# Elimination of Amino Acid Interferences in the Chiral Ligand-Exchange Chromatographic Analysis of Lactic Acid Enantiomers in Wine

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## Abstract

**Chiral ligand-exchange liquid chromatography is used to identify and quantitate lactic acid enantiomers in wines that have or have not undergone malolactic fermentation. The stationary phase is (*R*)-penicillamine, which is bound lipophilically to a $C_{18}$ bonded silica matrix. The mobile phase is 1mM copper sulfate, and the detection mode is ultraviolet. Serious interference from (*S*)-aspartic acid and other amino acids is eliminated by the use of propanesulfonic acid-type cation exchange solid-phase extraction cartridges prior to chromatographic analysis. Lactic acid enantiomers in wine are quantitated in the range of 10 to 500 mg/L. The detection limit is 3 mg/L. The method is also successful in the determination of lactic acid enantiomers in certain beers (e.g., lambic beers), kim-chi, sauerkraut, and various yogurts.**

## Introduction

Chiral ligand-exchange chromatography (CLEC) is a powerful technique for the separation of enantiomers whose molecules are relatively highly polar. The method was first described by Davankov and Rogozhin (1) in 1971 and has recently been reviewed by Davankov (2). CLEC depends on the differential chelate complex formation between enantiomeric bidentate analyte ligands and a metal, often Cu(II), bound to a chiral bidentate ligand, such as (*S*)-proline or (*R*)-penicillamine (called the chiral selector).Typically, α-amino acids, α-hydroxy acids, and α-amino alcohols (analytes that are not readily accessible by other types of chiral liquid chromatography without derivatization) have been investigated by CLEC (3–9). The chiral selector can either be in solution as a mobile phase additive (3,4), or it can be permanently bound to the stationary phase (5–9). Derivatized enantiomeric analytes can be separated using other types of chiral stationary phase (10) or by the use of a reversed-phase mode (11). Apart from these, underivatized enantiomeric α-hydroxy acids have been analyzed by reversed-phase high-performance liquid chromatography (HPLC) using a circular dichroism detector (12), and enantiomeric α-amino acids have been separated on chiral zeolite phases (13) and ionically-bound chiral stationary phases (14). Separation of these highly polar analytes by gas chromatography is difficult without derivatization, but there is a recent report of the direct separation of longer chain carboxylic acid enantiomers using a chiral stationary phase (15).

CLEC lends itself well to the analysis of the acids in fruit juices and wine, because many important acids (e.g., malic, lactic, and tartaric acids) are α-hydroxy acids (16,17). This paper describes a simple CLEC method for the specific analysis of (*R*)- and (*S*)-lactic acids in grape juice and wine. In particular, it describes a rapid solid-phase extraction (SPE) procedure for the removal of serious interferences in the detection and quantitation of these two analytes by the CLEC method.

## 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 was Fisons (Loughborough, U.K.) analytical grade material, and copper sulfate pentahydrate was from BDH Analytical Grade (Poole, U.K.). The grape juice was from Stute (Paderborn, Germany) White Grape Juice cartons, pooled together to ensure homogeneity, and was fermented with pure wine yeast (strain 71B) from Gervin (Reading, U.K.). The commercial red wine used for Figures 1E and 1F was from 1989 Yearlstone (Devon, U.K.).

The SPE cartridges were Isolute PRS (100-mg sorbent, 1-mL reservoir volume) obtained from Jones Chromatography (Hengoed, U.K.). A Ciba Corning (Fernwald, Germany) Checkmate 90 pH meter fitted with a Corning pH electrode was used to monitor solution pH.

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**Chromatographic system and HPLC operating conditions**

The HPLC instrumentation consisted of a LDC/Milton Roy (Stone, Staffs, U.K.) Constametric III pump, a Rheodyne (Cocati, CA) injector unit with a 20-µL loop, a Jasco 875-UV ultraviolet detector, and a Hewlett-Packard (Palo Alto, CA) 3392A integrator. The precolumn system was a Waters (Watford, Herts, U.K.) Guard Pak fitted with a Resolve $C_{18}$ insert. The analytical column was either a Daicel Chiralpak MA(+) (5 cm × 4.6-mm i.d.) column obtained from Anachem (Luton, U.K.) or a Phenomenex (Macclesfield, U.K.) Chirex D-penicillamine column of the same dimensions. The mobile phase was 1mM $CuSO_4$ run at a flow rate of 1 mL/min at room temperature or 30°C using a Jones Chromatography column heater. The detector wavelength was set at 254 nm.

All solutions for injection were either made up in or diluted with the mobile phase and filtered through Phenonenex 0.2-µm PTFE syringe sample membrane filters directly into the Rheodyne loop.

**SPE procedure**

The HPLC mobile phase (1 mL) was added to an Isolute PRS cartridge, drawn through at a rate of 1 mL/min, and discarded. The sample (1 mL) was then introduced, drawn through at the same rate, and subjected to chromatographic analysis as previously described. Standards (made up in 1mM $CuSO_4$) were added without further dilution, but the grape juice and wine samples were accurately diluted tenfold with 1mM $CuSO_4$ prior to SPE treatment.

## Results and Discussion

**Analysis of (*R*)- and (*S*)-lactic acids and the effect of intererences from (*S*)-aspartic acid and other α-amino acids**

Grape juice and wine contain a wide range of constituents, many of which are chiral and some of which can be analyzed by CLEC. Principal among these are α-amino acids and various α-hydroxy acids. For example, alanine, arginine, aspartic acid, glutamic acid, leucine, lysine, and proline are among the more common α-amino acid constituents of grape juice and wine (17). Of the α-hydroxy acids, (*S*)-malic acid is found in juice and also in wine that has not experienced significant malolactic fermentation (MLF), (*R*)-lactic acid is found in wine but not juice, and (*S*)-lactic acid has a significant presence only in wines that have undergone MLF (16,18,21). Malolactic fermentation is a secondary fermentation of wine that converts the sharp-tasting (*S*)-malic acid to the softer (*S*)-lactic acid. It is a useful means of reducing the overall acidity of wine and, because it also produces volatile byproducts, the wine generally acquires a more complex flavor (21). The winemaker can encourage MLF (as in most red wines) or discourage it (as in many white wines) by carefully choosing conditions (21). Either way, it is often important to assess the extent of MLF.

The CLEC method described in this paper results in good resolution of (*R*)- and (*S*)-lactic acids with retention times generally between 6 and 10 min and with resolution factors ($R_s$) in the region of 2. Figure 1A is a typical chromatogram of (*R*)- and (*S*)-lactic acid standards. Retention times were observed to vary by a considerable amount (approximately 25% decrease with 10°C increase in temperature) from day to day, depending on ambient temperature. Consequently, it is advisable to carry out the CLEC analyses using a column heater to maintain a steady column temperature of 30°C. However, satisfactory analyses can still be performed without a column heater, provided frequent injections of standards are included between injections of samples.

Details of the performance of the CLEC method are given in Table I.

Figure 1B shows a typical CLE chromatogram of grape juice. The presence of (*S*)-aspartic acid was verified by both separate injection of an (*S*)-aspartic acid standard and co-injection of sample and standard. Comparison of Figures 1A and 1B shows that (*R*)-lactic acid is not present in grape juice.

Figure 1C is a CLE chromatogram of the same grape juice in Figure 1B after fermentation by wine yeast in the absence of malolactic bacteria. The presence of (*R*)-lactic acid is evident but somewhat obscured by a broad interfering peak (unidentified). More serious is the presence of (*S*)-aspartic acid, which elutes close to (*S*)-lactic acid (compare Figures 1A and 1C). Thus, (*S*)-aspartic acid may be mistaken for (*S*)-lactic acid in non-MLF wines or may obscure (or at least interfere with) the quantitation of (*S*)-lactic acid in MLF wines. The latter situation is illustrated by the CLE chromatogram of a wine that has undergone MLF (Figure 1D). Once again, the (*R*)-lactic acid peak is seen clearly, but the (*S*)-lactic acid and (*S*)-aspartic acid peaks overlap. Both (*S*)-lactic acid and (*S*)-aspartic acid were verified by coinjection of the wine sample and the appropriate standard.

Two interesting features of this CLEC method will now be discussed. First, it was noted that the extent of interference between the (*S*)-aspartic acid and (*S*)-lactic acid peaks varied considerably with temperature according to different changes in retention times. However, overlap was always present in the range of 20–40°C. The situation was not improved by the inclusion of 10% acetonitrile in the mobile phase. Although retention times were decreased, the resolution of (*R*)- and (*S*)-lactic acids was much poorer ($R_s$ ~1.2 at 30°C). More than this quantity of organic component in the mobile phase can damage the stationary phase, because the chiral selectors are only lipophilically bonded to the ODS phase.

Secondly, the two other major α-hydroxy acids in grape juice and wine, (*S*)-malic and (*R*,*R*)-tartaric acids, are not easily analyzed by this CLEC method. The former appears as a broad asymmetric peak at approximately 55 min under standard analytical conditions. Its peak shape can be somewhat improved and its retention time can be approximately halved by the inclusion of 10% acetonitrile in the mobile phase, but as mentioned previously, separation of lactic acid enantiomers (the analytes of primary importance in this study) is compromised. Hence, if quantitative analysis of malic acid is needed, an alternative procedure should be used (8,19,20). (*R*,*R*)-Tartaric acid is not detected at all by this method. We are presently preparing a column-switching procedure that may allow the analysis of all three major wine acids, (*S*)-malic acid, (*R*,*R*)-tartaric acid, and both lactic acid enantiomers, simultaneously by the

combination of the CLEC method described in this paper and a reversed-phase method described in the literature (20).

The late, broad peaks for (*S*)-malic acid of earlier analyses do not pose a serious interference problem until 4 or 5 determinations have been performed (and even then only by unfortunate timing of the injection). However, the column can be cleared every 5 injections by running through mobile phase at 2 mL/min for half an hour. Thus, it is still normally possible to perform 4 analyses per hour, including the time needed for each manual SPE procedure (approximately 3 min). Also, at the end of a series of analyses, the column is cleared satisfactorily by running through 2mM $CuSO_4$ for half an hour. In any case, the column is stored in this medium.

**Removal of amino acid interferences using SPE**

Although the amount of overlap between the interfering peak and the peaks of interest varies according to temperature and mobile phase composition as described previously, it was

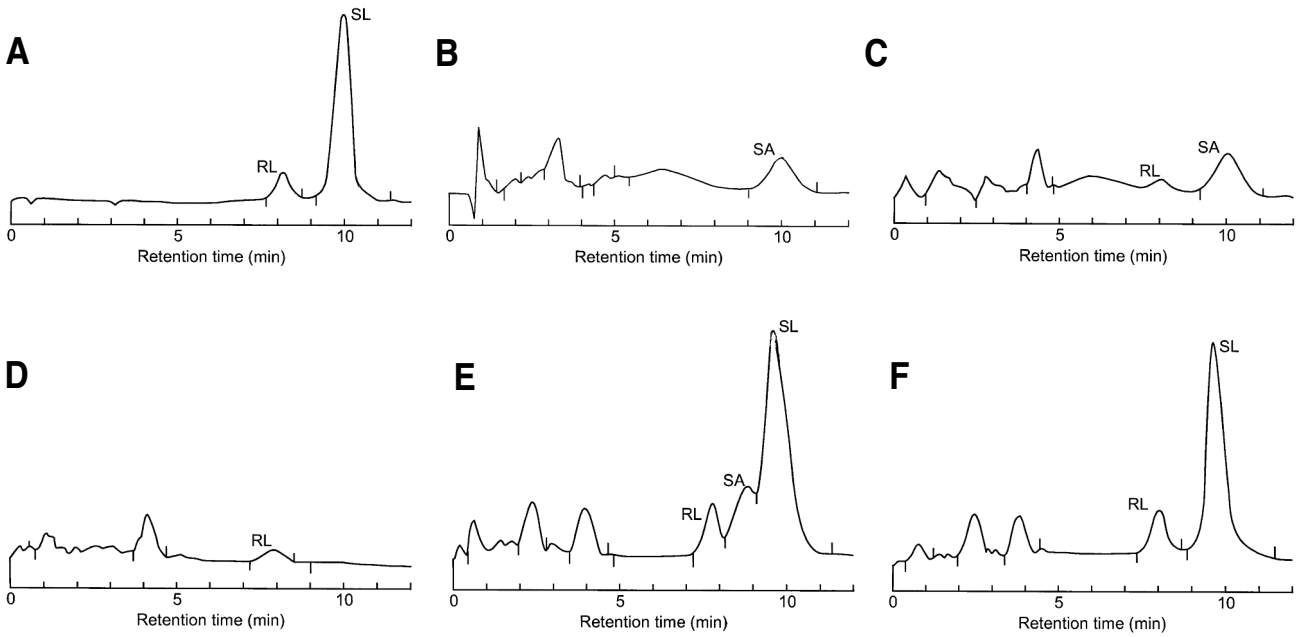


**Figure 1.** Chiral ligand-exchange chromatograms: (*R*)- and (*S*)-lactic acid standards, lithium (*R*)-lactate and sodium (*S*)-lactate (29 and 307 mg/L, respectively) (A); white grape juice (B); white wine (the white grape juice of B fermented by *Saccharomyces cereviseae*, strain 71B) (C); white wine in C after SPE processing (D); commercial red wine (Yearlstone 1989) (E); red wine in E after SPE processing (F). RL is (*R*)-lactic acid, SL is (*S*)-lactic acid, and SA is (*S*)-aspartic acid.

**Table I. Accuracy and Precision Data for the CLEC Determination of (*R*)- and (*S*)-Lactic Acids (without SPE)**

| Analyte | Linearity range (mg/L)* | Coefficient of correlation *r* | Coefficient of determination $r^2$ | Reproducibility CV (%) |
|---|---|---|---|---|
| (*R*)-Lactic acid | 20–500 | 0.999 | 0.999 | 0.5 |
| (*S*)-Lactic acid | 20–500 | 0.999 | 0.998 | 0.7 |

* Limit of detection ($n$ = 5): 3 mg/L, calculated as twice the value of the general baseline variation.
† Coefficient of variation ($n$ = 5) determined by multiple injections of mixed standards: (*R*)-isomer 353 mg/L, (*S*)-isomer 324 mg/L.

**Table II. Accuracy and Precision Data for the CLEC Determination of (*R*)- and (*S*)-Lactic Acids (with SPE)**

| Analyte | Linearity Range (mg/L, $n$ = 5) | Coefficient of correlation *r* | Coefficient of determination $r^2$ | SPE recoveries* | SPE reproducibilities CV (%) | |
|---|---|---|---|---|---|---|
| | | | | | 48 mg/L | 414 mg/L |
| (*R*)-Lactic acid | 10–500 | 1.000 | 1.000 | 97–103% (mean 99%) | 1.5 | 2.1 |
| (*S*)-Lactic acid | 10–500 | 1.000 | 1.000 | 96–101% (mean 99%) | 0.8 | 1.7 |

* Range given in linearity range column ($n$ = 5), calculated by comparison of triplicate chromatograms of mixed standards before and after SPE procedure.
† Coefficient of variation ($n$ = 7) determined by multiple injections of mixed standards.

felt that complete removal of the interferences was the best option. To this end, it was decided to use the SPE method in which the interferences are retained on the cartridge and the analytes are allowed through. Aspartic acid and other α-amino acids are essentially zwitterionic in aqeous solution, so when in a solution whose pH is close to their isoelectric points, the amino acid molecules will have cationic sites. The pH of the solutions used in the SPE procedure for juice and wine analysis is typically close to 4, so that under these conditions, the α-amino acids have cationic sites, whereas the two analytes, (*R*)- and (*S*)-lactic acids, do not. It was therefore decided to use a cation-exchange SPE procedure. Because of the nonaromatic nature of (*S*)-aspartic acid, it was felt that a strongly acidic phase of the PRS (propanesulfonic acid) type would give the best performance. The use of the Isolute brand of this phase was very successful, giving good recoveries, linearities, and reproducibilities for both (*R*)- and (*S*)-lactic acid standards; neither analyte is retained to any significant extent in the concentration range of 10–500 mg/L phase (Table II). Furthermore, the whole procedure, carried out manually on one sample, takes approximately 3 min. When juice and wine samples were subjected to the SPE method using Isolute PRS cartridges, the CLE chromatograms of the eluent were completely clear of the (*S*)-aspartic acid peak, leaving both the (*R*)- and (*S*)-lactic acid peaks (where appropriate) unobscured. Figure 1D illustrates the complete removal of (*S*)-aspartic acid from a non-MLF wine (compared with Figure 1C) using the SPE method. It can be seen that the baseline is smoother, the (*R*)-lactic acid peak is more easily (and more accurately) quantitatable, and there is no measurable amount of (*S*)-lactic acid as expected of a non-MLF wine. Figure 1F shows the CLE chromatogram of the same wine as in Figure 1E after the wine has undergone the SPE treatment. This wine has experienced MLF, as shown by the (*S*)-lactic acid peak, free of the interference from (*S*)-aspartic acid; both the (*R*)- and (*S*)-lactic acid peaks can be more satisfactorily quantitated.

The basic method described in this paper has been used in the successful determination of lactic acid enantiomers in beers (e.g., lambic and related beers), kim-chi, sauerkraut, and yogurts (22).

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