ASEV Honorary Research Lecture 2008

Application of Countercurrent Chromatography for Wine Research and Wine Analysis

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Abstract: One of the few liquid chromatographic techniques that can be predictably scaled up from analytical to process scale is countercurrent chromatography (CCC). Countercurrent chromatography furthermore enables a 100% recovery of the sample and, because of its gentle separation conditions, is ideally suited to the analysis of various groups of wine constituents. In this review, CCC instrumentation and its application to the analysis of labile wine aroma precursors, antioxidants, and pigments are examined. Moreover, novel centrifugal precipitation chromatography instrumentation for the fractionation of polymeric wine constituents and the scale-up of the technique for separations in the 10 to 100 g range are described.

Key words: aroma precursors, antioxidants, pigment, analysis, preparative isolation, countercurrent chromatography, centrifugal precipitation chromatography

Countercurrent chromatography (CCC) is a descriptive term for continuous liquid-liquid partition methods—generally known as "Craig distribution"—that do not use any solid supporting matrix. In principle, liquid-liquid partitioning methods have been available for many years, such as the well-known Craig apparatus. However, because of time requirements, inconvenience, and enormous solvent consumption, the application of these methods has been restricted. In recent years, the introduction of innovative CCC techniques has led to a renaissance in their use for the separation of natural products (cf. Table 1) (Conway 1990, Ito and Conway 1996). In many applications these methods offer additional or alternative procedures to the more extensively used chromatographic separations on solid adsorbents. There are several key advantages of CCC. The first is the absence of solid adsorbents, that is, adsorption losses and the formation of artifacts caused by active surfaces are eliminated. Second, CCC techniques rely exclusively on inexpensive solvent mixtures instead of solid packing materials, which in many cases are very costly. Third, only small amounts of solvents are required. Fourth, large sample loads can be applied. And, finally, a total recovery of the sample material is guaranteed. For a successful separation, all that is required is basically an immiscible solvent pair, in which the components of the mixture have different partition coefficients according to the Nernst distribution law (Conway 1990). The following is a description of developments in and applications of CCC for isolation and the identification of a variety of natural products from grapes and wines.

Early Experiments with Droplet CCC

In the course of studies on the generation of wine aroma compounds from nonvolatile precursors, CCC techniques have been shown to be indispensable tools for the separation and purification of glycosidic progenitors from complex natural mixtures. Such glycosidic mixtures can be isolated from fruit juices and wine by selective retention of the precursors on C_{18}-reversed-phase adsorbent (Williams et al. 1982) or alternatively on Amberlite XAD-2 resin (Günata et al. 1985). However, for a long period the polarity and complexity of the so-obtained precursor concentrates prevented a breakthrough in the further analysis of these mixtures, as with the identification of C_{13}-norisoprenoid aroma compounds in Riesling wine.

Table 1 Development of countercurrent chromatography (CCC) since 1970.

Technique	Author/year
Craig systems	Craig et al. 1951
Helix CCC	Ito and Bowman 1970
Rotation locular CCC	Ito and Bowman 1970
Droplet CCC (DCCC)	Tanimura et al. 1970
Flowthrough coil planet centrifuge	Ito and Bowman 1971
Toroidal coil centrifuge	Ito 1980
Centrifugal droplet-CCC	Murayama et al. 1982
Coil planet centrifuge (Multilayer coil CCC) (High-speed CCC)	Ito et al. 1982
Low-speed rotary CCC (LSRCCC)	Du et al. 2000
Spiral-coil LSRCCC	Köhler et al. 2004

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*Dr. Winterhalter presented this lecture at the 2008 ASEV Annual Meeting, Portland, Oregon, on 18 June 2008.

Manuscript submitted Jan 2009, accepted Feb 2009

Norisoprenoid compounds considered to be important for the aroma of bottle-aged Riesling wine include inter alia isomeric vitispiranes (**1**), 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) (**2**), and ß-damascenone (**3**) (Figure 1). Initial research into the origin of these norisoprenoids indicated a formation via acid-catalyzed degradation of glycosidically bound progenitors (Strauss et al. 1986, Williams et al. 1982, Winterhalter et al. 1990a). However, the complex nature and large diversity of glycosidically bound precursors made isolation and characterization of specific precursors from actual grape and wine samples difficult. In a first approach, the all-liquid chromatographic technique of droplet countercurrent chromatography (DCCC) has been used for the fractionation of the precursor concentrate (Strauss et al. 1987). A further development, the so-called two-dimensional GC-DCCC analysis of Riesling wine glycosides (Winterhalter et al. 1990b), revealed the presence of almost 100 various glycosylated constituents in Riesling wine, including several progenitors **4**–**12** of the target compounds **1**–**3** (Figure 1) (Winterhalter 1991, 1992, Waldmann and Winterhalter 1992).

DCCC is a rather simple technique by which 300 to 500 vertically arranged glass tubes are filled with a stationary phase. A second immiscible phase is then pumped through the apparatus in the form of droplets. Separation is achieved by partitioning the solutes between the stationary phase and the steady stream of droplets. The chief disadvantage of DCCC was its low maximum flow rate, which resulted in rather long separation times (~24 hr). In addition, the poor mixing of the mobile and the stationary phase caused relatively low separation efficiency.

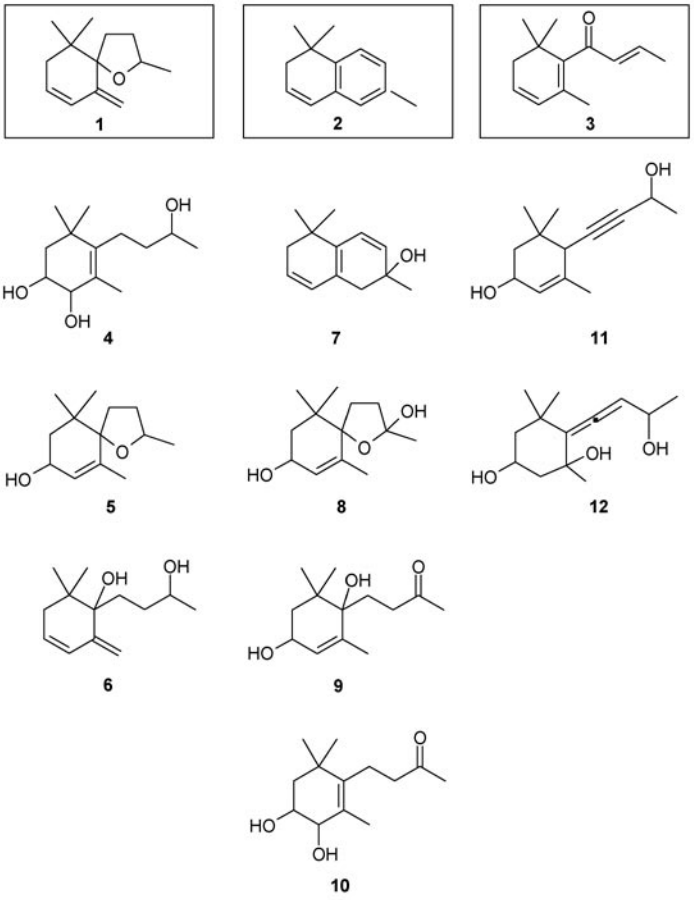

Figure 1 Structures of aglycones (**4–12)** involved in the formation of vitispirane (**1**), TDN (**2**), and ß-damascenone (**3**) (Winterhalter 1991, 1992, Waldmann and Winterhalter 1992, Winterhalter et al. 1990b).

An advancement over DCCC involved a centrifuge to enhance gravity. Centrifugal DCCC, more commonly known as centrifugal partition chromatography, is increasingly used in natural product analysis and preparative separations are obtained within several hours (Foucault 1995).

High-Speed Countercurrent Chromatography

The most versatile and most powerful CCC technique, however, is high-speed countercurrent chromatography (HSCCC) (Conway 1990, Ito and Conway 1996). Separation in HSCCC takes place in a so-called multilayer coil that is made by wrapping inert Teflon tubing around a holder in multiple layers. This technique is also known as multilayer coil countercurrent chromatography (MLCCC). The tubing usually has an inner diameter between 1.6 and 2.6 mm and a length of up to 160 m. Multiple coils can be connected in series to increase the total volume of the instrument and the sample capacity. During separation the coil rotates at 800 to 1000 rpm around its own "planetary" axis and simultaneously around the "solar" axis. This planetary motion has two effects: retention of stationary phase and partitioning of solutes.

Retention of stationary phase. During rotation of a coil filled with immiscible liquids, the two phases move toward opposite ends of the coil, the head and the tail. Generally the less dense phase displaces the heavier phase toward the tail, but the orientation is also influenced by viscosity and interfacial tension. This phenomenon, known as hydrodynamic equilibrium, requires a user to choose the elution mode carefully, and it gives the analyst the choice to select either the lighter or the heavier layer as mobile or stationary phase. In any case, one of the two immiscible phases is retained in the instrument while the second phase is pumped through the column as mobile phase.

Partitioning of solutes. The planetary rotation creates a fluctuating acceleration field that enables vigorous mixing of the two phases followed by settling within the coil. In areas of the coil that are close to the center of rotation, the force field is weak. As a consequence, the phases are mixed. At a further point of their orbit, when they are far away from the center of rotation, the force field becomes stronger and the two phases are separated. Alternate mixing and settling is repeated with each rotation such that up to 70,000 partitioning steps per hour can be achieved.

The crucial point for a successful separation by HSCCC is the choice of a suitable solvent system. The basic requirement for such a system is that it consists of two immiscible phases in which the target compounds have dif-

ferent partition coefficients, P. In recent years, numerous solvent mixtures have been successfully used for the separation of a wide range of natural products. It is therefore recommended to start with one of these approved systems and to optimize it according to the specific requirements of the actual separation. This requires a so-called partition study, which involves dissolution of a small amount of the mixture in the biphasic solvent system (e.g., a few milligrams of the sample in 2 mL of each of the two immiscible phases in a test tube), shaking the mixture, and allowing the system to equilibrate (recommended time <30 sec). The concentration of the analytes in each of the layers is then determined by spectrophotometry, high-performance liquid chromatography (HPLC), gas chromatography (GC), or thin-layer chromatography (TLC). Alternatively, the solvents can be evaporated and the masses of the residues determined by gravimetry. The partition coefficient P is then calculated by dividing the concentration of the target compounds in the upper phase by the concentration in the lower phase. The optimum partition coefficients range from 0.5 to 1.0. In the case of lower P values, compounds are eluted too quickly and not well separated, whereas higher values will give long retention times and considerable peak broadening. Depending on the outcome of the "partition study," the polarity of the two-phase mixture has to be modified in order to obtain a successful fractionation by CCC. Useful information about the optimization of CCC solvents systems can be found in the literature (Ito 2005, Oka et al. 1991).

HSCCC/MLCCC in Wine Research

High-speed countercurrent chromatography has already been used for the fractionation and/or purification of numerous constituents of different classes of wine constituents, such as labile aroma precursors, antioxidants, and red wine pigments.

Aroma precursor studies. The outcome of earlier studies in which CCC enabled the isolation and characterization of aroma precursors from wine and grape vine leaves is reported in the literature (Baderschneider et al. 1997a, 1997b, Roscher and Winterhalter 1993, Skouroumounis and Winterhalter 1994, Winterhalter and Skouroumounis 1997, Winterhalter et al. 1998). A masterpiece of analytical chemistry involving CCC was the isolation of the precursors of wine lactone **15** (3a,4,5,7a-tetrahydro-3,6-dimethyl-3*H*-benzofuran-2-one) from Riesling wine. Wine lactone (Figure 2)—a most potent aroma component in wine (flavor threshold 0.00002 ng/L of air)—was found to increase in concentration six-fold during wine maturation. This increase clearly indicated the presence of labile progenitors, which during wine aging are chemically degraded to the odoriferous lactone **15**. In view of the extremely low concentration of **15** in wine, an attempt to isolate the respective precursors required the workup of a substantial amount of wine to ensure a complete structural characterization by NMR techniques (Winterhalter et al. 1997). From 100 L of a German Riesling wine, the precursor fraction was isolated by adsorption on XAD-2 resin. After elution with methanol, a total of 20 g of a precursor concentrate was obtained. Countercurrent chromatography was the only method ensuring a gentle separation of this mixture on a preparative scale. Fractionation by MLCCC using the solvent system $CHCl_3$/MeOH/H_2O (7:13:8) combined with a screening of the separated fractions by simultaneous distillation extraction (SDE; pH 3.2) allowed the determination of precursor fractions by measuring the amount of liberated wine lactone by GC. Wine lactone-producing fractions were further purified by preparative HPLC and ~1.0 mg of each of the compounds **13** and **14** were obtained in a pure state. In order to prove the precursor function of (*E*)-2,6-dimethyl-6-hydroxyocta-2,7-dienoic acid **13** and its glucose ester **14**, both compounds were synthesized and chemically (SDE; pH 3.2) degraded to wine lactone **15**. In addition, storage of acid **13** in a model wine medium clearly demonstrated its role as precursor of the odoriferous lactone **15** (Figure 2) (Winterhalter and Bonnländer 2001).

Antioxidants in white wine. Another application that highlights the potential of CCC in wine research is the study on antioxidants in white wine. From 100 L German Riesling, a total of 101 substances have been isolated and completely characterized by mass spectrometry and NMR spectroscopy. Seventy percent of the isolated compounds were (poly)phenols, and for the majority of them the antioxidant capacity could be determined. More than half of the isolated components (a total of 54) were reported by us as new wine constituents and 15 of them were reported for the first time as

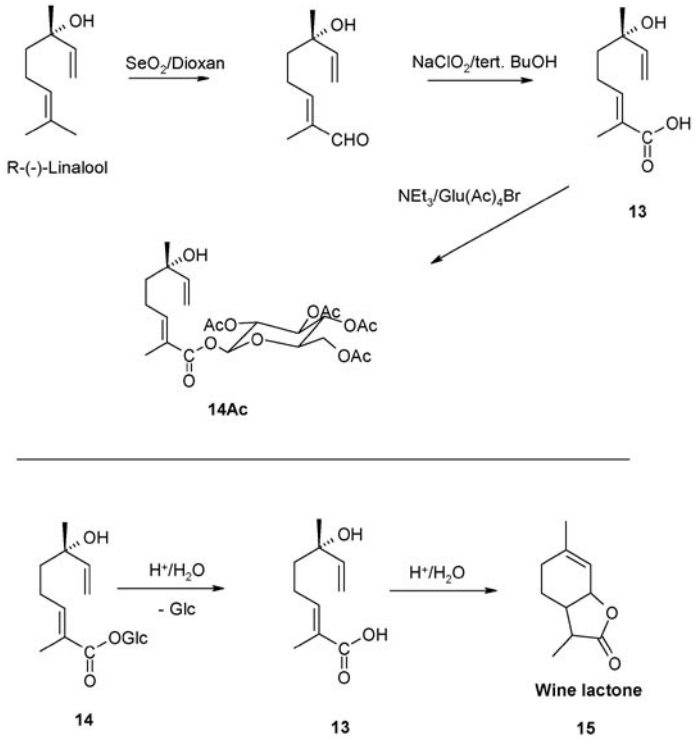

Figure 2 Synthesis of wine lactone precursor **13** and **14** (top) and acid-catalyzed degradation giving rise to the odoriferous lactone **15**.

natural products (Baderschneider 2000, Baderschneider and Winterhalter 2000, 2001). This application clearly shows the advantage of CCC over conventional chromatographic techniques, as it provides a rapid preparative fractionation tool for crude mixtures, which leads to an enrichment of minor constituents in the CCC fractions. Rechromatography with CCC or preparative HPLC then gives ready access to sufficient amounts of pure compounds, thus enabling structure elucidation of unknown minor or even trace constituents by application of modern NMR techniques. The structures of some of the newly isolated wine constituents belonging to the classes of stilbenes **16**–**21**, lignans and neolignans **22**–**26**, and indol derivatives **27**–**32** are shown (Figure 3).

Analysis of red wine pigments. Analysis of anthocyanin composition from red wine is routinely used in authenticity control (Ebeler et al. 2007). The ratio of acetylated and coumaroylated anthocyanins ($R_{ac/coum}$) can be used to determine the grape varietal composition of red wines (Holbach et al. 1997). In order to ensure an accurate analysis, reference compounds are required, which can be isolated from red wine by CCC. Because of the complexity of the anthocyanin fraction in wine, a whole set of different CCC solvent mixtures is applied for the purification of

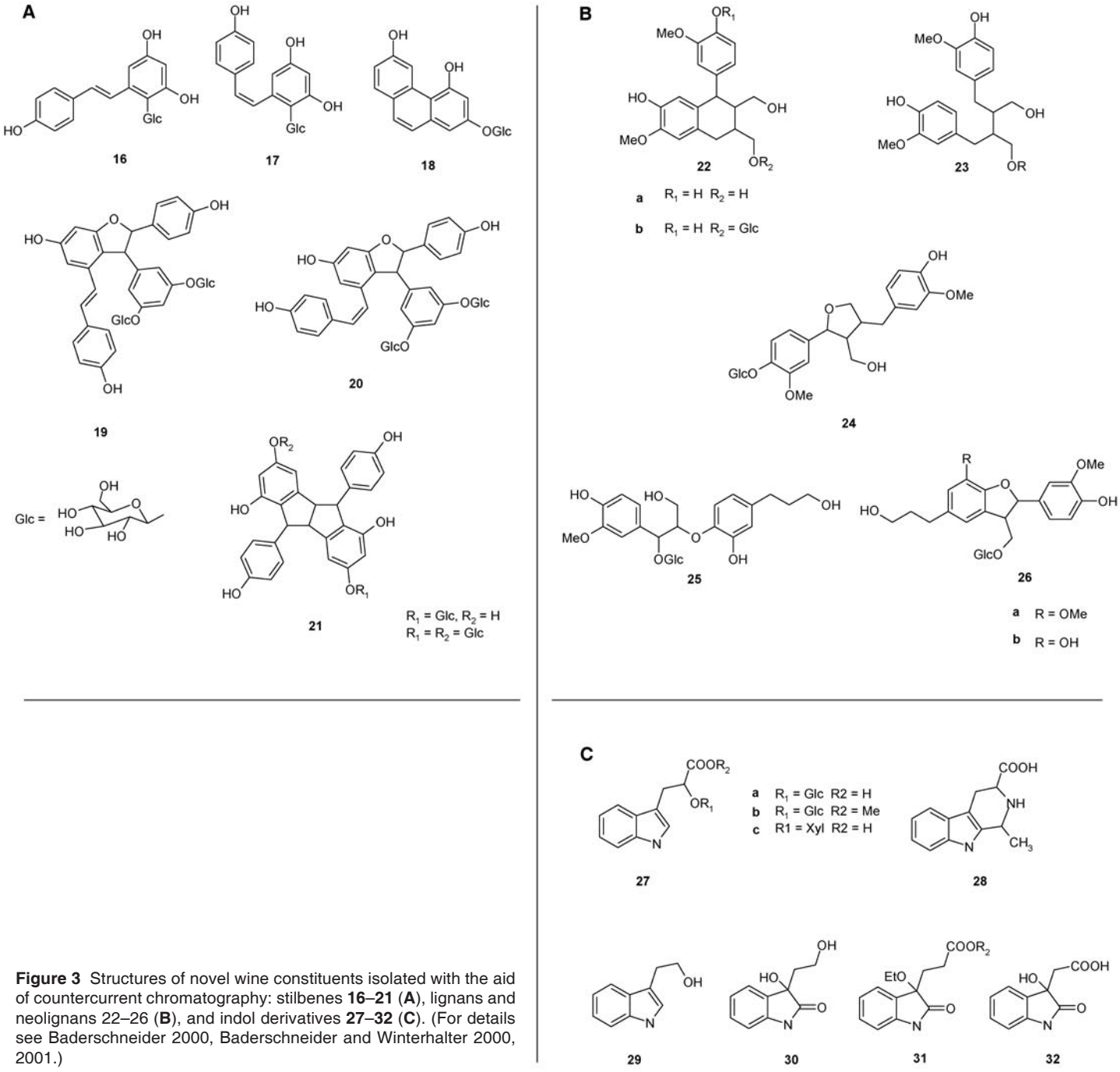

Figure 3 Structures of novel wine constituents isolated with the aid of countercurrent chromatography: stilbenes **16**–**21** (**A**), lignans and neolignans 22–26 (**B**), and indol derivatives **27**–**32** (**C**). (For details see Baderschneider 2000, Baderschneider and Winterhalter 2000, 2001.)

individual pigments. The anthocyanins are first isolated by adsorption on Amberlite XAD-7, and after elution and concentration are separated by HSCCC (Schwarz et al. 2003a). A first fractionation of the XAD-7 isolate can be achieved with the solvent system MTBE-*n*-butanol-acetonitrile-water (2:2:1:5, v/v/v/v, acidified with 0.1% TFA, less dense layer acting as stationary phase). This system is optimized for the isolation/separation of monoglucosylated anthocyanins (e.g., malvidin-3-glucoside). More polar pigments, such as the diglucosides and the polymeric fraction, are separated in a second step by application of either ethyl acetate/*n*-butanol/water (2:3:5, v/v/v, acidified with 0.1% TFA) or a modified MTBE-*n*-butanol-acetonitrile-water system (1:3:1:5, v/v/v/v, acidified with 0.1% TFA). For the less polar acylated anthocyanins, successful separations have been obtained using ethyl acetate/water (1:1, v/v, acidified with 0.1% TFA) (Degenhardt et al. 2000, Schwarz et al. 2003a).

With the isolated pure standards of anthocyanins, it was for the first time possible to determine the visual detection limits of each of the wine pigments (Degenhardt et al. 2000). This was the basis for the application of the so-called color activity concept that allows the determination of the contribution of individual pigments to the overall color of red wines. An important outcome of this study was a first determination of the contribution of pyranoanthocyanins and of the polymeric pigment fraction to the color of aged wines. It could be demonstrated that the contribution of polymeric pigments is by far superior to the contribution of either genuine monomeric anthocyanins or pyranoanthocyanins that are present in only very low concentrations (Schwarz and Winterhalter 2004).

High-speed countercurrent chromatography methods have also been successfully applied in the structure elucidation of pyranoanthocyanins (Schwarz et al. 2003b, 2003c), flavanol-anthocyanin adducts (Salas et al. 2005a, 2005b), and the large-scale isolation of flavanol phloroglucinol adducts (Köhler and Winterhalter 2005) as well as dimeric and oligomeric procyanidins (Köhler et al. 2008a, 2008b).

Centrifugal Precipitation Chromatography

Additional research has focused on analysis of the composition of polymeric pigments of wine. After isolation of a crude fraction of the polymers by HSCCC, additional separation steps are required before the different classes of polymers can be further characterized. For the first time, the newly developed technique of centrifugal precipitation chromatography was applied to the separation of wine pigments (Degenhardt et al. 2001). Centrifugal precipitation chromatography generates solvent gradients through a long separation channel under a centrifugal force field. The wine polymers are first precipitated with MTBE and subsequently exposed to a gradually increasing ethanol concentration, which causes a repetitive precipitation and dissolution of the polymers along the separation channel. Eventually, the polymers are eluted in the order of their solubility in the organic solvent. The monomers and oligomers elute first, followed by different classes of fractionated polymers. Further structural data can then be obtained by matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) analyses or phloroglucinolysis.

Scale-up of CCC

There are multiple approaches for the scale-up of CCC. One possibility is simply the enlargement of existing high-speed countercurrent chromatographs. In these centrifugal CCC systems, scaling-up of the column capacity for industrial separations is limited by the high speed of rotation (up to 1200 rpm) that is required for retaining the stationary phase in the column. Therefore, commercial high-speed countercurrent chromatographs are mainly equipped with one helical column (~350 mL capacity) or three helical columns connected in series (~1000 mL capacity) and are made of 0.8–2.6 mm inner diameter (i.d.) teflon tubing. By replacing these tubings with a 5-mm i.d. teflon tube, a preparative high-speed countercurrent chromatograph has been developed (Du et al. 2002). The sample load of the instrument is in the range of 40 g per separation, and the columns have a total capacity of 2460 mL. However, a different strategy has been followed for the further scale-up of CCC to kilogram scale.

Low-Speed Rotary Countercurrent Chromatography

One of the most promising developments so far is low-speed rotary countercurrent chromatography (LSRCCC), in which a cylindrical column rotates slowly around a single axis. The use of a special convoluted tubing enabled sufficient retention of stationary phase at a rotational speed of only 50 to 100 rpm (Du et al. 2000, 2003). In experiments with an instrument equipped with a 10-L column, sample load was in the range of 150 g per separation and gram amounts of pure anthocyanins have been obtained. The instrument is readily scaled-up to industrial separations by using longer columns and/or by increasing the inner diameter of the convoluted tubing. Instruments that are suitable for separation in the kilogram scale and with improved stationary phase retention are under development (Köhler et al. 2004).

Conclusions

Countercurrent chromatography gives the wine chemist an efficient tool for the gentle and rapid separation of the various classes of wine constituents. The use of CCC is particularly effective where scale-up is causing problems with separation performance or time consumption. Separation conditions are so gentle that even reactive aroma precursors are obtained in an intact form. Moreover, the technique is inexpensive because it only relies on the partitioning of the analytes between two immiscible solvents. Separation systems for most classes of wine constituents have been developed very recently, and novel applications will only require slight changes in the ratios of the known

solvent mixtures in order to obtain a successful separation. Instrumentation is becoming more robust, and the scale-up to the 100 g (and even kilogram) scale will be feasible within the near future.

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