. Thus they also act against infections and aggression by microorganisms. A common response is the incorporation of phenylpropanoids into the cell wall, however, the precise role of this reinforcement remains to be elucidated. In addition, phenolic compounds may serve as a protection against herbivorous insects and mammals by acting as feeding deterrents. However, there is only a narrow dividing line between attraction and repellence.

Generally, flavonoid biosynthesis is enhanced in response to increased light exposure, especially UV-B rays, which might be indicative of

their UV-absorbing function. This explains why most phenolic compounds are found in external and aerial tissues. Thus, higher concentrations are found in foods characterized by a high skin : volume ratio such as grapes (BRAVO, 1998; ESCARPA and GONZALEZ, 2001). In some cases, flavonoid function may also be related to primary metabolism by an indirect effect on plant growth, however, only few attention has been paid to these interactions so far.

The evaluation of the occurrence of phenolic compounds in plant food has changed significantly during recent years and decades. In the past, they have most often been considered a drawback due to a number of adverse effects. Polyphenols may lead to discoloration of processed food by enzymatic browning involving the action of endogenous polyphenol oxidases and peroxidases which produce quinones that can polymerize into brown products (FRIEDMAN, 1996). High polyphenol concentrations are responsible for astringency and bitterness (GAWEL, 1998) and they contribute to sediment formation by polyphenol-protein interactions causing undesirable hazes in products such as wine, beer, and fruit juices (SIEBERT, 1999). In the same way especially highly polymerized tannins can bind endogenous enzymes in the intestinal tract, thus affecting digestibility *in vivo* not only of proteins but also of other macronutrients such as starch and lipids (ROBARDS and ANTOLOVICH, 1997). By chelating metal ions chronic pharmacological doses might exacerbate iron deficiency, and some adverse effects on copper, zinc, sodium and aluminium bio-availability have also been reported (BRAVO, 1998). Obvious concerns have been raised when prooxidant activity of some isolated compounds and mutagenicity related to flavonoid-mediated oxidative damage were detected *in vitro* (BEKHIT et al., 2004; STAN and HUNI, 1984).

However, in recent years increasing attention has been paid to the physiological activities of plant phenolics and the potential health-beneficial effects associated with these attributes. Beginning with the 'French Paradox' observations more than ten years ago (RENAUD and DE LORGERIL, 1992), research interest has focused on plant phenolics since epidemiological studies revealed a correlation between the dietary intake of phenolic compounds and a reduced risk of certain diseases such as coronary heart diseases, stroke and cancer. This has mainly been attributed to the antioxidant activity and radical scavenging capacity *in vivo* which render reactive oxygen species, such as superoxide and hydroxyl radicals, hydrogen peroxide and singlet oxygen, harmless. These are formed during the reduction process transforming oxygen into water (ANTOLOVICH et al., 2002). A wide variety of *in vitro* test systems has been developed for assessing these properties. However, the determination of antioxidant activities of complex mixtures cannot be evaluated satisfactorily by non-specific one-dimensional methods, since the results are dependent on a multitude of factors. Approved, standardized methods are still lacking and results are often inconsistent and sometimes contradictory (FRANKEL and MEYER, 2000). In contrast, studies on *in vivo* effects of polyphenolics and their metabolites are still scarce. Furthermore, the comparability of *in vitro* activities with *in vivo* studies is doubtful since polyphenols are generally present in humans as conjugates of glucuronate or sulfate or in methylated form which are likely to possess biological properties and distribution patterns

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within tissues and cells different from those of the phenolics present in the dietary source (KROON et al., 2004; LOTITO and FREI, 2004). Some promising results have been reported concerning inhibition of cancer cell growth *in vitro* or in animals studies, however, systematic clinical studies still need to be performed. A wide range of further observations has been described in the literature, such as hypocholesterolemic, antithrombotic and neuroprotective effects, antiviral and anticarcinogenic properties, but the underlying mechanisms for most of these activities are still unknown (CLIFFORD, 2000; HAVSTEEN, 1983; SANTOS-BUELGA and SCALBERT, 2000; YODIM et al., 2002). Based on these findings and due to an improved health-awareness concerning food and nutrition, numerous research activities have been initiated dealing with the search for sources of plant secondary metabolites. These may be used in food for technological purposes, e.g. anthocyanins as substitutes for synthetic and nature-identical food colorants, or polyphenolics in general as food lipid antioxidants. Even more interestingly, they are increasingly recovered for their health-beneficial effects as supplements to enriched or functional food. By-products of plant food processing have been recognized as an important and inexpensive source of these compounds (SCHIEBER et al., 2001). Therefore, in a series of studies reviewed here polyphenolics from grape pomace as a cheap by-product of winemaking were characterized and a process for enzyme-assisted extraction and the recovery using adsorption technology was developed on a pilot-plant scale.

Winemaking By-Products as a Source of Functional Compounds

Grapes (*Vitis vinifera* L.) belong to the world's largest fruit crops with an annual production of 65.5 million tons in 2004 (FAOSTAT, 2004). Taking into account that about 80 % of this crop is used in winemaking, large amounts of grape pomace are produced, with the data reported in the literature ranging from 5-7 million tons to 14.5 million tons solely in Europe (MEYER et al., 1998; SCHIEBER et al., 2001; TORRES and BOBET, 2001). Direct incorporation into vineyard soils has commonly been applied, however, its disposal is aggravated by stricter environmental regulations. Grape pomace may also be used as animal feed, but digestibility is low due to the presence of high amounts of polymeric polyphenols which are known to inhibit cellulolytic and proteolytic enzymes and the growth of some rumen bacteria (SCHURG et al., 1980). Besides these applications, a number of various valuable compounds may be recovered from grape pomace, such as tartaric, malic and citric acids (PALMA and BARROSO, 2002; HANG and WOODAMS, 1985). Grape seeds are a source of a high-quality culinary oil with very high linoleic acid levels, and the recovery can be managed by mechanical pressing or by solvent extraction (SCHIEBER et al., 2002). However, this process requires cost-intensive drying of the pomace and subsequent sieving. Moreover, grape pomace is rich in dietary fiber consisting mainly of hemicelluloses, cellulose and smaller proportions of pectins, the nutritive value of which has been highlighted since the fiber matrix is associated with both nonextractable polymeric procyanidins and extractable low-molecular phenolics exerting antioxidant activity (SAURA-CALIXTO, 1998). Additionally, grape seeds were shown to be a rich source of proteins which could be used as food ingredients (IGARTUBURU et al., 1991) and grape pomace has long been used for the economic recovery of ethanol (SILVA et al., 2000).

Despite all these potential uses of grape pomace, most attention has been paid to the qualitative and quantitative characterization of the phenolic fraction. Winemaking may be regarded as an incomplete aqueous or ethanolic extraction of phenolic compounds from the skins and seeds, since their particle size is not significantly reduced during grape mashing. Therefore, considerable amounts of polyphenols remain in the winery press residues. Numerous phenolic compounds

in grape pomace have been studied in great detail, especially from the anthocyanins, hydroxybenzoic and hydroxycinnamic acids, flavan 3-ols, flavonols and stilbenes (LU and FOO, 1999; ECTOR et al., 1996). The seeds are a source of a series of oligomeric and polymeric procyanidins, the structural elucidation of which has proven difficult and is usually performed by thiolysis and characterization of the reaction products (ESCRIBANO-BAILÓN et al., 1992).

Polyphenol Screening of Pomace from Red and White Grape Varieties

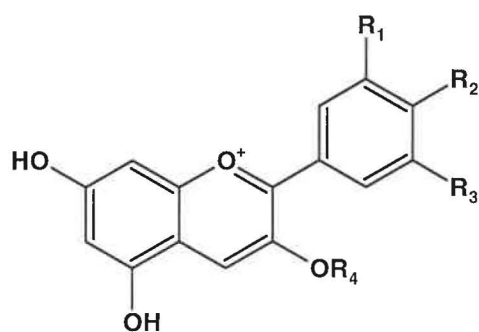
Despite the great number of studies quantitative data are most often given as total phenolic contents (MEYER et al., 1998; LARRAURI et al., 1996). Only in a few studies contents of a few representative individual phenolics were reported, however, comprehensive data on most phenolic compounds in grape pomace are still not available. Furthermore, most often only one or two pomace samples or even mixtures of different pomaces have been investigated. A systematic comparison of the press residues originating from the processing of several grape cultivars has not been performed. However, knowledge concerning total phenolic contents and those of individual compounds may facilitate the selection of suitable plant material for the extraction of phytochemicals (LU and FOO, 2001). Therefore, as a prerequisite for the development of a process for the recovery of polyphenolics from grape pomace, phenolic contents were screened in the pomace of several red and white grape cultivars (KAMMERER et al., 2004). The skins and seeds of 14 pomace samples originating from red and white winemaking (Tab. 1) were manually separated. An analytical system was developed for determining the qualitative and quantitative composition of the methanolic extracts of skins and seeds by HPLC. Due to the complexity of the phenolic profile, a fractionation of the crude extracts was inevitable to avoid peak coelution and inaccurate integration. Therefore, a method for the fractionation of phenolic compounds in red wine based on adsorption of polyphenols onto C₁₈ cartridges and subsequent elution with acidified water, ethyl acetate and acidified methanol was adapted (OSZMIANSKI et al., 1988). For red grape skins non-anthocyanin phenolics had to be pre-extracted from the crude extracts with ethyl acetate, due to the limited capacity of the cartridges and the quantitative predominance of anthocyanins. Three fractions were obtained, the phenolic compounds of which were separated by HPLC using volatile eluents. This allowed coupling to a mass spectrometer which proved to be inevitable, due to the limited availability of reference compounds. Up to 39 phenolic acids, non-anthocyanin flavonoids, stilbenes and anthocyanins were identified and quantified in the skins and seeds. Their structures are depicted in Fig. 1.

The study demonstrates that grape pomaces have generally very high polyphenolic contents, making their utilization worthwhile. Antho-

Tab. 1: Varieties and vintages of the grape pomace samples investigated in the polyphenol screening (KAMMERER et al., 2004).

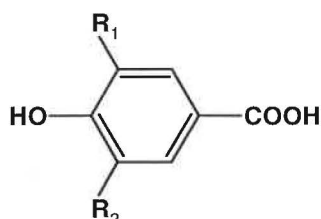
Red Grape Pomace	White Grape Pomace
Cabernet Mitoš 2002	Kerner 2002
Cabernet Mitoš 2001	Merzling 2001
Lemberger 2002	Müller-Thurgau 2002
Lemberger 2001	Weißbriesling 2002
Spätburgunder 2002	Weißbriesling 2001
Spätburgunder 2001	
Schwarzriesling 2002	
Trollinger 2002	
Trollinger 2001	

Fig. 1: Structures of phenolic compounds identified and quantified in grape pomace (KAMMERER et al., 2004).



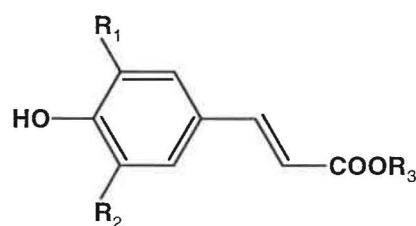
Anthocyanins

Compound	R ₁	R ₂	R ₃	R ₄
Cyanidin 3- <i>O</i> -glucoside	OH	OH	H	glc
Cyanidin 3- <i>O-p</i> -coumaroylglucoside	OH	OH	H	<i>p</i> -coumaroyl-glc
Delphinidin 3- <i>O</i> -glucoside	OH	OH	OH	glc
Delphinidin 3- <i>O</i> -acetylglucoside	OH	OH	OH	acetyl-glc
Malvidin 3- <i>O</i> -glucoside	OCH ₃	OH	OCH ₃	glc
Malvidin 3- <i>O</i> -acetylglucoside	OCH ₃	OH	OCH ₃	acetyl-glc
Malvidin 3- <i>O-p</i> -coumaroylglucoside	OCH ₃	OH	OCH ₃	<i>p</i> -coumaroyl-glc
Peonidin 3- <i>O</i> -glucoside	OCH ₃	OH	H	glc
Peonidin 3- <i>O</i> -acetylglucoside	OCH ₃	OH	H	acetyl-glc
Peonidin 3- <i>O-p</i> -coumaroylglucoside	OCH ₃	OH	H	<i>p</i> -coumaroyl-glc
Petunidin 3- <i>O</i> -glucoside	OCH ₃	OH	OH	glc
Petunidin 3- <i>O</i> -acetylglucoside	OCH ₃	OH	OH	acetyl-glc
Petunidin 3- <i>O-p</i> -coumaroylglucoside	OCH ₃	OH	OH	<i>p</i> -coumaroyl-glc



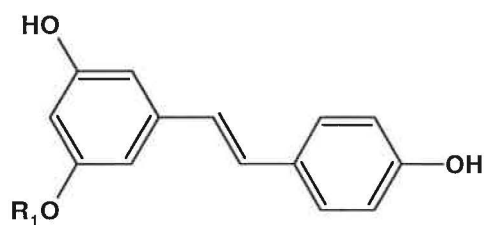
Hydroxybenzoic acids

Compound	R ₁	R ₂
Gallic acid	OH	OH
<i>p</i> -Hydroxybenzoic acid	H	H
Protocatechuic acid	OH	H
Syringic acid	OCH ₃	OCH ₃



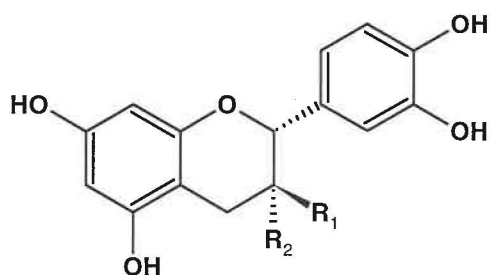
Hydroxycinnamic acids

Compound	R ₁	R ₂	R ₃
Caffeic acid	OH	H	H
Caftaric acid	OH	H	tartaric acid
<i>p</i> -Coumaric acid	H	H	H
Coutaric acid	H	H	tartaric acid
Fertaric acid	OCH ₃	H	tartaric acid
Ferulic acid	OCH ₃	H	H
Sinapic acid	OCH ₃	OCH ₃	H



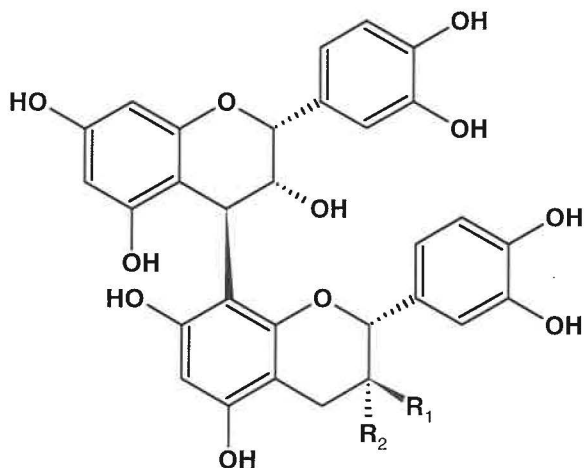
Stilbenes

Compound	R ₁
<i>trans</i> -Polydatin	glc
<i>trans</i> -Resveratrol	H



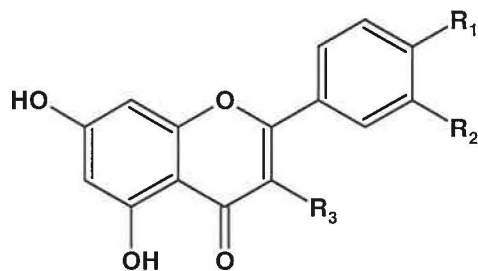
Monomeric flavan 3-ols

Compound	R ₁	R ₂
Catechin	OH	H
Epicatechin	H	OH
Epicatechingallat	H	<i>O</i> -gallic acid



Dimeric procyanidins

Compound	R ₁	R ₂
Procyanidin B1	OH	H
Procyanidin B2	H	OH



Flavonols

Compound	R ₁	R ₂	R ₃
Isorhamnetin 3- <i>O</i> -glucoside	OH	OCH ₃	glc
Kaempferol	OH	H	H
Kaempferol 3- <i>O</i> -glucoside	OH	H	glc
Quercetin	OH	OH	H
Quercetin 3- <i>O</i> -galactoside	OH	OH	gal
Quercetin 3- <i>O</i> -glucoside	OH	OH	glc
Quercetin 3- <i>O</i> -glucuronide	OH	OH	glcA
Quercetin 3- <i>O</i> -rhamnoside	OH	OH	rham

Abbreviations: gal, galactose; glc, glucose; glcA, glucuronic acid; rham, rhamnose

cyanins yielded up to 132 g/kg dry matter in the peels of a 'Cabernet Mitoš' pomace. Phenolic acids, non-anthocyanin flavonoids and stilbenes were present in considerably lower amounts compared to the anthocyanins, with the exception of the flavan 3-ols (catechin, epicatechin, oligomeric procyanidins) in the seeds which contained up to 18.76 g of polyphenols / kg dry matter. However, the data reveal great differences in the contents of individual phenolic compounds depending on cultivar and vintage. Furthermore, differences in the ripening stage of the grapes and in microclimatic and phytosanitary conditions may be responsible for varying polyphenol contents since some compounds, such as resveratrol, are phytoalexins which are only synthesized under certain biotic or abiotic stress conditions. Besides the lack of anthocyanins in white grape pomaces, no principal differences between red and white grape varieties were observed. Therefore, the view that white grape pomace is characterized by higher total phenolic contents, due to differences in the processes used for red and white winemaking (LARRAURI et al., 1996) should be revised. Furthermore, the results underscore the necessity of such a screening for future extraction procedures since extracts rich in polyphenols but of different composition may have different health attributes *in vivo*.

A Novel Process for the Recovery of Polyphenols from Grape Pomace

Anthocyanins are considered the most valuable compounds in grape pomace. Therefore, several methods have been developed for their extraction, most often using acidified alcohols or sulfited water or alcohols (BOCEVSKA and STEVCEVSKA, 1997; MANTELL et al., 2002; METIVIER et al., 1980). However, since sulfite cannot be removed quantitatively from the extracts and since pseudoallergic reactions have been reported after the consumption of sulfited foods (GASTAMINZA et al., 1995), its use is crucial in food processing and labelling of this suspected food allergen is now demanded by recent EEC regulations. Alternatively, cell-wall degrading enzymes have been applied for an enhanced release of phenolic compounds from grape

pomace (MEHRLÄNDER et al., 2004; MEYER et al. 1998; MUNOZ et al., 2004). However, the effects of enzymatic pectin and cellulose hydrolysis on the stability of individual phenolics was not studied or enzymatic digestion was performed on laboratory scale under conditions which do not allow a scale-up.

Therefore, after selecting grape pomaces with high amounts of phenolic compounds as indicated by the pomace screening, enzymatic hydrolysis was optimized in laboratory experiments, i.e. selection of pectinolytic and cellulolytic enzymes, enzyme : substrate ratio, and time-temperature regime of enzymatic treatment based on viscosity measurements and on the quantification of oligomeric pectin and cellulose degradation products released from cell wall material (KAMMERER et al., 2005a). Optimized conditions were then applied in pilot-plant scale experiments. The yields of individual polyphenolics were assessed based on the methodology used for the pomace screening. For a satisfactory digestion at 50 °C, 5,000 ppm (based on dry matter) of a pectinolytic and 2,500 ppm of a cellulolytic enzyme preparation were required, which is considerably high compared to fruit juice technology and was ascribed to the known enzyme inhibitory effect of high polyphenol contents. Enzymatic treatment significantly improved extraction efficiency for most phenolic compounds compared to aqueous extraction. Yields were increased more than fivefold for phenolic acids and about 1.7 fold for flavonoids and stilbenes. However, anthocyanin recovery rates were considerably lower both after enzymatic digestion of the grape skins and after sulfitation of the pomace suspension. An aqueous pre-extraction of the pomace prior to enzymatic digestion with pectinolytic and cellulolytic preparations significantly improved extraction since part of the phenolics, which are known to inhibit enzymes, were removed during the pre-extraction step. Relative changes of the contents of individual phenolic compounds in the extracts obtained after enzymatic liquefaction in comparison to aqueous pomace extracts demonstrated that quercetin glycosides were partly hydrolyzed, indicating the presence of a flavonoid glycosidase side activity. However, stability of anthocyanidin 3-*O*-glucosides was not affected (Fig. 2). The yields of most other compounds were increased. Therefore, enzyme-assisted extraction of grape pomace offers a valuable alternative to the recovery of polyphenolics using sulfite. Thus, the use of a suspected allergen can be avoided, however, applicability of this process on industrial scale and economic considerations should be evaluated in the future.

Recovery of Polyphenols Using Polymeric Adsorber Resins

Crude extracts of plants or by-products may be directly used for their health-beneficial effects or for technological reasons. However, most often, extracts are concentrated either by evaporation or, more recently, by the use of adsorber technology which allows the removal of hydrophilic compounds such as carbohydrates and a concentration under mild conditions since usually alcohols are used for desorption (DI MAURO et al., 2002). Even though this technology is part of patented processes for the recovery of pigments or other phenolics from grape pomace (HOWARD, 1998; SHRIKHANDE, 1983; SHRIKHANDE et al., 2003), little is known about recovery rates and the influence thereon when different solvents are used for desorption at various temperatures, thus making the process empirical and not allowing any predictions (SCORDINO et al., 2004).

As a consequence, the solutions obtained in the pilot-plant extraction experiments (KAMMERER et al., 2005a) were used for anthocyanin recovery rate experiments on laboratory and pilot-plant scale investigating the above-mentioned parameters when a styrene-divinylbenzene copolymerisate is used (KAMMERER et al., 2005b). Pigment losses during sample loading and washing of the column with water were far below 1 % and thus negligible, and were also not affected by a rise in temperature. The elution of anthocyanins was performed

with acidified methanol, ethanol and 2-propanol, respectively, with methanol showing highest recovery rates ranging up to 96-100 %. A change of temperature (25-50 °C) did not show significant effects on recovery rates. The adsorption and desorption process facilitates non-thermal pigment concentration since highly concentrated eluates were obtained at the beginning of the elution step. Scale-up experiments were promising since pigment loss during adsorption and washing and recovery rates were similar to those of laboratory experiments. Therefore, this process is particularly useful for the concentration of temperature-sensitive components because aqueous extracts can easily be converted into alcoholic concentrates by elution with low-boiling alcohols which allows further concentration under mild conditions.

Concluding Remarks

Due to their ubiquitous occurrence in the plant kingdom, phenolic compounds are an integral part of the human diet. There is now increasing evidence that plant secondary metabolites such as carotenoids and polyphenols together with dietary fiber and further plant constituents may exert certain health-beneficial effects. A series of epidemiological studies proved a correlation between the dietary intake of fruits and vegetables and a decreased incidence of cardiovascular diseases, stroke and certain forms of cancer. Therefore, increased attention has been paid to these micronutrients both among food producers and in academia. This goes along with the demand for 'healthy' food being 'natural' and devoid of synthetic additives and becomes manifest in the search for natural food colorants, i.e. preparations containing anthocyanins, betalains or carotenoids which apart from improving visual appearance of food may impart additional value by exerting antioxidant activity.

Extracts rich in polyphenols from apple have been shown to be safe in acute and subchronic animal toxicity tests (SHOJI et al., 2004). Similar results were obtained when feeding experiments were performed with grape seed and grape skin extracts (BENTIVEGNA and WHITNEY, 2002; YAMAKOSHI et al., 2002). Despite these findings more detailed studies including clinical trials will be needed in the future for obtaining results on physiological properties of phenolic extracts in humans. Some authors underscored that due to the high application levels in animal studies which did not show any negative effects and taking into account a per capita dietary uptake of up to 1 g of phenolics per day, these products may be regarded as safe. However, with the commercialization of highly concentrated dietary supplements this may no longer be valid for those persons trying to improve their health status by consuming larger amounts of such preparations.

Moreover, as a further evaluation of quality assurance, elimination of potential health risks must be an integral part of HACCP concepts developed for the extraction procedures using by-products of plant food processing (WROLSTAD, 2000). For grapes and grape products problems may evolve especially from mycotoxins such as ochratoxin A and from pesticides commonly used in viticultural practice. Degradation kinetics, extractability with the solvents used for polyphenol extraction and the potential to adsorb onto the polymer resins used for the purification and concentration of the plant secondary metabolites will be crucial points to avoid accumulation of these compounds. This again demonstrates the necessity for a detailed investigation of the by-products prior to their use for the recovery of phenolic compounds.

The most important need for further research activities is in the field of physiological activities of phenolic compounds. Numerous studies have revealed health-beneficial effects and indicate even anti-proliferative activities against cancer cells, inactivation of carcinogens or induction of apoptosis and of anticarcinogenic enzymes (BROWNSON et al., 2002; MANTHEY and GUTHRIE, 2002; REN et al.,

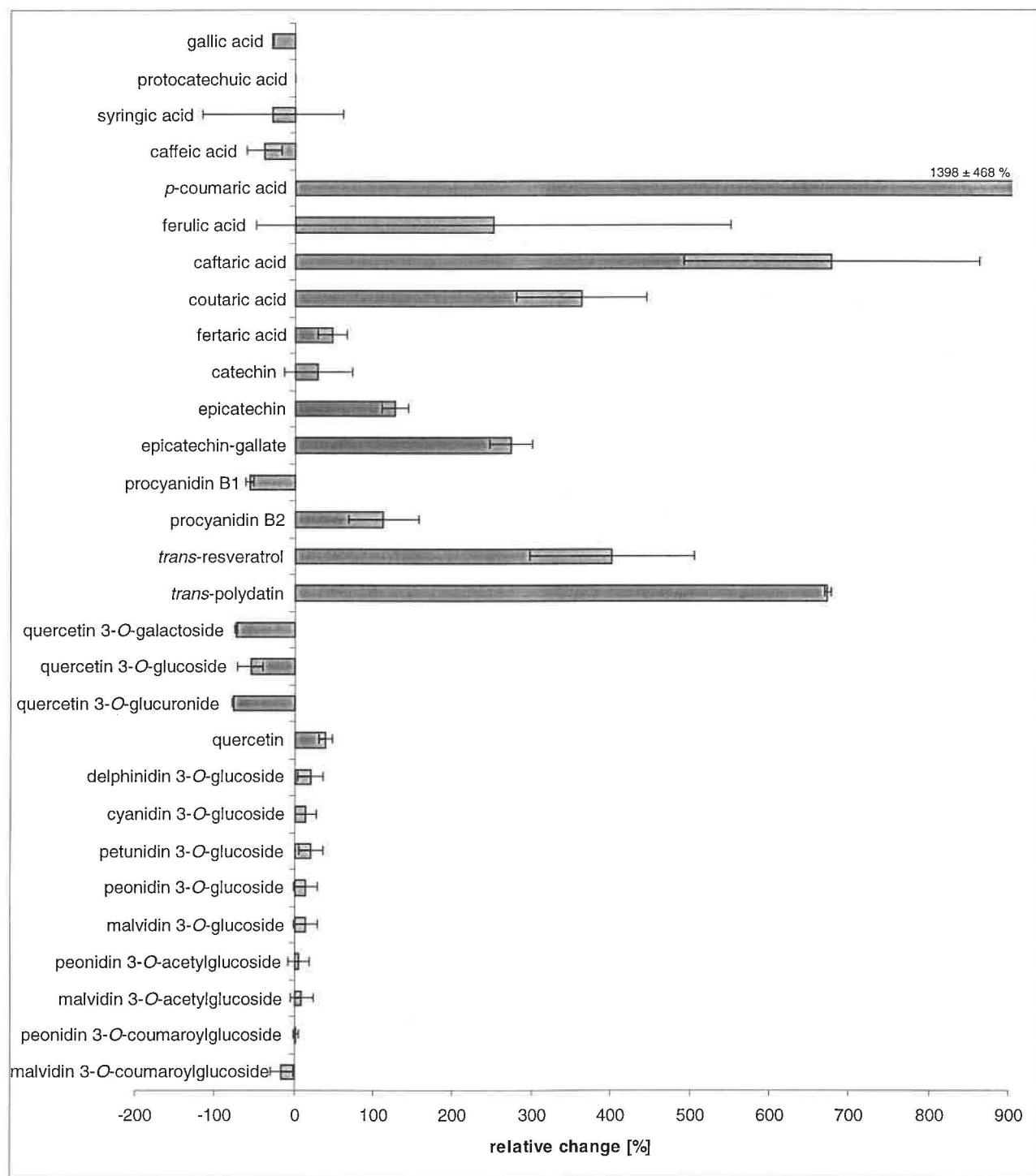


Fig. 2: Relative change in the contents of individual compounds in a 'Cabernet Mitos' extract after enzymatic treatment of the pomace compared to an aqueous pomace extraction (KAMMERER et al., 2005a).

2003). However, a lot of work still needs to be done. *In vitro* test systems are a valuable tool for screening potential bioactivities of plant extracts or of individual compounds, but even comparability of different test systems proves difficult since reactivity of compounds may vary significantly depending on the reaction medium. Thus, the data have to be evaluated carefully. Transferability of the results to *in vivo* conditions is hardly possible, and even contradictory results were obtained (LOTITO and FREI, 2004).

These findings demonstrate that more insight into the molecular

mechanisms of physiologically active compounds *in vivo* is urgently needed. To date, this has been hampered by the fact that the phenolic profile of most plants is usually very complex. Therefore, the consumption of plant crude extracts or of fruits or vegetables as a whole results in a multitude of metabolic reactions *in vivo* increasing the number of potentially bioactive compounds which even aggravates the situation. Further experiments will have to use individual compounds or well-defined mixtures of only a few substances, thus simplifying the detection and identification of the genuine compounds

and of the metabolites. The obvious lack of commercially available reference substances at all or in suitable amounts at affordable prices is a major limitation which has hitherto made such studies almost impossible. It was not until recently that human trials were performed with pure compounds (OLTHOF et al., 2000, 2001, 2003). With the development of more sophisticated separation techniques a number of further compounds will be available in the near future both as reference substances for identification and quantification purposes and for *in vivo* studies.

However, it must be kept in mind that plant extracts and preparations derived therefrom are usually complex mixtures of various compounds. Since both synergistic and antagonistic effects have even been observed in *in vitro* tests (PINELO et al., 2004; STINTZING et al., 2002), interactions are also expected *in vivo*. In addition, matrix compounds such as dietary fiber or other food components such as emulsifiers affect resorption and with it *in vivo* effects (AZUMA et al., 2002) or influence the efficacy of antioxidants, e.g. by interaction between polyphenolics and proteins (ARTS et al., 2002). Interindividual variations in the metabolic enzyme system may also contribute to differing results (HIGDON and FREI, 2003), thus underlining the need for further research activities in the field of polyphenolics.

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