Sequential Analysis of Malic Acid and Both Enantiomers of Lactic Acid in Wine Using a High-Performance Liquid Chromatographic Column-Switching Procedure

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Abstract

A liquid chromatographic column-switching method for the sequential determination of malic acid and both enantiomers of lactic acid in wine is described. The procedure involves the heart cutting of lactic acid enantiomers from a reversed-phase high-performance liquid chromatography chromatogram, retaining them, and back-flushing them through a chiral ligand-exchange column in which they are separated. The method is used to determine the concentration of lactic acid enantiomers in commercial wines. The results are in satisfactory agreement with those of other methods. The malic acid contents of various wines are also determined. The total analysis time for one experiment is approximately 10 min.

Introduction

(*R*)- and (*S*)-lactic acids, (*S*)-malic acid, and (*R*,*R*)-tartaric acid are some of the more widely studied aliphatic acid constituents of wine (1). Their relative concentrations in a particular wine depend upon the wine's microbiological history. For example, if the wine has experienced significant malolactic fermentation (MLF), the concentration of (*S*)-malic acid will be very low (e.g., < 100 mg/L) and that of (*S*)-lactic acid will be relatively high (e.g., > 2000 mg/L). This is because (*S*)-malic acid is converted to (*S*)-lactic acid during MLF. In non-MLF wines the situation is reversed. All wines contain (*R*,*R*)-tartaric acid and (*R*)-lactic acid, the latter generally being a minor constituent whose typical concentration is 100–300 mg/L (2).

There are many nonchiral high-performance liquid chromatographic (HPLC) methods described in the literature for the analysis of the aliphatic acid content of wines (3). A number of chiral HPLC methods are available for the analysis of aliphatic acid enantiomers, including a chiral ligand-exchange chromatographic (CLEC) method that determines the lactic acid enantiomer content of wine (4). However, there are no reports of the HPLC analysis of (*R*)- and (*S*)-lactic acids, malic acid, and tartaric acid in wine using a single injection.

Column-switching is the name for a family of techniques that are very useful in the analysis of particularly difficult samples. These methods employ at least two separations and involve the use of a switching valve, which can be manually or automatically operated. The separations can be performed on the same column or on different columns. A variety of tasks can be carried out using column-switching methods, including purification, clean-up ("stripping"), and "heart cutting". The latter involves selecting a particular portion of a chromatogram resulting from a separation on the first column, retaining it on a focusing column, and then rechromatographing that portion by back-flushing it through a second column. Heart cutting is a particularly useful column-switching procedure in the analysis of complex food samples, biological fluids, and pharmaceutical preparations.

Most column-switching methods involve two or more columns of different stationary phases. Recent examples include the analysis of flavonoid phenols in urine (5) and the use of a column-switching HPLC method for the measurement of hydrophobicity parameters of diarylpyrazines (6).

No references to the use of column-switching or heart-cutting chromatographic techniques in the analysis of acids in grape must or wine could be found in the literature. However, there is a method for the separation of complex mixtures of nonflavonoid polyphenols, which incidentally can be found in some oaked wines (7). Also of interest are two methods for the determination of substances in complex media: one for oxalic acid in water (8) and one for guanidine derivatives in plasma (9). Additionally, there is a ligand-exchange capillary electrophoresis method for the chiral separation of α-hydroxyacids and β-blockers (10).

This study describes an HPLC column-switching method that allows for the rapid determination of (*R*)- and (*S*)-lactic acids and other wine components (particularly malic acid and tartaric acid) using a single injection.

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Experimental

Materials

(*S*)-malic acid, lithium (*R*)-lactate, and sodium (*S*)-lactate were all from Sigma Aldrich (Gillingham, U.K.) and were used without further purification. (*R,R*)-tartaric acid of analytical-grade material was from Fisons (Loughborough, U.K.), and analytical-grade copper sulfate pentahydrate was from Sigma Aldrich. Sigma Aldrich 0.1N volumetric standard sulfuric acid was used to dilute the 0.5mM H_2SO_4 reversed-phase (RP) HPLC mobile phase. The commercial wines were either donations or purchased locally.

Chromatographic systems and HPLC operating conditions

The column-switching apparatus was composed of two separate liquid chromatographic systems—an RP system complete with a 20-µL-loop Rheodyne (Cocati, CA) injector unit and a CLEC system. A focusing column and a 2-position, 6-port Rheodyne switch valve connected the two chromatographic systems. PEEK (poly ether ether ketone) tubing and PEEK male finger-tight fittings were used as far as possible throughout the system.

The RP-HPLC system consisted of an LDC/Milton Roy (Stone, U.K.) Constametric III pump, a Jasco (Great Dunmow, U.K.) 875-UV detector set at 230 nm, and a Hewlett Packard (Palo Alto, CA) 3392A integrator. The mobile phase was 0.5mM H_2SO_4. The flow rate was between 0.75 and 2.00 mL/min, depending on the column dimensions (as will be discussed). The pump outlet was connected to a Rheodyne 7125 injector valve with a 20-µL sample loop. This in turn was connected to a Waters (Watford, U.K.) Guard Pak module containing a Resolve C_{18} insert. The outlet from this was connected to a Waters radial compression module containing a 100- × 8-mm-i.d. Resolve C_{18} cartridge. A Phenomenex (Macclesfield, U.K.) Aqua 75- × 4.6-mm-i.d. C_{18} column was also used on occasion. The outlet from the RP column was connected to the Rheodyne 3799 2-position, 6-port switch valve via port 1, and port 2 was connected to the Jasco detector.

The focusing column was a Waters Novapak 150- × 3.9-mm-i.d. C_{18} unit clamped next to the Rheodyne switch valve and connected to the valve via ports 3 and 6.

The CLEC system consisted of a Spectro-Physics (Altrincham, U.K.) SP8810 pump, a Shimadzu (Milton Keynes, U.K.) SPD-6A detector set at 254 nm, and a Spectro-Physics SP4290 integrator. The mobile phase was 2mM $CuSO_4$, and the flow rate was 1 mL/min. The pump outlet was connected to the Rheodyne switch valve at port 4. The remaining switch port (5) was con-

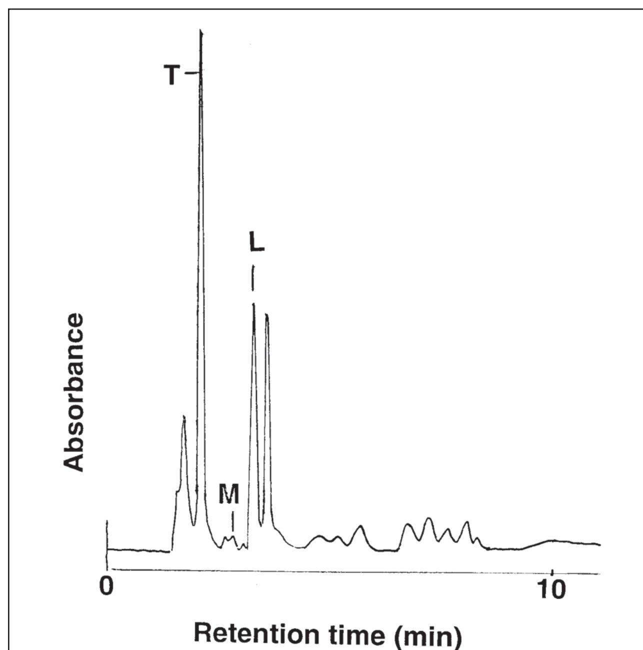

Figure 1. Radial compression RP chromatogram of a wine sample: (L) lactic acid, (M) malic acid, and (T) tartaric acid.

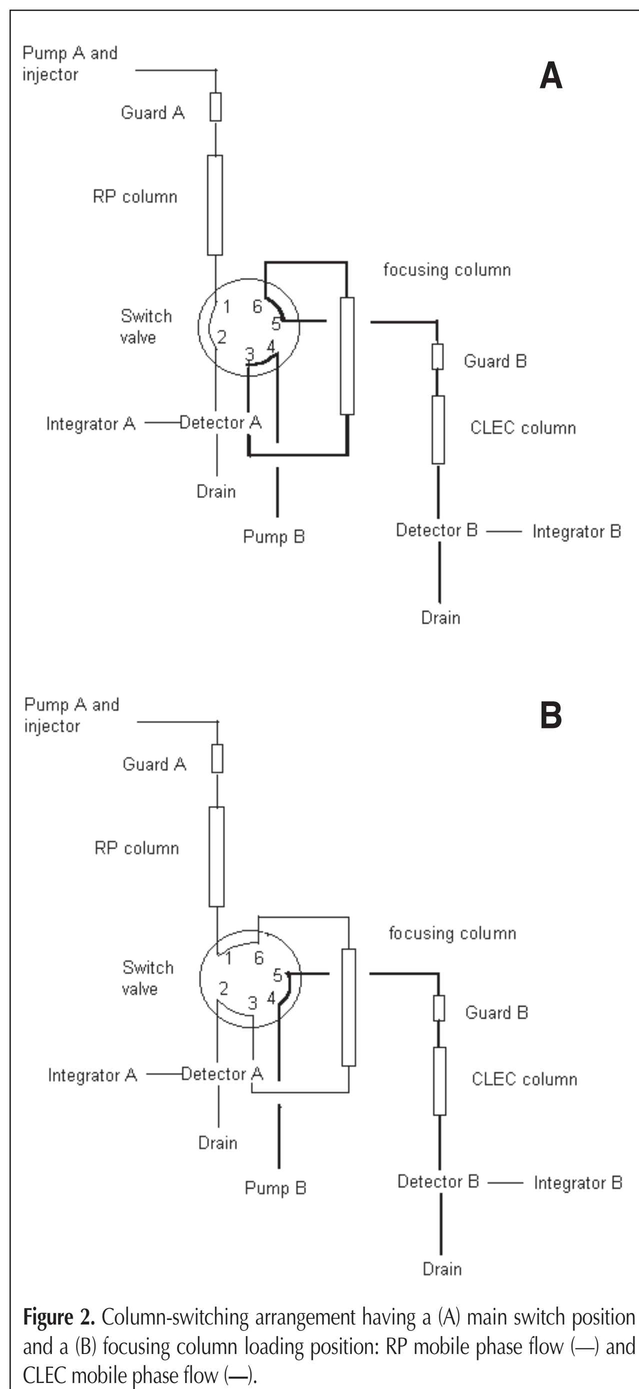

Figure 2. Column-switching arrangement having a (A) main switch position and a (B) focusing column loading position: RP mobile phase flow (—) and CLEC mobile phase flow (**—**).

nected to a Phenomenex Security Guard containing two C_{18} inserts. This in turn was connected to a 50- × 4.6-mm-i.d. Daicel Chiralpak MA(+) column. Finally, the column outlet was connected to the Shimadzu detector. The Rheodyne switch valve was always initially in the "main switch position" (see Figure 2A).

The sample was injected and its RP chromatogram was run with the switch valve in the "main" position. At a predetermined time, the CLEC pump was stopped and the column switch valve switched to the "focusing column loading position" (see Figure 2B) for a fixed time (usually 30 s). After this time, the valve was switched back to the main switch position and the CLEC pump was restarted. After another fixed time (usually 5 s), the CLEC integrator was started. This timing was necessary in order to give a reasonable consistency of retention times in the chiral ligand-exchange (CLE) chromatogram.

Standards dissolved in 0.5mM H_2SO_4 were injected as such, whereas all samples underwent accurate ten-fold dilution with 0.5mM H_2SO_4 prior to injection. All solutions were filtered through Phenomenex 0.2-µm PTFE syringe sample membrane filters before injection.

Results and Discussion

Column-switching arrangement

It was demonstrated in an earlier study (4) that a solid-phase extraction (SPE)–CLEC procedure is a good, rapid method for the quantitative analysis of (*R*)- and (*S*)-lactic acids in wine, even in the presence of (*S*)-malic acid. However, this latter acid was not satisfactorily analyzed under these conditions, and indeed it is a potential interference. Although this problem is easily overcome, it does lead to longer overall analysis times (4). It was thought worthwhile to devise a method that would allow for the measurement of all three acids (plus tartaric acid, if desired) in one experiment. In particular, it was decided to combine the RP-HPLC method (11) (which measures malic, tartaric, and lactic acids) with the CLEC method by heart-cutting the former and analyzing its (*R*)- and (*S*)-lactic acid composition in the latter. In doing so, it was possible to measure the malic acid content and the lactic acid enantiomer content without the former interfering with the analysis of the latter, because malic acid elutes before lactic acid in RP-HPLC conditions and can be "cut" from the CLE chromatogram (Figure 1).

The column-switching arrangement is shown in Figure 2 in which the left-hand side (pump A) represents the RP-HPLC setup and the right-hand side (pump B) is the CLEC system.

With the valve in the main switch position (Figure 2A), the sample was pushed by pump A onto the ODS RP column in which separation of the tartaric, malic, and lactic acids takes place and (via ports 1 and 2) the first two are subsequently detected by detector A set at 230 nm. At the same time, pump B pushed its mobile phase through port 4 and out of port 3 into the ODS focusing column, and from there it proceeded back to the valve via port 6 where it emerged from port 5 and went through the CLEC column and detector B set at 254 nm.

When the detector/integrator A indicated that the malic acid peak was almost finished, the valve was switched to the focusing column loading position (Figure 2B). Then, the (*R*)- and (*S*)-lactic acids in a plug of RP mobile phase were diverted to the focusing column via port 6. The RP mobile phase after it was mixed with some of the CLEC mobile phase after passing through the focusing column re-entered the valve at port 3 and left via port 2 to the detector. Meanwhile, pump B continued to push CLEC mobile phase through the CLEC column and detector B via valve ports 4 and 5.

After a predetermined time (usually 20–30 s), the valve was switched back to the main switch position. Then, pump B pushed the CLEC mobile phase via ports 3 and 4 and eluted the (*R*)- and (*S*)-lactic acids (still unresolved at this point) with their plug of the RP mobile phase from the focusing column via valve ports 6 and 5 onto the CLEC column in which they were resolved and detected by detector B. In the meantime, pump A pushed the RP mobile phase via ports 1 and 2 through the detector A, thus washing out the plug of the CLEC mobile phase from the previous valve switch position.

The RP mobile phase of 0.5mM H_2SO_4 (an approximate pH of 3.3) was chosen to minimize the pH change experienced by the CLEC column as the heart-cut plug of the RP solvent (carrying the lactic acid enantiomers) moved through it. The pH of the CLEC mobile phase (2mM $CuSO_4$) was approximately 4.3. Usually, the RP mobile phase would be either 0.2M phosphate buffer or 5mM H_2SO_4 (approximately pH 2.4) (11), but these were thought to be too aggressive for the delicate (and expensive) CLEC column. There was a slight compromise in resolution, but this did not appear to adversely influence the results.

Table I. Linearity and Reproducibility Data

	Correlation coefficient*	Coefficient of determination*	Standard deviation (mg/L)†,‡	Relative standard deviation (%)†	Mean recovery (%)§
(*R*)-lactic acid	0.998	0.996	4.0	3.8	94.0
(*S*)-lactic acid	0.998	0.996	3.8	3.6	94.4

* Determination from duplicate determinations of five standard solutions containing 25–500 mg/L of lithium (*R*)-lactate and sodium (*S*)-lactate. Limit of detection, defined as a peak height of twice the general baseline variation, is 5 mg/L.
† Determined from seven injections of a standard containing 104.5 mg/L each of lithium (*R*)-lactate and sodium (*S*)-lactate.
‡ $n = 7$.
§ Calculated from the separate additions of five standard solutions to a wine sample.

Validation

Because the RP side of the column-switching method is well-documented (11), the validation emphasis was placed upon the CLEC side of the procedure. Another reason for this emphasis was that the CLE chromatogram relied on the switch mechanism to accurately cut the appropriate section of the RP chromatogram and transfer that portion totally to the CLEC column via the focusing column.

The linearity and reproducibility data for both (*R*)- and (*S*)-lactic acid standards and (*R*)- and (*S*)-lactic acids in wine are given in Table I. The linearity parameters (r and r^2) were determined by

the method of least squares (12), but the calibration graphs were also judged by eye to show slight random scatter rather than a distinct curvature. The recoveries were determined by the separate accurate addition of each of the five sets of standards to a wine sample. It can be seen from Table I that all of the parameters were satisfactory.

Typical column-switch chromatograms of the standard mixture are illustrated in Figure 3A, which shows part of the RP chromatogram with the lactic acid enantiomers cut out along with the CLE chromatogram of D- and L-lactic acids cut out of the RP chromatogram. Similarly, Figure 3B shows column-switch chromatograms of a wine sample. The large positive absorption in the RP chromatogram corresponded with the situation when the switch valve was in the focusing column loading position (see Figure 2B) for 30 s. In this study, the CLEC mobile phase (which absorbs strongly at 230 nm) was passed through the RP system detector. At the same time, the lactic acid enantiomers were being loaded onto the retainer column with their plug of RP mobile phase. The rest of the RP chromatogram was with the switch valve in the normal position (see Figure 2A). The large negative absorption in the CLE chromatogram corresponded with the passage of the plug of 0.5mM H_2SO_4 that was cut out of the RP chromatogram along with the lactic acid enantiomers at the operation of the switch valve. For this short period of time (approximately 30 s), the CLEC mobile phase was depleted of much of its Cu^{2+} ions, hence the negative absorption at 254 nm.

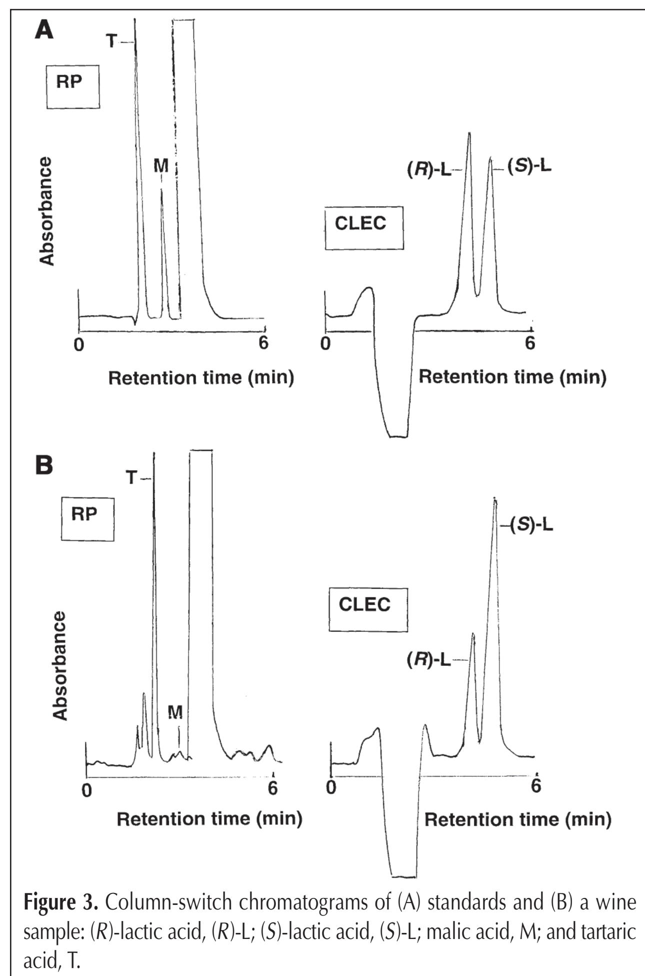

Figure 3. Column-switch chromatograms of (A) standards and (B) a wine sample: (*R*)-lactic acid, (*R*)-L; (*S*)-lactic acid, (*S*)-L; malic acid, M; and tartaric acid, T.

Determination of the (*R*)- and (*S*)-lactic acid content of selected commercial wines

A selection of commercial wines was investigated by the column-switching method. The results are displayed in Table II, where the symbol "±" defines the limits of the (*R*)- and (*S*)-lactic acid contents from mostly duplicate (but sometimes triplicate) determinations, as explained in the table. These particular samples were chosen from a much larger collection of data, because for these there is also data available from alternative methods, thus allowing a comparison. It can be seen from Table II that the data from this study's method were generally in reasonably good agreement with those from an SPE–CLEC method (4), an enzymatic method (13), and a headspace gas chromatographic (GC) method (14). Although a statistical analysis was not carried out, it can be seen that the column-switch data had no overall bias toward lower or higher values in comparison with most of the other data. There was generally a rather closer agreement

Table II. (*R*)- and (*S*)-Lactic Acid Content of Selected Commercial Wines and a Comparison of Results with Those of Other Methods

Wine	Mean lactic acid content by column-switching method (mg/L)*			Mean lactic acid content by SPE–CLEC method (mg/L)†			Mean lactic acid content by enzymatic method (mg/L)‡			Total mean lactic acid content by GC headspace method (mg/L)§
	(*R*)	(*S*)	Total	(*R*)	(*S*)	Total	(*R*)	(*S*)	Total	
Bardingley 1989 (English red)	264	2488	2732	253	2488	2741	210	2630	2840	–
Meonwara 1989 (English red)	422	2247	2669	417	2176	2593	520	2304	2824	–
St. Anne's 1989 (English red)	244	103	347	249	151	400	300	160	460	–
Yearlstone 1989 (English red)	660	3031	3691	510	2716	3226	530	3200	3730	3365
Westbury 1984 (English red)	826	3877	4703	821	3678	4491	1002	3680	4682	4720
Ruchottes-Chambertin 1985 (red Burgundy)	565	2511	3076	605	2392	2997	–	–	–	2980
Ch. Langoa Barton 1976 (red Bordeaux)	603	1493	2096	422	1385	1807	590	1505	2095	–
Dizy Rouge (red Coteaux champenois)	562	3663	4225	–	–	–	588	3747	4335	4050

* ± 2%, mean based on triplicate determinations.
† ± 2%, mean based on triplicate determinations from the method found in reference 4.
‡ ± 3%, mean based on duplicate determinations from the method found in reference 13.
§ Approximately ± 3%, mean based on duplicate determinations from the method found in reference 14.

between the (*S*)-lactic acid data than between the (*R*)-lactic acid data for reasons that were unclear. It is worth noting, however, that agreement was poorest overall for the St. Anne's wine sample, which (uniquely in this selection) had low concentrations of both of the enantiomers of lactic acid. The data for all the wines (except St. Anne's) were indicative of the occurrence of extensive MLF, which is a bacterial conversion of (*S*)-malic acid to (*S*)-lactic acid. This was supported by the observation of very low malic acid concentrations (< 100 mg/L) in the RP-HPLC chromatograms of these wines. The exception (St. Anne's) was seen to have a malic acid content of 2100 ± 40 mg/L. MLF was encouraged in the production of most red wines and some white wines (15). Ruchottes-Chambertin, Ch. Langoa Barton, and Dizy Rouge were from French viticultural regions in which MLF is more or less routine for red wines. It is of interest to note that the English red wines (except St. Anne's) had also experienced extensive MLF. These results were in contrast with those of the previous decade in which MLF was not a major feature in the production of English red wines (14). Although the data in reference 14 was very limited because English red wines were very rare at that time, the results in this study did support the notion that English wine making has become more strongly influenced by European and "New World" styles over the past ten years or so (16).

Various aspects of the operation of the column-switching method

Removal of interferences

It can be seen from Figure 1 that under RP conditions lactic acid elutes close to an unidentified component. Because of this and also because of the relatively low absorbance of lactic acid at 220 or 230 nm, it was often more difficult to quantitate lactic acid content using RP-HPLC. In the column-switching procedure, some or all of this component was cut from the RP chromatogram along with the lactic acid enantiomers (compare Figures 1 and 3B). However, there appeared to be no interference from this component in the CLE chromatogram, as shown in Figure 3B. Because the SPE–CLEC method is more selective than RP-HPLC (4), it is likely that this RP interference was not detected under CLEC conditions. It has already been stated that the potential interference of malic acid in the CLE chromatographic determination of (*R*)- and (*S*)-lactic acids was eliminated by the column-switching method (4). The malic acid content of wines that have experienced full MLF was very low (often close to the detection limit); therefore, the present method offered relatively little advantage over the SPE–CLEC method in these cases. The column-switching method is probably most useful in the analysis of wines that have undergone either no MLF at all or only partial MLF in which the malic acid content is relatively high. This includes wines blended from base wines of varying extents of MLF. However, the column-switching method allowed for the easy quantitation of malic acid and both enantiomers of lactic acid in all of the situations, the only limitation being sensitivity. Furthermore, tartaric acid and other components that eluted before and after lactic acid in the RP-HPLC chromatogram may also be quantitated at the same time.

Flexibility and robustness

The column-switching method demonstrated considerable flexibility in that columns and HPLC modules other than those previously mentioned were successfully employed. In particular, RP columns from a variety of companies with a variety of stationary phases (e.g., endcapped and nonendcapped C_{18} and C_8) and various dimensions were tested using different flow rates, and most were found to be satisfactory. It was originally thought that a short (75 mm or 100 mm) RP column would be essential in minimizing back-pressure. Although back-pressure was lower with these columns, longer columns have performed just as well with only slightly higher back-pressure. The limiting factor was resolution; most of the RP columns tested showed inferior resolution to either the Waters or Phenomenex systems. However, although this factor affected the quantitation of malic acid (and other components) in the RP-HPLC chromatogram, it appeared to have little effect on the quantitation of lactic acid enantiomers in the CLE chromatogram.

The column-switching method appeared to be robust in that both the RP-HPLC and the CLEC side of the method showed only a minor deterioration in performance after approximately 100 injections. Optimum RP-HPLC performance was regenerated by the implementation of a simple washing program, as outlined in reference 11. Optimum CLEC performance was achieved by running the mobile phase through the column at 2 mL/min for 30 min. The timing of the column-switch valve operation was critical, but it appeared that as long as the switch occurred before the lactic acid component reached the valve, there was some flexibility in the length of time allowed for the switch (typically 20–40 s under most conditions).

It has already been stated that use of the less aggressive (approximately pH 3.3) 0.5mM H_2SO_4 RP mobile phase as opposed to the usual (approximately pH 2.4) 5mM H_2SO_4 solvent (11) results in a slight compromise in resolution. For a limited number of cases in which optimum RP resolution was deemed desirable, the pH 2.4 solvent was used for a small batch (< 20) of analyses without noticeable damage to the CLEC column. However, more extensive use of this mobile phase was avoided for fear of causing irreversible damage to the CLEC column.

Comparison of the column-switching method with other methods

Compared with the enzymatic method (13) and the headspace GC procedure (14), this study's method was more robust and required less intensive attention by a less skilled operator. It gave more information in a shorter time (≤ 10 min) than either the SPE–CLEC method (approximately 15 min), the enzymatic procedure (approximately 20 min), or the headspace GC method (approximately 20 min). Furthermore, no extra sample preparation was required. These advantages were gained with only a slight compromise in the RP part of the procedure (as already discussed) and also with a corresponding minor compromise in CLEC performance. The method in this study gave a typical resolution factor of 1.2 for (*R*)- and (*S*)-lactic acids, compared with 2.0 for the SPE–CLEC method (4). The reproducibility (RSD) was 3.6% with recoveries of 94%, compared with 2.0% and 99%, respectively, for the SPE–CLEC method (4).

Finally, because the RP component of the present method was achiral, only the total concentrations of malic and tartaric acids

could be quantitated. Unadulterated wine contains only the (*S*)-isomer of malic acid and the (*R*,*R*)-isomer of tartaric acid, which means that the present method is unable to supply definitive proof of adulteration concerning these components. However, the most likely adulteration would include the addition of (*R*,*S*)-malic acid by wine makers in warm climates, in which (*S*)-malic acid levels drop to nearly zero because of almost unavoidable MLF. The column-switching method could be used to suggest the possibility of adulteration by the observation of a significant malic acid peak along with a significant (*S*)-lactic acid peak. Another method (17) would be needed to determine the malic acid enantiomers.

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Manuscript accepted July 24, 2001.