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Methods for Analyzing Phenolics in Research

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Abstract: Chromatographic and spectroscopic methods important to the analysis of grape and wine phenolics are described. The developmental history of these methods is presented, and associated enological applications are summarized.

> [C]hemists interested in polyphenols, in common with the majority of scientists, tackle today's problems with yesterday's tools, i.e., current problems are attacked with methods which are inadequate and to that extent are already out of date. . .[t]he discovery and quick application of new methods or developments and extensions of existing methods is therefore of first importance (Brown 1964).

Key words: phenolics, instrumental analysis, chromatography, spectroscopy, spectrometry

There is often an observed disconnect between those facets of science deemed "research-oriented" versus those deemed "applied." These differences are further exacerbated in occupations such as the wine industry in which there is a large investment in or requirement for applied technologies. The situation is not improved by inherent differences among the practitioners; the wine industry is largely populated by those with limited scientific training, while the research community is characterized by those with advanced degrees but generally less practical experience. The cultures, and the concomitant needs and goals, thus often differ vastly. All too often, the industry perceives the researchers as out-of-touch denizens of the fabled ivory tower, while researchers perceive the industry as short-sighted (if not scientifically gullible) and focused exclusively on stop-gap solutions.

These differences are often exploited by neo-Luddite factions of the wine press that equate science with sterility, failing to recall the history of improvements resulting from enological discoveries. Indeed, the question has been posed as to whether research since the 1930s and 1940s has significantly improved wine quality when compared to the improvements made during that halcyon period. "Probably not," concludes H.W. Paul (1996), and with some justification. However, the comparison is inherently unfair, as the most tractable problems are solved first, often with the most dramatic results.

Emile Peynaud recognized both the dichotomy between theory and practice and the importance of both, stating that "Enology is not an abstract science. It has grown out of research into solutions to practical problems. But whereas the facts are observed during work at cellar level or in the winery, they can only be explained, rules laid down, and progress made at the higher level during study of these phenomena" (Peynaud 1984).

Historical Methods of Analysis

The importance of phenolic compounds to wine has been amply demonstrated (Singleton and Esau 1969, Ribéreau-Gayon 1972).The methods used to analyze these compounds can be broken down into two broad categories: separation and identification or, more specifically, chromatography and spectroscopy/spectrometry.

Chromatography. Chromatographic techniques are now celebrating the centenary of their first true application by Mikhail Tswett in the early 1900s. He first described his technique in 1901 and later published two articles regarding his work with chlorophyll (Tswett 1906a,b). Specifically, Tswett introduced the technique of column adsorption chromatography, when he used a column of ground chalk (calcium carbonate) to separate leaf pigments.

Given its later predominance as the analytical technique of choice (chromatographic instruments are the fastest-growing segment of characterization instruments; Cleaves and Lesney 2004), it is ironic that chromatography was not widely used in the first 20 years after its inception (Ettre and Horvath 1975). However, following A.J.P. Martin's work in the 1940s in which liquid-liquid partition chromatography was defined and paper chromatography developed (Martin and Synge 1941, Consden et al. 1944), chromatography underwent a rapid renaissance. The use of paper chromatography, its close relative thin-layer chromatography, and the development of gas chromatography (anticipated by Martin and Synge and later

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developed by James and Martin [1952]) revolutionized both analytical chemistry and biochemistry.

The importance of these techniques to the wine industry is best demonstrated by their rapid use for phenolic analyses. Four years after paper chromatography was described, the fledgling technique was used to separate anthocyanins and flavones in extracts of *Dahlia variabilis* petals (Bate-Smith 1948). Indeed, Bate-Smith credited paper chromatography with the eventual creation of the Plant Phenolics Group (the progenitor of the current Phytochemical Society of Europe), stating that, "the applications of this discovery have proved so immediately prolific and . . . the results obtained have so simplified the tasks of the chemist and botanist engaged in research" (Bate-Smith 1964). Beginning in 1953, Ribéreau-Gayon began a series of wine studies using paper chromatography, noting several advantages: phenolic compounds separated well on paper using minimal solvent types, the separated compounds were easily visualized (either by their intrinsic color or under ultraviolet light), and only micro quantities of compound were required (Ribéreau-Gayon 1972).

Although less common, column chromatography was still being used. Spaeth and Rosenblatt (1950) published a report on silicic acid-based column chromatography used to separate anthocyanidin mixtures as part of their ongoing study of grape and wine pigments. Carelli and colleagues reported using polyamides for separating various phenolic compounds in what they termed "sorption chromatography" (Carelli et al. 1955). Chandler and Swain (1959) later used polyamide columns to separate anthocyanins. Vuataz and colleagues used cellulose column chromatography to separate black tea polyphenols (Vuataz et al. 1959); Singleton and coworkers then used that approach to study phenolic compounds in grape seeds (Singleton et al. 1966). Porath and Flodin (1959) introduced the technique of gel-permeation chromatography using dextran gels; Somers (1966, 1967) would use these techniques to begin his studies of condensed tannins and polymeric pigments.

Spectroscopy/spectrometry. *Ultraviolet-visable spectrometry.* Since ultraviolet-visible (UV-vis) spectroscopic techniques are reviewed elsewhere (Harbertson and Spayd 2006), I will focus instead on infrared spectroscopy, nuclear magnetic resonance spectroscopy, and mass spectrometry. The importance of the UV-vis spectrum to identification cannot be overstated, however, and merits some brief comments. Flavonoid spectra typically consist of two maxima. The first (designated Band II) occurs in the spectral range from 240 to 285 nm and is attributed to the flavonoid A-ring configuration. The second maxima occurs in the range from 330 to 550 nm (Band I), and reflects the B-ring configuration (Santos-Buelga et al. 2003). Thus, the respective l_{max} and the relative intensities of the maxima are diagnostic of the phenolic compounds present, with varying degrees of success. For example, anthocyanins in the flavylium form have l_{max} between 490 and 540 nm, depending on the substitution pattern of the B ring. Flavan-3-ols, lacking conjugation between the A and B rings, have spectra dominated by Band I absorbance between 270 and 290 nm. As this absorbance is shared by all flavonoids, it has limited utility for identification (Santos-Buelga et al. 2003). This limitation has recently been addressed through the use of derivatization agents such as *p*-dimethylaminocinnamaldehyde (DMACA) (de Pascual-Teresa et al. 2000).

Infrared spectroscopy. Infrared (IR) spectroscopy is a powerful tool for structure identification as covalent bonds absorb electromagnetic radiation in the IR region. This region of the spectrum, discovered by Friedrich Wilhelm Herschel in 1800 (Herschel's "calorific rays"), extends from 0.8 to 100 μm, but the main region of historic interest is the vibrational portion between 2.5 and 15 μm (4000 to 650 cm^{-1}). Those bonds within a molecule containing a dipole moment can absorb IR energy, increasing the amplitude of the molecule's vibrational modes (Pavia et al. 1979). While some modes are associated with the whole molecule, localized vibrations specific to certain bond types are of diagnostic utility.

Infrared instrumentation was available as early as 1922 (Filmore 2004). However, the IR spectroscopy of phenolic compounds (particularly flavonoids) began in earnest in the mid-1950s (Wagner 1964), following the development of the first double-beam spectrophotometer, the Perkin-Elmer 21, in 1950. The applicability of IR was limited by the sample preparation required: most phenolic compounds are insoluble in the solvents used for solution IR (such as chloroform, carbon tetrachloride) and the mulls used for solid-state IR (such as Nujol). Wagner avoided these problems by preparing samples as compressed potassium bromide discs, obviating the need for solvents or mulling agents. The potassium bromide technique did require a crystalline preparation of the compound of interest, making it poorly suited for the common separation techniques of the day, notably paper chromatography (Ribéreau-Gayon 1972). In the end, the greater ease and power of UV-vis spectroscopy marginalized IR spectroscopy. It would take the emergence of advanced chemometric techniques and greater computing power to bring IR analyses back to the forefront.

Nuclear magnetic resonance spectroscopy. While IR analysis measures dipole moments, nuclear magnetic resonance (NMR) spectroscopy examines magnetic moments (of those atomic nuclei with an odd mass or an odd atomic number). These nuclear magnetic moments interact with an applied magnetic field, ultimately resulting in information regarding the immediate environment about the nuclei (most typically hydrogen, although ^{13}C, ^{19}F, and ^{31}P exhibit similar behavior).

Nuclear magnetic resonance was independently observed in the mid-1940s by researchers at Stanford (Bloch et al. 1946) and Harvard (Purcell et al. 1946). However, it was not until Packard and colleagues discovered the chemical shift within a molecule (ethanol) in 1951 that the utility of NMR to chemists was made clear (Arnold et al.

1951). The first commercial instrument was introduced by Varian in 1953, and NMR became established as a standard analytical tool after the release of the Varian A-60 in 1961.

While the utility of NMR was clear, its application to early phenolic (and wine) analysis was limited, as protons in the complex flavonoid structures were difficult to assign. At best the technique was able to identify those protons shifted to definitive regions of the spectrum. The continuous-wave instrumentation available at the time simply did not give very high signal-to-noise ratios. The development of Fourier transform (FT) NMR by Richard Ernst improved the situation (Ernst and Anderson 1966).

Abraham (1964) summarized the state-of-the-art application of NMR to phenolics analysis. The studies of Webb and Kepner on anthocyanin acylation using a Varian A-60 are typical of the application of NMR to enology during this period (Anderson et al. 1970, Gueffroy et al. 1971).

Mass spectrometry. Mass spectrometry (MS) had its genesis in the laboratory of J.J. Thomson, the discoverer of the electron. Thomson studied the deflection of ions in a cathode ray tube and deduced that the characteristic parabolic path of each ion was dependent upon its charge to mass ratio (Thomson 1913). Francis Aston, a student of Thomson, further refined the "parabola" or "positive ray" spectrograph, culminating in the discovery of isotopes of non-radioactive elements (Aston 1919).

Following this initial fertile period, there was little advancement until the first commercially successful mass spectrometer, the Consolidated Engineering Corporation 21-101, was introduced in the early 1940s (Lesney 2004). Other developments occurred in rapid succession: time-of-flight (TOF) MS (1946), ion cyclotron MS (1948), quadrupole filters and ion-trap detectors (1953), and gas chromatography-TOF MS (1956). The first commercial quadrupole MS became available in 1962 (Lesney 2004).

Electron ionization MS was used to study flavans reduced from chalcones (Brown 1964), demonstrating its applicability to phenolic structure elucidation. There were few applications in enology during these initial years, as the low volatility of polyphenols precluded easy analysis by the MS techniques then available (Flamini 2003).

Modern Techniques

The mid-1960s to early 1970s witnessed major advances in both chromatographic and spectroscopic systems, many driven by increased computer capabilities combined with the development of improved Fourier transform and other chemometric algorithms. Some of these advances were also driven by improved support technologies such as pellicular packings for high-performance liquid chromatography (HPLC) or supermagnets for NMR.

Chromatography. It is interesting, if not humbling, that Martin and Synge not only discussed partition chromatography and anticipated gas liquid chromatography in their seminal 1941 paper but also outlined the requirements for HPLC. In discussing the height equivalent to a theoretical plate (HETP), the authors stated that "the smallest HETP should be obtainable by using very small particles and a high pressure difference across the length of a column" (Martin and Synge 1941). However, the technical problems inherent in providing a uniform flow under such conditions precluded further development of the technique at that time. Martin's subsequent developments of paper and gas chromatography obviated the need to develop liquid chromatographic techniques further (Ettre 2005).

Twenty-five years after Martin's prescient comments, a research associate at Yale led the development of the first high-performance liquid chromatograph (Horváth and Lipsky 1966). Csaba Horváth was uniquely qualified for this project because of his previous research in creating uniformly coated pellicular particles (Halász and Horváth 1964). Although HPLC has some inherent limitations (Karchesy 1988), its importance in phenolics research is crucial. As Somers and Vérette (1988) noted, "Before HPLC, quantitation of individual phenolic components from grapes or wines had been laborious and uncertain; from a few µL wine sample, HPLC enables resolution and quantitative analysis of numerous phenolic monomers in 30 mins!"

Two early pioneers of HPLC in enology were Charles Nagel and Larry Wulf, who published a series of articles that were among the first applications of HPLC to phenolics analysis (Wulf and Nagel 1976, 1978, Nagel and Wulf 1979). While initial HPLC studies tended to emphasize the novelty of the new method, particularly its speed, enologists soon began to apply the technique in earnest, including changes in flavonoid content during fermentation and aging of red wines (Nagel and Wulf 1979); the application of HPLC to the procyanidin content of ciders and wines (Lea 1979, 1980); and the anthocyanin profile in wines across 10 years of aging (McCloskey and Yengoyan 1981). Other studies examined malvidin 3-glucoside condensation kinetics (Baranowski and Nagel 1983); the characterization of young Bordeaux wines produced from different cultivars (Salagoïty-Auguste and Bertrand 1984); caftaric and coutaric acids in grapes and wines (Singleton et al. 1984, 1985, 1986); anthocyanin profiles of port grapes and wines (Bakker and Timberlake 1985a,b); and changes in anthocyanin content over ripening in three Syrah clones (Roggero et al. 1986).

The introduction by Hewlett Packard (now Agilent Technologies) of a diode array detector (DAD) for HPLC in 1982 (the HP 1040A) further improved the utility of HPLC for phenolics analysis. The advantages to obtaining UV-vis spectral data for each component were pronounced, as the spectrum allowed for improved identification, increased sensitivity because of detection at the absorbance maximum, and peak purity assessments (Santos-Buelga et al. 2003). The new detector was soon adopted for phenolic and enological research and was used to study anthocyanins in berries (Andersen 1985, 1987); for diode array detection to characterize the anthocyanins in both Tempranillo and a hybrid cultivar (Hebrero

et al. 1988, 1989); and to examine nonenzymic oxidation of caffeic acid (Cilliers and Singleton, 1989, 1990, 1991). A HPLC-DAD technique was developed in the Pacific Northwest to characterize anthocyanins from a variety of sources (Hong and Wrolstad 1990a,b).

An important analysis dependent upon HPLC as a separation tool is the structural characterization of procyanidins, whether by acid-catalyzed degradation in the presence of a nucleophilic trapping agent (Thompson et al. 1972, Rigaud et al. 1991, Prieur et al. 1994, Kennedy and Jones 2001), or by normal phase (Rigaud et al. 1993, Waterhouse et al. 2000), or gel permeation chromatography (Bae et al. 1994, Kennedy et al. 2001, Kennedy and Taylor 2003).

Spectroscopy/spectrometry. *Mass spectrometry.* While electron impact ionization (EI) techniques were used successfully to characterize monomeric phenolics, the applicability of EI to oligomers and polymers was limited because it required derivatization to increase their volatility. Weinges and colleagues acylated fruit procyanidins for EI analysis (Weinges et al. 1968, cited in Barofsky 1988), while Dadic and Belleau (1976) characterized acylated procyanidins from beer. However, the limited mass information available from this ionization technique severely limited its usefulness (Lazarus et al. 2003).

The development of liquid secondary ion mass spectrometry (LSI-MS) (Benninghoven and Sichtermann 1978) and fast atom bombardment mass spectrometry (FAB-MS) (Barber et al. 1981) marked a new era for structural identification. While earlier techniques had been developed, notably field desorption and plasma desorption (Matsuo and Seyama 2000), the newer techniques rapidly superseded them. LSI typically uses cesium ions as the particle beam source, whereas FAB uses a neutral inert gas (argon or xenon). Several micrograms of sample is mixed into a liquid matrix (typically 1 to 2 µL of glycerol) and applied to the tip of a sample probe, which is then introduced into the ion source chamber. The subsequent bombardment causes the ejection of a desorbed secondary ion beam containing positive and negative ions in addition to neutral species (the relative abundances are controlled by the source potentials, the analyte itself, and the nature of the support matrix). The primary advantage to these techniques is that no chemical derivatization is required: the sample preparation and manipulation are minimal. This relatively "soft" ionization produces abundant molecular ions with minimal structural fragmentation.

In one of the earliest enological applications, a xenon beam FAB-MS was used to obtain molecular weight data of anthocyanins from port wine cultivars (Bakker and Timberlake (1985a). Xenon FAB-MS was also used to sequence procyandin oligomers from a variety of natural sources (Karchesy et al. 1986); to identify catechin-gallate, catechin-catechin-gallate, and ß-1,3,6-tri-*O*-galloyl-D-glucose in Niagara grapes (Lee and Jaworski 1990); to characterize a variety of dimers and trimers from grape seeds (Ricardo da Silva et al. 1991); to identify the nonenzymic autoxidation products of caffeic acid (Cilliers and Singleton 1991); and to identify the acetaldehyde-bridged anthocyanin/catechin dimer in a model wine (Bakker et al. 1993). Bakker and colleagues also used xenon FAB-MS to characterize vitisin A and related pigments in wine (Bakker et al. 1997, Bakker and Timberlake 1997).

A disadvantage to these techniques is that the spectrum must typically be obtained from relatively pure samples. While FAB-MS has been applied to complex mixtures, it has been the exception rather than the rule (Lazarus et al. 2003, Cheynier and Fulcrand 2003). This problem was successfully circumvented with the development of matrix-assisted laser desorption ionization (MALDI) mass spectrometry (Karas et al. 1987, Tanaka et al. 1988). Laser desorption techniques had existed since the early 1960s; however, their mass cut-off was relatively low. Low-energy laser light (typically nitrogen at 337 nm), in conjunction with a suitable matrix (a UV-absorbing organic molecule or a metal powder) could protect the analyte from degradation during the vaporization step, enabling the mass spectrometry of large biomolecules. In addition to allowing an increased mass range, MALDI has proven robust against sample contamination (Lazarus et al. 2003, Flamini 2003), although sample clean-up is still recommended (Mano and Goto 2003). MALDI also primarily produces singly charged ions, allowing for the analysis of complex samples. When coupled to time-of-flight (TOF) spectrometers, which have no *m/z* limits, MALDI is an especially powerful mapping tool (Mano and Goto 2003).

While the first enological applications of MALDI were for protein determinations (Szilágyi et al. 1996, Weiss et al. 1998), the technique was soon applied to phenolic compounds. Anthocyanins were analyzed from a variety of grape skin extracts (Sugui et al. 1999) and were studied in wines and Concord grape juice (Wang and Sporns 1999). Procyanidins were studied from grape seed extracts (Krueger et al. 2000, Yang and Chien 2000). In both cases oligomers up to nonamers were characterized. MALDI-TOF was used to analyze grape tannin fractions (Perret et al. 2003); the spectrum acquired ranged up to 4000 amu, representing a degree of polymerization up to ~18.

While some authors have speculated that quantitation should be possible with a judiciously chosen internal standard (Wang and Sporns 1999), Lazarus and colleagues have noted that quantitation "remains a challenge" (Lazarus et al. 2003). Another inherent disadvantage to MALDI is the inability to conveniently couple it to chromatography. Flow FAB systems have been successfully constructed, such as frit-FAB and continuous-flow FAB (Abian 1999). Techniques to couple liquid chromatography to MALDI have proven less successful, although various approaches have been devised (Nagra and Li 1995, Zhang et al. 2004).

An alternative soft ionization method to MALDI, electrospray ionization, was developed by Yamashita and Fenn (Yamashita and Fenn 1984a,b). Electrospray techniques had been developed earlier by Dole and colleagues, who

had also anticipated ESI-MS (see Dülcks and Juraschek 1999); however, Fenn's work marks the first successful coupling of the ionization mode to mass spectrometry. Fenn shared the 2002 Nobel Prize in chemistry with Koichi Tanaka for their work on structural analysis of biological macromolecules.

The electrospray process can be succinctly summarized. First, nebulization of the sample produces electrically charged droplets via an electrophoretic mechanism. Second, ions are liberated from the droplets in a combined process of solvent evaporation and Coulombic repulsion. Finally, the ions thus produced are swept from the atmospheric source region into the mass analyzer (Bruins 1998, Smyth 1999). The gradual thermal desolvation leads to electrospray being a very "soft" ionization method. Unless the potential difference between the transfer capillary and the analyzer is increased (resulting in collision-induced dissociation,) there is minimal fragmentation of the analytes (Dülcks and Juraschek 1999). An additional, unique advantage to ESI is that the process leads to the formation of both singly and multiply charged ions. Multiple charging of larger biomolecules results in a distribution of *m/z* ratios and allows for accurate determination of molecular weights (Mano and Goto 2003). As most of the resulting signals fall below *m/z* = 2000, less expensive quadrupole mass filters may be used in place of more expensive time-of-flight systems (Dülcks and Juraschek 1999). Finally, in contrast with FAB and MALDI, no matrices are required for analysis: ESI allows for direct interfacing of the LC to the MS (Kebarle and Tang 1993).

One of the earliest enological applications of HPLC-ESI-MS was in a study of anthocyanin extracts (Baldi et al. 1995). It was also used to study reactions of acetaldehyde in solution with flavan-3-ols (Fulcrand et al. 1996) and to characterize flavan-3-ol oligomers and polymers (Cheynier et al. 1997). In the past 10 years, ESI techniques have dominated the enological literature; as noted in one study, HPLC-ESI-MS enjoys a contemporary success analogous to that of HPLC-DAD in the 1980s (Tomás-Barberán et al. 2003). A comprehensive review of the literature through 2002 is available (Flamini 2003), and ESI as a stand-alone ionization method has been addressed (Cheynier and Fulcrand 2003).

Several recent reviews of flavonoid research discuss the use of tandem mass spectrometry (MS-MS, $MS^{(n)}$) techniques, in which a parent ion is further fragmented into daughter ions for improved structural characterization (Wolfender et al. 2000, Cuyckens and Claeys 2004). Examples from enology include the study of flavonoids and stilbenes in wine (Stecher et al. 2001) and oligomeric pigment in grape skins (Vidal et al. 2004).

Nuclear magnetic resonance and electron paramagnetic resonance spectroscopy. The development of pulse excitation Fourier transform nuclear magnetic resonance (FTNMR) by Richard Ernst and Weston Anderson (Ernst and Anderson 1966) led to increases of sensitivity between one and two orders of magnitude over continuous wave instruments. This sensitivity increase in turn enabled the study of low-abundance isotopes, notably ^{13}C. The concomitant improvements in magnet designs, computers, and pulse transmitters further increased the sensitivity and resolution of the spectra (Ferreira and Brandt 1988).

The combination of these technological improvements and the availability of FTNMR instruments led to the development of the one-dimensional nuclear Overhauser effect (n.O.e.) difference techniques. Overhauser predicted that nuclear spin resonance intensity could be altered by unpaired electrons (Overhauser 1953a,b). It was later demonstrated that the Overhauser effect could occur between two nuclei because of dipolar interactions (Solomon 1955). Anet and Bourn (1965) were the first to apply the information provided by n.O.e. in their structural analysis of ß,ß-dimethylacrylic acid. The n.O.e. is dependent on spatial geometry (that is, the "through space" distance), so it is possible to obtain information on intramolecular steric relationships. Difference techniques subtract the normal NMR spectra from the doubly irradiated (enhanced) spectra. Ferreira and Brandt (1988) provide a comprehensive overview of n.O.e. difference techniques applied to flavonoid structures.

The development of FTNMR also allowed for the development of the so-called two-dimensional (2-D) techniques. Inspired by a lecture of Jean Jenner, Ernst and colleagues outlined a general approach to 2-D NMR (Aue et al. 1976). The experiment is defined by a series of radio-frequency pulses and four distinct intervals: preparation (initial pulse distortion), evolution (the period of which changes experiment to experiment), mixing (the next series of pulses), and detection. The detected signal intensity depends on the length of the evolution period and the point in time during the detection period. The Fourier transform is performed on both of these parameters, yielding a 2-D frequency spectrum in which both axes depict chemical shifts and the intensity is topographically represented. Three important 2-D techniques to result from this work are correlation spectroscopy (COSY), nuclear Overhauser effect spectroscopy (NOESY), and exchange spectroscopy (EXSY). COSY determines all first-order couplings in a single experiment; NOESY provides a diagram of the Overhauser interactions; and EXSY provides a diagram of those sites undergoing chemical exchange (Lambert et al. 1987). More recent advancements in 2-D NMR include heteronuclear correlated spectroscopy methods. Heteronuclear single quantum coherence (HSQC) and heteronuclear multiple quantum coherence (HMQC) experiments produce 2-D spectra in which the ^{1}H and ^{13}C (or ^{15}N, and so on) spectra represent the axes (that is, a ^{1}H – heteronuclei COSY). Correlation between protons and their attached heteronuclei is obtained. Heteronuclear multiple-bond correlation spectroscopy (HMBC) diagrams the long-range coupling of ^{1}H and heteronuclei signals through two or more bonds.

Finally, proton NMR has been successfully coupled to LC to provide a very powerful tool for separating and characterizing compounds. A comprehensive review of this

technique as it applies to polyphenol analysis has recently been published (Wolfender et al. 2003), and multihyphenated techniques have also been reviewed (Wilson 2000).

There are numerous reports on one-dimensional FT-NMR. It has been used at 200 MHz to study a malvidin 3,5-diglucoside/catechin condensation product (Bishop and Nagel 1984); at 300 MHz to elucidate the structure of grape reaction product, 2-*S*-glutathionylcaftaric acid (Cheynier et al. 1986); at 360 MHz to characterize the flavonols in Cinsault skins (Cheynier and Rigaud 1986); also at 360 MHz to study the nonenzymic autoxidation products of caffeic acid (Cilliers and Singleton 1991); at 400 MHz to characterize grape seed procyanidin dimers and trimers (Ricardo da Silva et al. 1991); and ^{13}C NMR spectroscopy was used to determine procyanidin structural unit composition for a variety of plant materials, including Gamay Beaujolais fruit (Czochanska et al. 1979, 1980).

Although Cui and colleagues first applied 2-D techniques to procyanidins (Cui et al. 1991), it was the work of Balas and Vercauteren (1994) that unambiguously determined the correct interflavan linkages for the catechin-(4a-8)-catechin and catechin-(4a-6)-catechin dimers. One-dimensional (1-D) and 2-D NMR (COSY, HMQC, and HMBC) were used to propose a structure for vitisin A (Bakker et al 1997). Fulcrand and colleagues also used 1-D and 2-D techniques to characterize this reaction product of pyruvic acid and anthocyanins; they used 2-D heteronuclear techniques (HSQC and HMBC) in addition to 1-D NMR and 1-D n.O.e. to propose an alternative structure for vitisin A (Fulcrand et al. 1998). A malvidin 3-glucoside dimer bridged via acetaldehyde that formed in winelike model solution was characterized with 1-D and 2-D techniques (COSY, NOESY, HSQC, and HMBC) (Atanasova et al. 2002). Mateus and colleagues used 1-D and 2-D NMR to identify three new pigments from port wines (Mateus et al. 2002); they ran COSY, HSQC, and HMBC experiments to characterize malvidin 3-glucososide-vinyl-(+)-catechin-(+)-catechin, malvidin 3-glucoside-vinyl-(+)-catechin, and malvidin 3-glucoside-vinyl-(-)-epicatechin. Recently, the structures of malvidin 3-glucoside/catechin pigments bridged via a variety of aldehydes were elucidated with COSY, HSQC, HMBC, and NOESY (Pissarra et al. 2004).

The electron analog to proton NMR, electron paramagnetic resonance (EPR), discovered in 1944 (Zavoisky 1945), has also been applied to phenolics analysis. The technique has been used to monitor the oxidative changes in seed polyphenols during development (Kennedy et al. 2000), to study the radical chemistry of polyphenolics (Bors et al. 2000, Hagerman et al. 2003), and to study oxygen-induced phenolic radical formation in wine (Laurie et al. 2004).

Infrared spectroscopy. With the development of fast Fourier transform (FT) algorithms and advances in multivariate calibration techniques, most notably partial least squares regression analysis (Martens and Næs 1992), the ability to perform high-speed IR analyses became feasible. The renaissance of near infrared (NIR) since Karl Norris' groundbreaking research has been reviewed (McClure 1994). Current NIR technology, whether FT or dispersive, allows for rapid, nondestructive, multicomponent analysis of samples with minimal or no sample preparation (Herrera et al. 2003; Versari et al. 2004). Even given the power of multivariate statistics to simplify complex spectra, wine analyses present unique difficulties. High concentrations of water, ethanol, and occasionally sugars are particularly problematic, as their absorbance masks signals from other constituents (Patz et al. 1999).

Patz and colleagues attempted a multivariate calibration of FTIR versus the Folin assay to measure wine phenolics (Patz et al. 1999). However, the accuracy and repeatability of the derived model were unacceptable, which the authors speculated was due to the nature of the reference method and/or the multiplicity of wine types examined. More likely the 83 wines used did not sufficiently span product variability, so that a stable model could not be derived. The success of these analyses is highly dependent upon the training set (Pasquini 2003). A later study with a larger sample set resulted in a predictive model of total phenolics with $R^2 = 0.96$ (Patz et al. 2004). Edelmann and colleagues used MIR spectroscopy of phenolic wine extracts to build a discriminant model which could classify wines based upon cultivar (Edelmann et al. 2001) and later reported the binding of tannins with proline-rich proteins with FTIR (Edelmann and Lendl 2002).

NIR reflectance spectroscopy was used to develop predictive models for color (red wine only) and phenolics (red, rosé, and white wines); predictive equations were $R^2 = 0.82$ and 0.97, respectively, with standard errors close to the reference methods (spectrophotometric absorbance and Folin) (Urbano-Cuadrado et al. 2004). NIR was also used to develop predictive models of malvidin 3-glucoside, polymeric pigment, and tannins in red wine fermentations, with $R^2 = 0.83$ for tannin and $R^2 = 0.91$ for malvidin; the reference method was analysis by HPLC (Cozzolino et al. 2004). MIR spectroscopy was used to develop models for total color, total anthocyanins, copigmented color, and polymeric pigment; R^2 ranged from 0.89 to 0.97 (Versari et al. 2004). Both NIR and FT-MIR data were used to develop predictive models for total polyphenols; for the NIR, MIR, and mixed model (NIR/MIR), $R^2 = 0.92$, 0.89, and 0.89, respectively (Urbano-Cuadrado et al. 2005).

Conclusion

It is important to revisit the quotation in the abstract of this article (Brown 1964); the fact that the techniques described here have been applied so successfully, and have resulted in such an extensive literature, indicates that these are, in a sense, yesterday's methods. That is not to suggest that there is no longer a place for these techniques: paper chromatography can still play an important role in a research laboratory, as can LC-ESI-MS-MS. The discovery of new analytical techniques is a continuous process, however, and the industry should both antici-

pate and welcome the application of these techniques to enological and viticultural problems.

For each technique described, there were techniques that were left out, either because of the sparseness of enological literature or the insufficiency of space. These tools available to enologists will undoubtedly find application in the future, and include advanced hyphenated techniques, such as HPLC-NMR-MS, SEC-NMR-IR (Wilson 2000) and CE-MS (Schmitt-Kopplin and Frommberger 2003); new variations on old techniques, such as ESI-FT-ICR (Cooper and Marshall 2001) and SNIF-NMR, IRMS (Ogrine et al. 2003); new technologies, such as monolithic LC columns (Castellari et al. 2002); and wholly new developments (Borman 2005, Stevenson 2005).

Over 50 years ago Bate-Smith (1948) first separated anthocyanins on sheets of paper. Today the advanced analytical techniques available to researchers are being applied to a variety of questions, whether qualitative (the phenolic composition of Champagnes; Chamkha et al. 2003), applied (the evolution of phenolics during aging; Pérez-Magariño and González-San José 2004), or basic (the chemistry of red wine color; Brouillard et al. 2003). These advances are not an excuse for complacency, however. The conclusions of Singleton and Esau in the late 1960s remain true today: "Much remains to be done in determining the effects of various agronomic and processing variables on both the specific classes and individual phenols. Complex as the potential phenol reactions in grapes and wines are . . . probable dividends of such study would be better color, longer shelf life, quicker aging, and optimum quality" (Singleton and Esau 1969).

Literature Cited

Abian, J. 1999. The coupling of gas and liquid chromatography with mass spectrometry. J. Mass Spectrom. 34:157-168.

Abraham, R.J. 1964. Nuclear magnetic resonance spectroscopy. *In* Methods in Polyphenol Chemistry. J.B. Pridham (Ed.), pp. 49-57. Macmillan, New York.

Andersen, O.M. 1985. Chromatographic separation of anthocyanins in cowberry (lingonberry) *Vaccinium vites-idaea* L. J. Food Sci. 50:1230-1232.

Andersen, O.M. 1987. Anthocyanins in fruits of *Vaccinium uliginosum* L. (bog whortleberry). J. Food Sci. 52:665-666.

Anderson, D.W., D.E. Gueffroy, A.D. Webb, and R.E. Kepner. 1970. Identification of acetic acid as an acylating agent of anthocyanin pigments in grapes. Phytochemistry 9:1579-1583.

Anet, F.A.L., and A.J.R. Bourn. 1965. Nuclear magnetic resonance spectral assignments from nuclear Overhauser effects. J. Am. Chem. Soc. 87:5250-5251.

Arnold, J.T., S.S. Dharmatti, and M.E. Packard. 1951. Chemical effects on nuclear induction signals from organic compounds. J. Chem. Phys. 19:507.

Aston, F.W. 1919. The constitution of the elements. Nature 104: 393.

Atanasova, V., H. Fulcrand, C. Le Guernevé, V. Cheynier, and M. Moutounet. 2002. Structure of a new dimeric acetaldehyde malvidin 3-glucoside condensation product. Tetrahedron Lett. 43:6151-6153.

Aue, W.P., E. Bartholdi, and R.R. Ernst. 1976. Two-dimensional spectroscopy. Application to nuclear magnetic resonance. J. Chem. Phys. 64:2229-2246.

Bae, Y.S., L.Y. Foo, and J.J. Karchesy. 1994. GPC of natural procyanidin oligomers and polymers. Holzforschung 48:4-6.

Bakker, J., and C.F. Timberlake. 1985a. The distribution of anthocyanins in grape skin extracts of port wine cultivars as determined by high performance liquid chromatography. J. Sci. Food Agric. 36:1315-1324.

Bakker, J., and C.F. Timberlake. 1985b. The distribution and content of anthocyanins in young port wines as determined by high performance liquid chromatography. J. Sci. Food Agric. 36:1325-1333.

Bakker, J., and C.F. Timberlake. 1997. Isolation, identification, and characterization of new color-stable anthocyanins occurring in some red wines. J. Agric. Food Chem. 45:35-43.

Bakker, J., A. Picinelli, and P. Bridle. 1993. Model wine solutions: Color and composition changes during aging. Vitis 32:111-118.

Bakker, J., P. Bridle, T. Honda, H. Kuwano, N. Saito, N. Terahara, and C.F. Timberlake. 1997. Identification of an anthocyanin occurring in some red wines. Phytochemistry 44:1375-1382.

Balas, L., and J. Vercauteren. 1994. Extensive high-resolution reverse 2D NMR analysis for the structural elucidation of procyanidin oligomers. Magn. Reson. Chem. 32:386-393.

Baldi, A., A. Romani, N. Mulinacci, F.F. Vincieri, and B. Casetta. 1995. HPLC/MS application to anthocyanins of *Vitis vinifera* L. J. Agric. Food Chem. 43:2104-2109.

Baranowski, E.S., and C.W. Nagel. 1983. Kinetics of malvidin-3-glucoside condensation in wine model systems. J. Food Sci. 48:419-429.

Barber, M., R.S. Bordoli, R.D. Segwick, and A.N. Tyler. 1981. Fast atom bombardment of solids (F.A.B.): A new ion source for mass spectrometry. J. Chem. Soc., Chem. Commun. 325-327.

Barofsky, D.F. 1988. FAB-MS applications in the elucidation of proanthocyanidin structures. *In* Chemistry and Significance of Condensed Tannins. R.W Hemingway and J.J. Karchesy (Eds.), pp. 175-195. Plenum Press, New York.

Bate-Smith, E.C. 1948. Paper chromatography of anthocyanins and related substances in petal extracts. Nature 161:835-836.

Bate-Smith, E.C. 1964. Paper chromatography of phenolics. *In* Methods in Polyphenol Chemistry. J.B. Pridham (Ed.), pp. 73-79. Macmillan, New York.

Benninghoven, A., and W.K. Sichtermann. 1978. Detection, identification and structural investigation of biologically important compounds by secondary ion mass spectrometry. Anal. Chem. 50:1180-1184.

Bishop, P.D., and C.W. Nagel. 1984. Characterization of the condensation product of malvidin 3,5-diglucoside and catechin. J. Agric. Food Chem. 32:1022-1026.

Bloch, F., W.W. Hansen, and M. Packard. 1946. Nuclear induction. Phys. Rev. 69:127.

Borman, S. 2005. Pittcon 2005 takes Orlando by storm. Chem. Eng. News 83:39-67.

Bors, W., C. Michel, and K. Stettmaier. 2000. Electron paramagnetic resonance studies of radical species of proanthocyanidins and gallate esters. Arch. Biochem. Biophys. 374:347-355.

Brouillard, R., S. Chassaing, and A. Fougerousse. 2003. Why are grape/fresh wine anthocyanins so simple and why is it that red wine color lasts so long? Phytochemistry 64:1179-1186.

Brown, B.R. 1964. General introduction. *In* Methods in Polyphenol Chemistry. J.B. Pridham (Ed.), pp. 1-11. Macmillan, New York.

Bruins, A.P. 1998. Mechanistic aspects of electrospray ionization. J. Chromatogr., A 794:345-357.

Carelli, V., A.M. Liquori, and A. Mele. 1955. Sorption chromatography of polar substances on polyamides. Nature 176:70-71.

Castellari, M., E. Sartini, A. Fabiani, G. Arfelli, and A. Amati. 2002. Analysis of wine phenolics by high-performance liquid chromatography using a monolithic type column. J. Chromatogr., A 973:221-227.

Chamkha, M., B. Cathala, V. Cheynier, and R. Douillard. 2003. Phenolic composition of Champagnes from Chardonnay and Pinot noir vintages. J. Agric. Food Chem. 51:3179-3184.

Chandler, B.V., and T. Swain. 1959. Separation of anthocyanins from plant extracts. Nature 183:989.

Cheynier, V., and J. Rigaud. 1986. HPLC separation and characterization of flavonols in the skins of *Vitis vinifera* var. Cinsault. Am. J. Enol. Vitic. 37:248-252.

Cheynier, V., and H. Fulcrand. 2003. Analysis of polymeric proanthocyanidins and complex polypheonls. *In* Methods in Polyphenol Analysis. C. Santos-Buelga and G. Williamson (Eds.), pp. 284-313. Royal Society of Chemistry, Cambridge.

Cheynier, V.F., E.K. Trousdale, V.L. Singleton, M.J. Salgues, and R. Wylde. 1986. Characterization of 2-*S*-glutathionylcaftaric acid and its hydrolysis in relation to grape wines. J. Agric. Food Chem. 34:217-221.

Cheynier, V., T. Doco, H. Fulcrand, S. Guyot, E. Le Roux, J.M. Souquet, J. Rigaud, and M. Moutounet. 1997. ESI-MS analysis of polyphenolic oligomers and polymers. Analusis 25:32-37.

Cilliers, J.J.L., and V.L. Singleton. 1989. Nonenzymic autoxidative phenolic browning reactions in a caffeic acid model system. J. Agric. Food Chem. 37:890-896.

Cilliers, J.J.L., and V.L. Singleton. 1990. Nonenzymic autoxidative reactions of caffeic acid in wine. Am. J. Enol. Vitic. 41:84-86.

Cilliers, J.J.L., and V.L. Singleon. 1991. Characterization of the products of nonenzymic autoxidative phenolic reactions in a caffeic acid model system. J. Agric. Food Chem. 39:1298-1303.

Cleaves, K.S., and M.S. Lesney. 2004. Capitalizing on chromatography. *In* Chronicles of Chemistry II: Enterprise of the Chemical Sciences. J.F. Ryan (Ed.), pp. 75-82. Am. Chemical Society, Washington, DC.

Consden, R., A.H. Gordon, and A.J.P. Martin. 1944. Qualitative analysis of proteins: A partition chromatographic method using paper. Biochem. J. 38:224-232.

Cooper, H.J., and A.G. Marshall. 2001. Electrospray ionization Fourier transform mass spectrometric analysis of wine. J. Agric. Food Chem. 49:5710-5718.

Cozzolino, D., M.J. Kwiatkowski, M. Parker, W.U. Cynkar, R.G. Dambergs, M.Gishen, and M.J. Herderich. 2004. Prediction of phenolic compounds in red wine fermentation by visible and near infrared spectroscopy. Anal. Chim. Acta 513:73-80.

Cui, C.B., Y. Tezuka, T. Kikuchi, H. Nakano, T. Tamoaki and J.H. Park. 1991. Davallin, a new tetrameric proanthocyanidin from the rhizomes of *Davallia mariesii* Moore. Chem. Pharm. Bull. 39:2179-2181.

Cuyckens, F., and M. Claeys. 2004. Mass spectrometry in the structural analysis of flavonoids. J. Mass Spectrom. 39:1-15.

Czochanska, Z., L.Y. Foo, R.H. Newman, L.J. Porter, W.A. Thomas, and W.T. Jones. 1979. Direct proof of a homogeneous polyflavan-3-ol structure for polymeric proanthocyanidins. J. Chem. Soc., Chem. Comm. 375-377.

Czochanska, Z., L.Y. Foo, R.H. Newman, and L.J. Porter. 1980. Polymeric proanthocyanidins. Sterochemistry, structural units, and molecular weight. J. Chem. Soc., Perkin Trans. 1:2278-2286.

Dadic, M., and G. Belleau. 1976. Analytical methods for polyphenols in brewing. V. Chromatography and spectroscopy of tanninogens in malt and beer. J. Am. Assoc. Brew. Chem. 34:158-165.

de Pascual-Teresa, S., C. Santos-Buelga, and J.C. Rivas-Gonzalo. 2000. Quantitative analysis of flavan-3-ols in Spanish foodstuffs and beverages. J. Agric. Food Chem. 48:5331-5337.

Dülcks, T., and R. Juraschek. 1999. Electrospray as an ionization method for mass spectrometry. J. Aerosol Sci. 30:927-943.

Edelmann, A., and B. Lendl. 2002. Toward the optical tongue: Flow-through sensing of tannin-protein interactions based on FTIR spectroscopy. J. Am. Chem. Soc. 124:14741-14747.

Edelmann, A., J. Diewok, K.C. Schuster, and B. Lendl. 2001. Rapid method for the discrimination of red wine cultivars based on mid-infrared spectroscopy of phenolic wine extracts. J. Agric. Food Chem. 49:1139-1145.

Ernst, R.R., and W.A. Anderson. 1966. Application of Fourier transform spectroscopy to magnetic resonance. Rev. Sci. Instrum. 37:93-102.

Ettre, L.S. 2005. Csaba Horváth and the development of the first modern high performance liquid chromatograph. LC/GC N. Am. 23:486-495.

Ettre, L.S., and C. Horváth. 1975. Foundations of liquid chromatography. Anal. Chem. 47:422A-446A.

Ferreira, D., and E.V. Brandt. 1988. New NMR experiments applicable to structure and conformation analysis. *In* Chemistry and Significance of Condensed Tannins. R.W Hemingway and J.J. Karchesy (Eds.), pp. 153-173. Plenum Press, New York.

Filmore, D. 2004. Seeing with spectroscopy. *In* Chronicles of Chemistry II: Enterprise of the Chemical Sciences. J.F. Ryan (Ed.), pp. 87-91. Am. Chemical Society, Washington, DC.

Flamini, R. 2003. Mass spectrometry in grape and wine chemistry. Part I. Polyphenols. Mass Spectrom. Rev. 22:218-250.

Fulcrand, H., T. Doco, N. Es-Safi, V. Cheynier, and M. Moutounet. 1996. Study of the acetaldehyde induced polymerization of flavan-3-ols by liquid chromatography-ion spray mass spectrometry. J. Chromotogr., A 752:85-91.

Fulcrand, H. C. Benabdeljalil, J. Rigaud, V. Cheynier, and M. Moutounet. 1998. A new class of wine pigments generated by reaction between pyruvic acid and grape anthocyanins. Phytochemistry 47:1401-1407.

Gueffroy, D.E., R.E. Kepner, and A.D. Webb. 1971. Acylated anthocyanin pigments in *Vitis vinifera* grapes: Identification of malvidin-3-(6-*p*-coumaroyl) glucoside. Phytochemistry 10:813-819.

Hagerman, A.E., R.T. Dean, and M.J. Davies. 2003. Radical chemistry of epigallocatechin gallate and its relevance to protein damage. Arch. Biophys. Biochem. 414:115-120.

Halász, I., and C.S. Horváth. 1964. Porous layer glass bead column packing in gas adsorption layer chromatography. Anal. Chem. 36:2226-2229.

Harbertson, J.F., and S. Spayd. 2006. Measuring phenolics in the winery. Am. J. Enol. Vitic. 57:280-288.

Hebrero, E., C. Santos-Buelga, and J.C. Rivas-Gonzalo. 1988. High performance liquid chromatography-diode array spectroscopy identification of anthocyanins of *Vitis vinifera* variety Tempranillo. Am. J. Enol. Vitic. 39:227-233.

Hebrero, E., C. Carcia-Rodriguez, C. Santos-Buelga, and J.C. Rivas-Gonzalo. 1989. Analysis of anthocyanins by high performance liquid chromatography-diode array spectroscopy in a hybrid grape variety (*Vitis vinifera* × *Vitis berlandieri* 41B). Am. J. Enol. Vitic. 40:283-291.

Herrera, J., A. Guesalaga, and E. Agosin. 2003. Shortwave-near infrared spectroscopy for non-destructive determination of maturity of wine grapes. Meas. Sci. Technol. 14:689-697.

Hong, V., and R.E. Wrolstad. 1990a. Characterization of anthocyanin-containing colorants and fruit juices by HPLC/photodiode array detection. J. Agric. Food Chem. 38:698-708.

Hong, V., and R.E. Wrolstad. 1990b. Use of HPLC separation/photodiode array detection for characterization of anthocyanins. J. Agric. Food Chem. 38:708-715.

Horváth, C.G., and S.R. Lipsky. 1966. Use of liquid ion exchange chromatography for the separation of organic compounds. Nature 211:748-749.

James, A.T., and A.J.P. Martin. 1952. Gas-liquid partition chromatography: the separation and microestimation of volatile fatty acids from formic acid to dodecanoic acid. Biochem. J. 50:679-690.

Karas, M., D. Bachmann, U. Bahr, and F. Hillenkamp. 1987. Matrix-assisted ultraviolet laser desorption of non-volatile compounds. Int. J. Mass Spectrom. Ion Processes 78:53-68.

Karchesy, J.J., R.W. Hemingway, Y.L. Foo, E. Barofsky, and D.F. Barofsky. 1986. Sequencing procyanidin oligomers by fast atom bombardment mass spectrometry. Anal. Chem. 58:2563-2567.

Karchesy, J.J. 1988. Analytical methods: An overview. *In* Chemistry and Significance of Condensed Tannins. R.W Hemingway and J.J. Karchesy (Eds.), pp. 197-202. Plenum Press, New York.

Kebarle P., and L. Tang. 1993. From ions in solution to ions in the gas phase. The mechanism of electrospray mass spectrometry. Anal. Chem. 65:972A-986A.

Kennedy, J.A., and G.P. Jones. 2001. Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. J. Agric. Food Chem. 49:1740-1746.

Kennedy, J.A., and A.W. Taylor. 2003. Analysis of proanthocyanidins by high-performance gel permeation chromatography. J. Chromatogr., A 995:99-107.

Kennedy, J.A., G.J. Troup, J.R. Pilbrow, D.R. Hutton, D.Hewitt, C.R. Hunter, R. Ristic, P.G. Iland, and G.P. Jones. 2000. Development of seed polyphenols in berries from *Vitis vinifera* L. cv. Shiraz. Aust. J. Grape Wine Res. 6:224-254.

Kennedy, J.A., Y. Haysaka, S. Vidal, E.J. Waters, and G.P. Jones. 2001. Composition of grape skin proanthocyanidins at different stages of berry development. J. Agric. Food Chem. 49:5348-5355.

Krueger, C.G., N.C. Dopke, P.M. Treichel, J. Folts, and J.D. Reed. 2000. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of polygalloyl polyflavan-3-ols in grape seed extract. J. Agric. Food Chem. 48:1663-1667.

Lambert, J.B., H.F. Shurvell, D.A. Lightner, and R.G. Cooks. 1987. Introduction to Organic Spectroscopy. Macmillan, New York.

Laurie, F., I. Reeve, J. Voss, and A.L. Waterhouse. 2004. Oxygen induces free radical formation in red wine. Abstract. Am. J. Enol. Vitic. 55:313A-314A.

Lazarus, S.A., M.A. Kelm, G.A. Wächter, J.F.Hammerstone, and H.H. Schmitz. 2003. Analysis and purification of proanthocyanidin oligomers. *In* Methods in Polyphenol Analysis. C. Santos-Buelga and G. Williamson (Eds.), pp. 267-283. Royal Society of Chemistry, Cambridge.

Lea, A.G.H. 1979. High performance liquid chromatography of cider procyanidins. J. Sci. Food Agric. 30:833-838.

Lea, A.G.H. 1980. Reversed-phase gradient high-performance liquid chromatography of procyanidins and their oxidation products in ciders and wines, optimized by Snyder's procedures. J. Chromatogr. 194:62-68.

Lee, C.Y., and A.W. Jaworski. 1990. Identification of some phenolics in white grapes. Am. J. Enol. Vitic. 41:87-89.

Lesney, M.S. 2004. Spectacular spectrometry. *In* Chronicles of Chemistry II: Enterprise of the Chemical Sciences. J.F. Ryan (Ed.), pp. 93-97. Am. Chemical Society, Washington, DC.

Mano, N., and J. Goto. 2003. Biomedical and biological mass spectrometry. Anal. Sci. 19:3-14.

Martens, H., and T. Næs. 1992. Multivariate Calibration. Wiley & Sons, New York.

Martin, A.J.P., and R.L.M. Synge. 1941. A new form of chromatogram employing two liquid phases. Biochem. J. 35:1358-1368.

Mateus, N., A.M.S. Silva, C. Santos-Buelga, J.C. Rivas-Gonzalo, and V. de Freitas. 2002. Identification of anthocyanin-flavanol pigments in red wines by NMR and mass spectrometry. J. Agric. Food Chem. 50:2110-2116.

Matsuo, T., and Y. Seyama. 2000. Introduction to modern biological mass spectrometry. J. Mass Spectrom. 35:114-130.

McCloskey, L.P., and L.S. Yengoyan. 1981. Analysis of anthocyanins in *Vitis vinifera* wines and red color versus aging by HPLC and spectrophotometry. Am. J. Enol. Vitic. 32:257-261.

McClure, W.F. 1994. Near-infrared spectroscopy: The giant is running strong. Anal. Chem. 66:43A-53A.

Ogrine, N., I.J. Košir, J.E. Spangenberg, and J. Kidric. 2003. The application of NMR and MS methods for detection of adulteration of wine, fruit juices, and olive oil. A review. Anal. Bioanal. Chem. 376:424-430.

Nagel, C.W., and L.W. Wulf. 1979. Changes in anthocyanins, flavonoids and hydroxycinnamic acid esters during fermentation and aging of Merlot and Cabernet Sauvignon. Am. J. Enol. Vitic. 30:111-116.

Nagra, D.S., and L. Li. 1995. Liquid chromatography-time-of-flight mass spectrometry with continuous-flow matrix-assisted laser desorption ionization. J. Chromatogr., A 711:235-245.

Overhauser, A.W. 1953a. Paramagnetic relaxation in metals. Phys. Rev. 89:689-700.

Overhauser, A.W. 1953b. Polarization of nuclei in metals. Phys. Rev. 92:411-415.

Pasquini, C. 2003. Near infrared spectroscopy: fundamentals, practical aspects and analytical applications. J. Braz. Chem. 14:198-219.

Patz, C.D., A. David, K. Thente, P. Kürbel, and H. Dietrich. 1999. Wine analysis with FTIR spectrometry. Vitic. Enol. Sci. 54:80-87.

Patz, C.D., A. Blieke, R. Ristow, and H. Dietrich. 2004. Application of FT-MIR spectrometry in wine analysis. Anal. Chim. Acta 513:81-89.

Paul, H.W. 1966. Science, Vine and Wine in Modern France. University Press, Cambridge.

Pavia, D.L., G.M. Lampman, and G.S.Kriz Jr. 1979. Introduction to Spectroscopy. Saunders, Philadelphia.

Pérez-Magariño, S., and M.L. González-San José. 2004. Evolution of flavanols, anthocyanins, and their derivatives during the aging of red wines elaborated from grapes harvested at different stages of ripening. J. Agric. Food Chem. 52:1181-1189.

Perret C., R. Pezet, and R. Tabacchi. 2003. Fractionation of grape tannins and analysis by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. Phytochem. Anal. 14:202-208.

Peynaud, E. 1984. Knowing and Making Wine. Wiley & Sons, New York.

Pissarra, J., S. Lourenço, A.M. Gonzáles-Paramás, N. Mateus, C. Santos Buelga, A.M.S. Silva, and V. de Freitas. 2004. Structural characterization of new malvidin 3-glucoside-catechin aryl/alkyl-linked pigments. J. Agric. Food Chem. 52:5519-5526.

Porath, J., and P. Flodin. 1959. Gel filtration: a method for desalting and group separation. Nature 183:1657-59.

Prieur, C., J. Rigaud, V. Cheynier, and M. Moutounet. 1994. Oligomeric and polymeric procyanidins from grape seeds. Phytochemistry 36:781-784.

Purcell, E.M., H.C. Torrey, and R.V. Pound. 1946. Resonance absorption by nuclear magnetic moments in a solid. Phys. Rev. 69:37-38.

Ribéreau-Gayon, P. 1972. Plant Phenolics. Hafner Publishing, New York.

Ricardo da Silva, J.M., J. Rigaud, V. Cheynier, A. Cheminat, and M. Moutounet. 1991. Procyanidin dimers and trimers from grape seeds. Phytochemistry 30:1259-1264.

Rigaud, J., J. Perez-Ilzarbe, J.M. Ricardo da Silva, and V. Cheynier. 1991. Micro method for the identification of proanthocyanidin using thiolysis monitored by high-performance liquid chromatography. J. Chromatogr. 540:401-405.

Rigaud, J., M.T. Escribano-Bailon, C. Prieur, J.M. Souquet and V. Cheynier. 1993. Normal-phase high-performance liquid chromatographic separation of procyanidins from cacao beans and grape seeds. J. Chrom. A 654:255-260.

Roggero, J.P., S. Coen, and B. Ragonnet. 1986. High performance liquid chromatography survey on changes in pigment content in ripening grapes of Syrah. An approach to anthocyanin metabolism. Am. J. Enol. Vitic. 37:77-83.

Salagoïty-Auguste, M.H., and A. Bertrand. 1984. Wine phenolics–analysis of low molecular weight components by high performance liquid chromatography. J. Sci. Food Agric. 35:1241-1247.

Santos-Buelga, C., C. García-Vugurea, and F.A. Tomás-Barberán. 2003. On-line identification of flavonoids by HPLC coupled to diode array detection. *In* Methods in Polyphenol Analysis. C. Santos-Buelga and G. Williamson (Eds.), pp. 92-127. Royal Society of Chemistry, Cambridge.

Schmitt-Kopplin, P., and M. Frommberger. Capillary electrophoresis-mass spectrometry: 15 years of developments and applications. Electrophoresis 24:3837-3867.

Singleton, V.L., and P. Esau. 1969. Phenolic Substances in Grapes and Wines and Their Significance. Academic Press, New York.

Singleton, V.L., D.E. Draper, and J.A. Rossi Jr. 1966. Paper chromatography of phenolic compounds from grapes, particularly seeds, and some variety-ripeness relationships. Am. J. Enol. Vitic. 17:206-217.

Singleton, V.L., J. Zaya, E. Trousdale, and M. Salgues. 1984. Caftaric acid in grapes and conversion to a reaction product during processing. Vitis 23:113-120.

Singleton, V.L., M. Salgues, J. Zaya, and E. Trousdale. 1985. Caftaric acid disappearance and conversion to products of enzymic oxidation in grape must and wine. Am. J. Enol. Vitic. 36:50-56.

Singleton, V.L., J. Zaya, and E.K.Trousdale. 1986. Caftaric and coutaric acids in fruit of *Vitis*. Phytochemistry 25:2127-2133.

Smyth, W.F. 1999. The use of electrospray mass spectrometry in the detection and determination of molecules of biological significance. Trends Anal. Chem. 18:335-346.

Solomon, I. 1955. Relaxation processes in a system of two spins. Phys. Rev. 99:559-565.

Somers, T.C. 1966. Wine tannins—isolation of condensed flavonoid pigments by gel filtration. Nature 209:368-370.

Somers, T.C. 1967. Resolution and analysis of total phenolic constituents of grape pigment. J. Sci. Food Agric. 18:193-196.

Somers, T.C., and E. Vérette. 1988. Phenolic composition of natural wine types. *In* Wine Analysis. H.F. Linskens and J.F. Jackson (Eds.), pp. 219-157. Springer-Verlag, New York.

Spaeth, E.C., and D.H. Rosenblatt. 1950. Partition chromatography of synthetic anthocyanidin mixtures. Anal. Chem. 22:1321-1326.

Stecher, G., C.W. Huck, M. Popp, and G.K. Bonn. 2001. Determination of flavonoids and stilbenes in red wine and related biological products by HPLC and HPLC-ESI-MS-MS. Fresenius J. Anal. Chem. 371:73-80.

Stevenson, R. 2005. New instruments for separation science at Pittcon '05. Am. Lab. 37:9-36.

Sugui, J.A., K.V. Wood, Z. Yang, C.C. Bonham, and R.L. Nicholson. 1999. Matrix-assisted laser desorption ionization mass spectrometry analysis of grape anthocyanins. Am. J. Enol. Vitic. 50:199-203.

Szilágyi, Z., G. Vas, G. Mády, and K. Vékey. 1996. Investigation of macromolecules in wines by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 10:1141-1143.

Tanaka, K., H. Waki, Y. Ido, S. Akita, Y. Yoshida, T. Yoshida, and T. Matsuo. 1988. Protein and polymer analyses up to *m/z* 100 000 by laser ionization time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 2:151-153.

Thompson, R.S., D. Jacques, E. Haslam, and R.J.N. Tanner. 1972. Plant proanthocyanidins. Part I. Introduction: The isolation, structure, and distribution in nature of plant procyanidins. J. Chem. Soc., Perkin Trans. 1:1387-1399.

Thomson, J.J. 1913. Rays of positive electricity. Proc. Roy. Soc. A. 89:1-20.

Tomás-Barberán, F.A., A. Gil-Izquierdo, F. Ferreres, and M.I. Gil. 2003. Analysis and purification of flavanones, chalcones and dihydrochalcones. *In* Methods in Polyphenol Analysis. C. Santos-Buelga and G. Williamson (Eds.), pp. 359-371. Royal Society of Chemistry, Cambridge.

Tswett, M.S. 1906a. Physical chemical studies on chlorophyll adsorptions. Ber. Deut. Botan. Ges. 24:316-323. *Translated and excerpted in* Source Book in Chemistry 1900-1950. 1968. H.M. Leicester (Ed.), pp. 23-28. Harvard University Press, Cambridge.

Tswett, M.S. 1906b. Adsorption analysis and chromatographic method. Application to the chemistry of chlorophyll. Ber. Deut. Botan. Ges. 24:385-393. *Translated and excerpted in* A Documentary History of Biochemistry, 1770-1940. 1992. M. Teich and D.M. Needham (Eds.), pp. 92-93. Leicester University Press, Leicester, UK.

Urbano-Cuadrado, M., M.D. Luque de Castro, P.M. Pérez-Juan, J. García-Olmo, and M.A. Gómez-Nieto. 2004. Near infrared reflectance spectroscopy and multivariate analysis in enology. Determination or screening of fifteen parameters in different types of wines. Anal. Chim. Acta 527:81-88.

Urbano-Cuadrado, M., M.D. Luque de Castro, P.M. Pérez-Juan, and M.A. Gómez-Nieto. 2005. Comparison and joint use of near-infrared spectroscopy and Fourier transform mid infrared spectroscopy for the determination of wine parameters. Talanta 66:218-224.

Versari, A., R. Boulton, and J. Thorngate III. 2004. The prediction of the color components of red wines using FTIR, wine analyses, and the method of partial least squares. *In* Red Wine Color: Revealing the Mysteries. A.L. Waterhouse and J.F. Kennedy (Eds.), pp. 53-67. Am. Chemical Society, Washington, DC.

Vidal, S., E. Meudec, V. Cheynier, G. Skouroumounis, and Y. Hayaska. 2004. Mass spectrometric evidence for the existence of oligomeric anthocyanins in grape skins. J. Agric. Food Chem. 52:7144-7151.

Vuataz, L., H. Brandenberger, and R.H. Egli. 1961. Plant phenols. I. Separation of the tea leaf polyphenols by cellulose column chromatography. J. Chromatogr. 2:173-187.

Wagner, H. 1964. Infrared spectroscopy of flavonoids. *In* Methods in Polyphenol Chemistry. J.B. Pridham (Ed.), pp. 37-48. Macmillan, New York.

Wang, J., and P. Sporns. 1999. Analysis of anthocyanins in red wine and fruit juice using MALDI-MS. J. Agric. Food Chem. 47:2009-2015.

Waterhouse, A.L., S. Ignelzi, and J.R. Shirley. 2000. A comparison of methods for quantifying oligomeric proanthocyanidins from grape seed extracts. Am. J. Enol. Vitic. 51:383-389.

Weiss, K.C., T. Yip, T.W. Hutchens, and L.F. Bisson. 1998. Rapid and sensitive fingerprinting of wine proteins by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Am. J. Enol. Vitic. 49:231-239.

Wilson, I.D. 2000. Multiple hyphenation of liquid chromatography with nuclear magnetic resonance spectroscopy, mass spectrometry and beyond. J. Chromatogr., A 892:315-327.

Wolfender, J.L., P. Waridel, K. Ndjoko, K.R. Hobby, H.J. Major, and K. Hostettmann. 2000. Evaluation of Q-TOF-MS/MS and multiple stage IT-MS^n for the dereplication of flavonoids and related compounds in crude plant extracts. Analusis 28:895-906.

Wolfender, J.L., K. Ndjoko, and K. Hostettmann. 2003. Application of LC-NMR in the structure elucidation of polyphenols. *In* Methods in Polyphenol Analysis. C. Santos-Buelga and G. Williamson (Eds.), pp. 128-156. Royal Society of Chemistry, Cambridge.

Wulf, L.W., and C.W. Nagel. 1976. Analysis of phenolic acids and flavonoids by high-pressure liquid chromatography. J. Chromatogr. 116:271-279.

Wulf, L.W., and C.W. Nagel. 1978. High-pressure liquid chromatographic separation of anthocyanins of *Vitis vinifera*. Am. J. Enol. Vitic. 29:42-49.

Yamashita, M., and J.B. Fenn. 1984a. Electrospray ion source. Another variation on the free-jet theme. J. Phys. Chem. 88:4451-4459.

Yamashita, M., and J.B. Fenn. 1984b. Negative ion production with the electrospray ion source. J. Phys. Chem. 88:4671-4675.

Yang, Y., and M. Chien. 2000. Characterization of grape procyanidins using high-performance liquid chromatography/mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J. Agric. Food Chem. 48:3990-3996.

Zavoisky, E.K. 1945. Paramagnetic relaxation of liquid solutions for perpendicular fields. J. Phys. USSR 45:211-217.

Zhang, B., C. McDonald, and L. Li. 2004. Combining liquid chromatography with MALDI mass spectrometry using a heated droplet interface. Anal. Chem. 76:992-1001.