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Problems and Pitfalls in the Analysis of Amygdalin and Its Epimer

M. Farooq Wahab,[†] Zachary S. Breitbach,[†] Daniel W. Armstrong,[*,†] Rick Strattan,[§] and Alain Berthod[#]

[†]Department of Chemistry, University of Texas at Arlington, 700 Planetarium Place, Arlington, Texas 76019, United States

[§]CTD Holdings, Inc., 14120 N.W. 126th Terrace, Alachua, Florida 32615, United States

[#]Institut des Sciences Analytiques, CNRS, University of Lyon 1, 5 Rue de la Doua, 69100 Villeurbanne, France

ABSTRACT: α-[(6-*O*-β-D-Glucopyranosyl-β-D-glucopyranosyl)oxy]-(α*R*)-benzeneacetonitrile, or *R*-amygdalin, is the most common cyanogenic glycoside found in seeds and kernels of the Rosaceae family and other plant genera such as *Passiflora*. Many commercially important seeds are analyzed for amygdalin content. In "alternative medicine", amygdalin has been sold as a treatment for cancer for several decades without any rigorous scientific support for its efficacy. We have found that there are some inconsistencies and possible problems in the published analytical chemistry of amygdalin. It is shown that some analytical approaches do not account for the presence of the *S*-isomer; therefore, a fast reliable method was developed using a chiral stationary phase and HPLC. This approach allows "real-time" monitoring and complete and highly efficient separations. It is found that the *S*-amygdalin continuously forms in aqueous solutions. A striking result is that the conversion of amygdalin is glassware dependent. "Clean" vials from various vendors can show drastically different reaction rates of the conversion to the isomer (*S*-amygdalin, also called neo-amygdalin). The epimerization kinetics are dependent on the solvent, temperature, pH, and the nature of the container. For example, epimerization in water was complete in <15 min in a new glass vial taken from the box, whereas it can take >1 h in specially cleaned glassware. Conversely, epimerization can be significantly delayed at high temperature if high-density polyethylene is used as the container. Hence, inert plastic containers are recommended for storage of aqueous amygdalin solutions. Commercial preparations of *R*-amygdalin actually contain greater quantities of *S*-amygdalin and ∼ 5% of other degradation products.

KEYWORDS: *amygdalin, cyanogenic glycoside, epimerization, analysis, food safety*

INTRODUCTION

Amygdalin is one of the most common members of the cyanogenic glycoside family. Cyanogenic glycosides are defined as glycosides of α-hydroxynitriles. In nature, their role is thought to be that of a natural toxicant having the potential to generate hydrogen cyanide, thereby defending the plant against insects and herbivores.[1] Amygdalin has the chemical name D-mandelonitrile-β-D-gentiobioside (Figure 1). It was first isolated in 1830 by French biochemists Robiquet and Boutron-Chalard and studied in detail by German chemist Emil Fisher.[2] It occurs naturally in the seeds of the Rosaceae family, which includes almond, apple, apricot, cherry, peach, pear, plum, and quince, and recently was discovered in passion fruits (*Passiflora* species).[3] Among all of the natural cyanogenic glycosides, amygdalin is the center of attention of food chemists as the cause of bitterness in seeds and regulatory agencies due to its controversial nature in alternative medicine for cancer treatment. Amygdalin is also present in many commercial apple juices at parts per million levels.[4]

When seeds or nuts are eaten, amygdalin comes in contact with hydrolytic enzymes. They first produce prunasin (D-mandelonitrile-β-D-glucoside) and glucose and, in a second step, prunasin (Figure 1) can be converted into benzaldehyde and hydrogen cyanide; the mixture of these chemicals produces the well-known pleasant almond-like aroma.[5] Hydrogen cyanide is a well-known poison that blocks mitochondrial cytochrome *c* oxidase, halting cellular respiration. Hence, it is important to be able to quantify the amount of its precursor, amygdalin, present in food and supplements.[6] Amygdalin is still sold under various names such as "vitamin B17" and laetrile, although the name laetrile actually is a different compound, D-mandelonitrile-β-D-glucuronic acid (Figure 1).[7]

This work focuses on amygdalin's mandelonitrile stereogenic center. Natural amygdalin has exclusively the *R*-configuration of the stereogenic mandelonitrile carbon (Figure 1). It has long been known that in the presence of water and weak bases, epimerization of this stereogenic carbon occurs.[8] *S*-Amygdalin or neo-amygdalin is then formed having the *S*-configuration on the mandelonitrile carbon (Figure 1). *R*-Amygdalin and *S*-amygdalin are epimers and not enantiomers because they maintain the same configuration on the 10 stereogenic centers of their gentiobiose moieties. As diastereomers, *R*-amygdalin and *S*-amygdalin can be separated by achiral stationary phases in gas or liquid chromatography. Recently, we noted a revival in the analysis of amygdalin in food chemistry publications in this journal and elsewhere.[5,6,9−13] The amygdalin epimerization mechanism was studied as early as 1934, but the problem is often overlooked in recent works on amygdalin analyses. A recently proposed enzyme-linked immunosorbent assay (ELISA) for the quantification of amygdalin completely ignored the possible epimerization of amygdalin when the immunogen conjugate is prepared in a carbonate buffer.[11] Today, there is little excuse for not having reliable and efficient liquid

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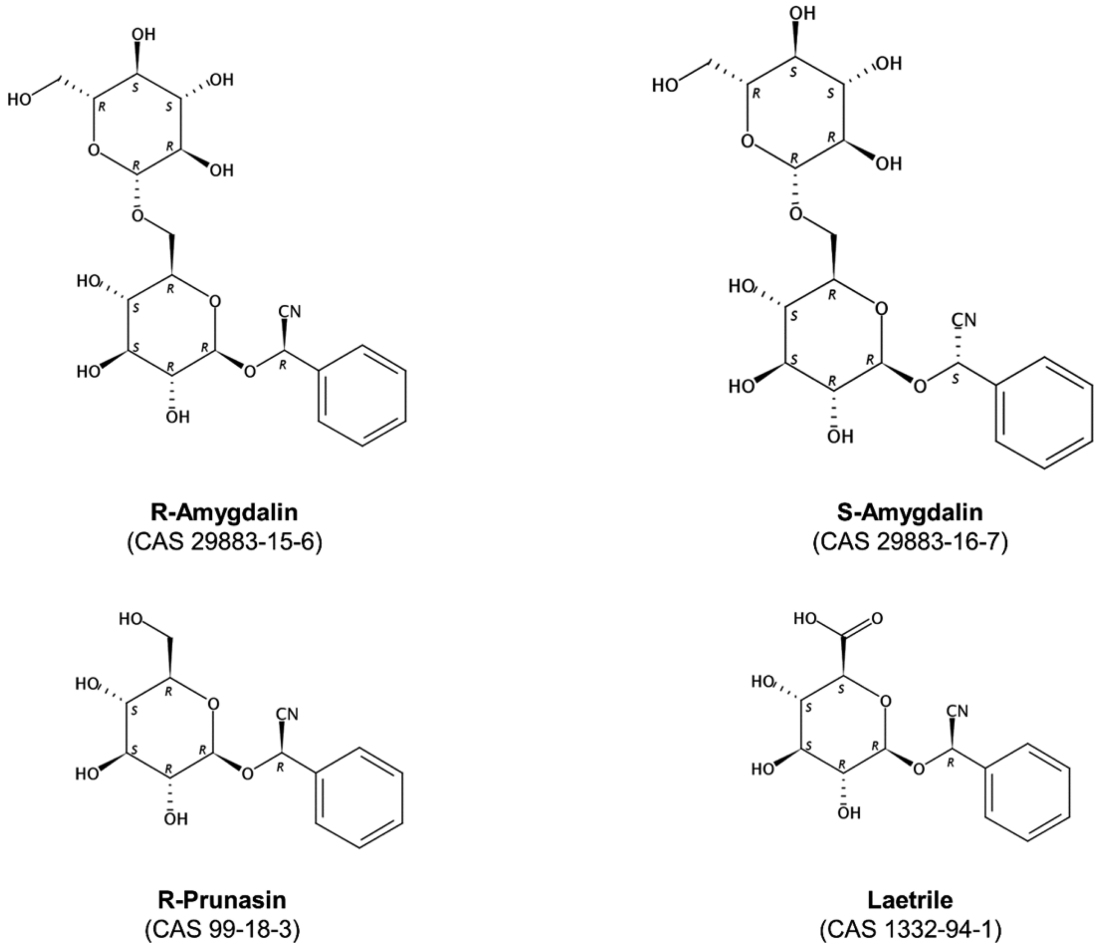

Figure 1. Chemical structures of amygdalin, D-mandelonitrile-β-D-gentiobioside with the *R*-configuration of the labeled carbon, and its *S*-amygdalin (neo-amygdalin) epimer or L-mandelonitrile- β-D-gentiobioside. Prunasin is the D-mandelonitrile-β-D-glucoside, and laetrile is the D-mandelonitrile-β-D-glucuronic acid.

chromatographic methods for the separation and quantification of amygdalin epimers, because the *R*- and *S*-configurations can have significantly different biochemical and physiological properties. Herein we outline the fastest and most reliable approaches for doing such analyses. Subsequently, the developed analytical approaches are used to evaluate the factors affecting amygdalin epimerization.

MATERIALS AND METHODS

Chemicals. Amygdalin, $C_{20}H_{27}NO_{11}$, mol wt 457.4, CAS Registry No. 29883-15-6, was obtained from Sigma-Aldrich (St. Louis, MO, USA) as ≥97% pure (BioXtra) and from Trappsol (from CTD Holdings, Shanghai DND Pharm-Technology Co. Inc., lot 20140103). Both were used as received. *S*-Amygdalin is not commercially available. It was formed by epimerization of *R*-amygdalin. We note that *Sigma-Aldrich also lists incorrectly the laetrile name as a synonym for amygdalin, whereas laetrile is a very different compound* as seen in Figure 1. Additionally, *Beilstein's Handbuch der Organischen Chemie* mentions that the dextro- and laevo- notations were interchangeably used for the naturally occurring amygdalin CAS Registry No. 29883-15-6.[2] Ten sealed ampules of commercial injectable amygdalin were purchased from www.cytopharma.com. The solutions are sold as "vitamin B17" in sealed amber glass ampules.

Methanol and acetonitrile were HPLC grade solvents obtained from Sigma-Aldrich. Ammonium hydroxide was a 29.5% w/v (about 17 M) solution from Fisher Scientific (Fair Lawn, NJ, USA). Water (18.2 MΩ) was produced by a Millipore Synergy 185 system (EMD, Billerica, MS, USA). The following glassware/vials were studied for epimerization of *R*-amygdalin: Wheaton 120 mL (catalog no. 225546, batch 1542790), Restek 20 mL vials (catalog no. 23082), VWR 8 dram glass vials (66011-165, lot 052A06), Supelco vials with a screw cap with septum (~23 mL), a 25 mL Chemglass round-bottom flask (19/22 standard taper) with a reflux condenser, and boiling glass pieces made by crushing 9″ Pasteur pipets (Fisher brand, catalog no. 13-678-6B). Two sets of glassware were studied, one as received, and the other set was soaked in KOH/2-propanol base bath, followed by detergent washing, rinsing with copious amounts of deionized water, and oven-drying before use. As an experimental control, a 250 mL high-density polyethylene bottle (HDPE) from Wheaton was used to boil amygdalin. Plastic syringes with stainless steel needles were used for transferring water and sampling.

Chromatographic Columns and Systems. The following 25 × 0.46 cm i.d. commercial columns packed with fully porous 5 μm particle size were obtained from Supelco (Bellefonte, PA, USA): Chirobiotic V, Cyclobond I 2000 RSP, and Cyclobond I 2000 HP-RSP. Porous graphitic carbon (Hypercarb, Thermo Scientific, 15 × 0.46 cm, 5 μm fully porous particle with 25 nm pore diameter and 170 m^2/g surface area) was a gift from Dionex, Thermo Scientific (Waltham, MA, USA). Additionally, hydroxypropyl-β-cyclodextrin was purchased from Sigma and bonded on 2.7 μm superficially porous silica particles with a surface area of 120 m^2/g and pore size of 120 Å according to a proprietary procedure. The particles were provided by Agilent Technologies (Wilmington, DE, USA). The core is 1.7 μm in diameter, and the surrounding shell thickness is 0.5 μm. The column was slurry packed into a 5 cm × 0.46 cm i.d. column. Two chromatographic systems from Agilent (Santa Clara, CA, USA) were used. The first one was a model Infinity 1260 Quaternary LC system

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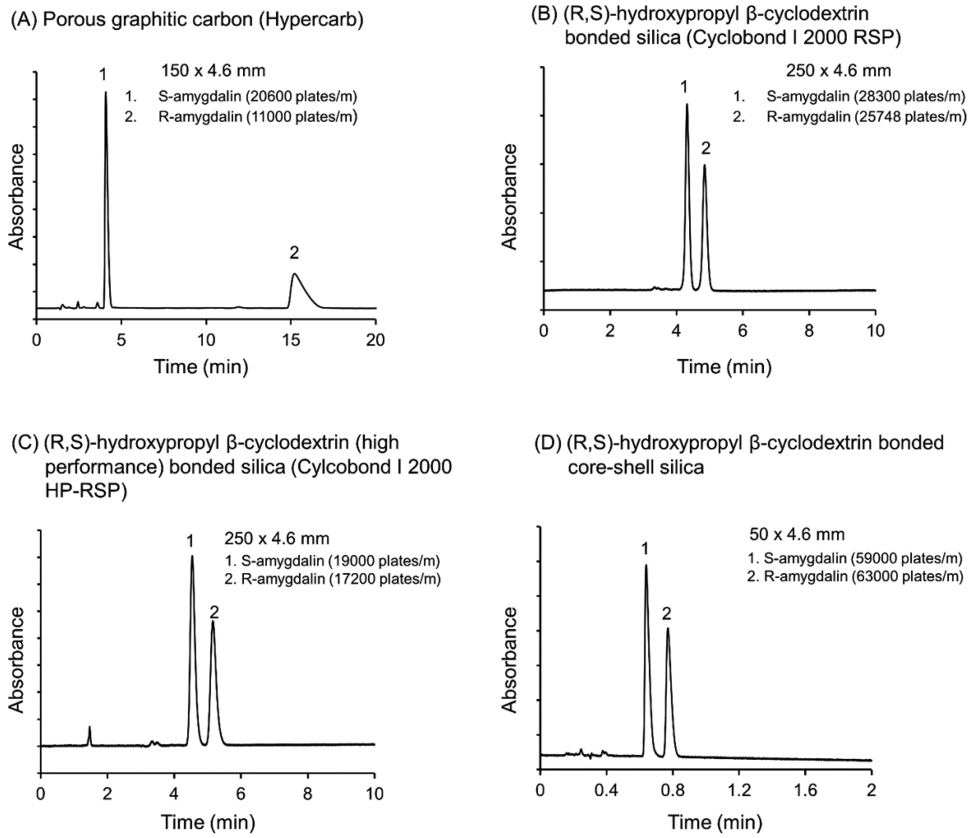

Figure 2. Amygdalin epimer LC analyses: (A) porous graphitic carbon column (15 × 0.46 cm i.d.) with 90:10 MeOH/H_2O at 1.5 mL/min; (B) Cylcobond I 2000 RSP (25 × 0.46 cm i.d.) with 20:80 MeOH/H_2O at 1.0 mL/min; (C) Cyclobond I 2000 HP-RSP (25 × 0.46 cm i.d.) with 30:70 MeOH/H_2O with 1.0 mL/min; (D) the same column chemistry as in (C) bonded on 2.7 μm core−shell particles packed in a 5 cm × 0.46 cm i.d. column 10:90 MeOH/H_2O at 1.8 mL/min. Detection wavelength = 254 nm. All analyses were done at ambient temperature.

with a gradient capable quaternary pump with a maximum pressure of 600 bar (8600 psi), an autosampler, a column oven, and a diode array detector. The second system was a model Infinity 1290 with an ultrahigh-pressure binary pump (UHPLC, maximum pressure of 1200 bar or 17000 psi) with an autosampler with an ultrafast diode array detector. The detector setting on UHPLC was 160 Hz and 0.016 s response time. Amygdalin absorbs at 220 and 254 nm. Both wavelengths can be used for detection. All separations were performed at ambient temperature.

An external calibration curve was constructed by making standards from 76 to 1525 ppm of *R*-amygdalin (Sigma, BioXtra ≥97% pure) in 0.1% aqueous acetic acid solution. The analysis was done in triplicate at 220 nm with an injection volume of 1 μL on a 5 cm × 0.46 cm core−shell hydroxypropyl-β-cylcodextrin column. The mobile phase was 90%H_2O/10% methanol at a flow rate of 1.8 mL/min. Thoroughly washed glass volumetric flasks were used. The curve was linear with a correlation coefficient R^2 value of 0.9995. We note that traces of *S*-amygdalin are present in commercial *R*-amygdalin. The commercial samples were diluted to bring their concentration within the range of the calibration curve.

Procedure for Amygdalin Epimerization. Two approaches were used in the epimerization studies. The first procedure is more efficient and faster (∼15 min), but introduces a very small amount of ammonia. This method was used for chromatographic method development for the separation of the *S*-isomer. For the "ammonia added method", 0.1 g of amygdalin was dissolved by sonication in deionized water (18.2 MΩ) in 10 mL of deionized water, and 10 μL of ACS certified concentrated ammonia (29%) was added via glass syringe. Within 15−20 min, all of the amygdalin epimerized to a ratio of 57% *S*-amygdalin and 43% *R*-amygdalin at room temperature. In the second method, where we show the glassware effect, 0.1 g/10 mL of amygdalin was heated on an oil bath in deionized water in thoroughly cleaned glassware for 60 min. An approximately 0.5 mL aliquot was drawn via stainless steel needle in a plastic syringe and transferred into a 1.8 mL amber VWR glass autosampler vial (catalog no. 82030-974).

RESULTS AND DISCUSSION

Amygdalin has 11 stereogenic centers, 10 of which are contained in the two glucopyranose moieties, and these are not easily isomerized. The remaining stereogenic center (i.e., the carbon atom with the phenyl and nitrile substituents) is susceptible to inversion, which can give rise to the formation of *S*-amygdalin. For extraction and analytical purposes, amygdalin is extracted in polar solvents including ethanol, methanol, ethyl acetate, and water, from seeds or kernels.[12] It was noted recently that polar solvents give low recoveries of amygdalin because of conversion of the natural isomer into the *S*-amygdalin, where 35% of natural amygdalin converted into the isomer upon 24 h of standing.[6] It is therefore critical to know the relative concentrations of the isomers in any study of or with these compounds. We also note that amygdalin starts to degrade even after boiling in pure water for a short period of time (∼1 h). To develop a reliable analytical method and produce *S*-amygdalin (for which there is no known commercial source), we used a small quantity of ammonia to produce the other epimer. This procedure is very efficient in promoting epimerization provided that optimal levels of ammonia are used (10 μL/10 mL). Using higher concentrations of bases can hydrolyze amygdalin into many side products including ammonia and mandelic acid, and often the cyano group is hydrated to the amide.[2,14] Existing separation methods relying

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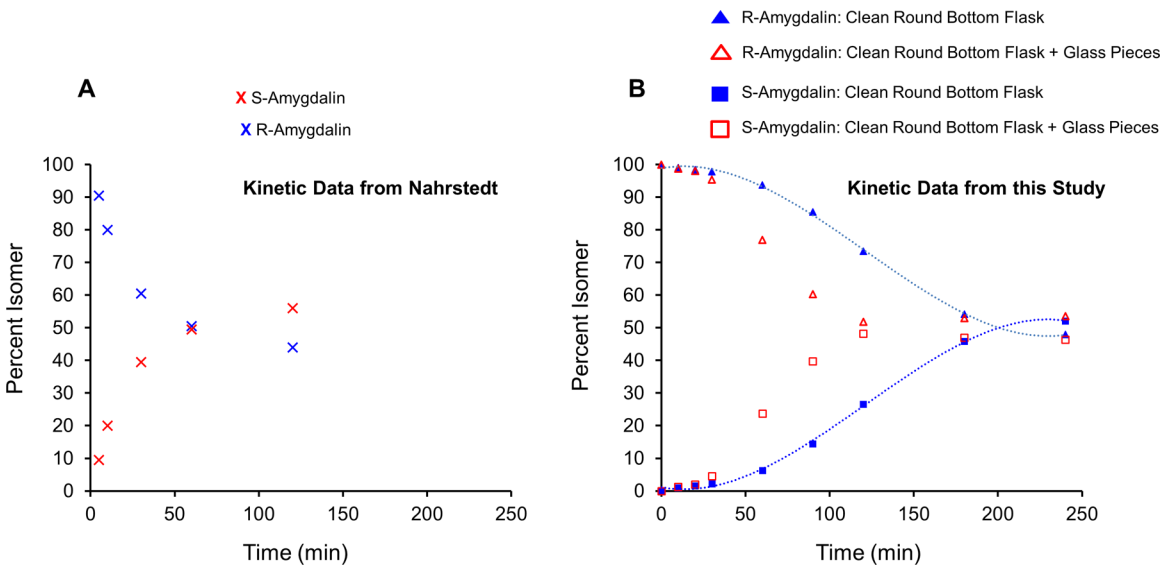

Figure 3. (A) Amygdalin concentration change due to epimerization under the Nahrstedt experiment of boiling amygdalin in water. (B) Heating amygdalin with deionized water in a 25 mL round-bottom flask (19/22 standard taper) with a reflux condenser (blue). Boiling glass pieces were made by crushing 9 in. Pasteur pipets (Fisher brand, catalog no. 13-678-6B) (red). The oil bath was kept at 107−110 °C.

on C_{18} stationary phases or other enzymatic approaches have a very strong potential to miss the *S*-isomer, thereby producing misleading information about the actual quantification of the naturally occurring *R*-amygdalin.[4,6,9−13,15−18] As a result, it is not surprising that many recent publications on amygdalin analysis do not mention/identify the *S*-amygdalin, which is always present to some extent depending on the storage conditions, extraction solvents, and analytical procedures. Although in a few specific cases, using advanced instrumentation such as MS-MS that may allow differentiation of epimers,[19,20] ideally it is desired and usually necessary that the epimers are separated chromatographically. Thus, the separation of the natural and its unwanted isomerized amygdalin is best done chromatographically.

Analytical Scale Separation of *R*- and *S*-Amygdalin. Early on, the separation of amygdalin epimers was accomplished by gas chromatography (GC) of their trifluoroacetylated derivatives on a semipolar capillary column (versilube F-50, a trichlorophenyl polysiloxane GC stationary phase) in 25 min at 180 °C.[7] Later, capillary electrophoresis was used to separate the epimers in 15 min with good selectivity using a cyclodextrin chiral additive to the electrolyte.[21] However, in most studies, liquid chromatography (LC) seems to have been the method of choice with a C_{18} phase, which produces overlapping/shoulder peaks for *R*- and *S*-amygdalin.[6]

In Figure 2, we show the utility of the preferred stationary phases for amygdalin using simple MS friendly mobile phases in the isocratic mode ($MeOH/H_2O$ without any additives). A number of achiral and chiral phases such as porous graphitic carbon, vancomycin, teicoplanin, cyclodextrin-bonded silica, and hydroxypropyl-*β*-cyclodextrin columns were studied. Porous graphitic carbon (PGC) is a very hydrophobic phase made of sheets of graphitized carbon. The flat surface of carbon allows for excellent shape selectivity of adsorbed molecules and, therefore, the *S*- and *R*-isomers have exceptionally high selectivity, which is not seen in any silica-based column. Thus, PGC can be employed when an exceptionally wide separation window is desired between the isomers (such as in preparative work). In Figure 2A, it can be seen that the *S*-amygdalin elutes earlier than the *R*-isomer with an impressive resolution of 12. The peak shapes and efficiencies for the two isomers are very different, showing that naturally occurring *R*-amygdalin has a very strong interaction with the carbon surface (3100 vs 1650 plates for the *S*- and *R*-isomers, respectively). The previous study,[9] which utilized PGC for the separation of amygdalin for the first time and used a methanol/water mobile phase, did not observe the formation of *S*-isomer. Given the very large selectivity, it is easy to miss the formation of *S*-amygdalin during the extraction process from natural products, or any biochemical studies on porous graphitic carbon. In Figure 2B,C, the separation of amygdalin epimers is shown on (*R*,*S*)-hydroxypropyl-modified *β*-cyclodextrin chiral columns. The key difference between the two columns is the nature of bonding to the silica surface. In general, the Cyclobond-I 2000 HP-RSP separates by extended H-bonding capability, offers broad chiral selectivity for chiral screening, and is most beneficial for basic and neutral compounds. Both chiral columns are able to separate the amygdalin epimers with an isocratic methanol/water mobile phase with baseline resolution and narrow symmetrical peaks showing more than 25000 plates/m. The *S*-amygdalin eluted first on both columns. It is interesting to note that *S*-amygdalin also eluted earlier on the PGC despite being a very different material from silica. A similar chiral column Cyclobond I 2000 HP-RSP produced a complete epimer separation in 5 min with 19000 plates/m (Figure 2C) but with higher methanol concentration.

To speed the same analysis, a recently introduced core−shell (*R*,*S*)-hydroxypropyl-modified *β*-cyclodextrin[22] column of 5 cm length was tested with a modern UHPLC chromatographic system. It was able to fully separate the amygdalin epimers under 50 s (Figure 2D) needing only 1.8 mL of 10/90 v/v methanol/water mobile phase. The measured efficiency was as high as 60000 plates/m with a resolution factor >2. Again, amygdalin, with its *R*-mandelonitrile stereogenic center, eluted after the *S*-amygdalin epimer (Figure 1).

Factors Influencing Amygdalin Epimerization. The Cyclobond I 2000 RSP with a 20:80 v/v methanol/water mobile phase was used for all kinetic studies on amygdalin

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Table 1. Epimerization Kinetics of Amygdalin in Different Glassware and Solvents

	expt with amygdalin	% *R*-amygdalin remaining[a] 10 min	30 min	60 min	comments and refs[b]
a	distilled water in glass at 100 °C	80.0	60.5	50.5	47% amygdalin remains intact after 2 h[24]
b	pure ethanol at 85 °C	negligible conversion			no conversion to *S*-amygdalin during 2 h of heating[24]
c	methanol/ethyl acetate azeotrope at 105 °C	100	100	100	no conversion to *S*-amygdalin during 2 h of heating[24]
d	distilled water in glass at 100 °C		66.6		45% amygdalin remains intact after 2 h minimum of boiling with a major unknown side product[29]
e	water + 0.05% citric acid at 100 °C		no conversion		negligible conversion to *S*-amygdalin in 2 h[29]
f	heating in dimethyl sulfoxide in glass		no conversion		the authors observed no conversion to the *S*-isomer (temperature and duration not mentioned)[27]
g	boiling water at in Pt vessel		no conversion		the authors observed no conversion to the *S*-isomer (boiling duration not mentioned)[27]
h	heating in deionized water in a VWR glass vial (as received)	44.5	39.7	35.0	ratio of *R*:*S* 47:53 after 1 h; oil bath temperature 109 °C
i	heating in deionized water in a washed VWR vial	93.0	68.3	55.2	ratio of *R*:*S* isomer 56:43 after 1 h; vial cleaned thoroughly before use
j	heating in deionized water in a Wheaton glass vial (as received)	43.3	41.9	35.0	ratio of *R*:*S* isomer 46:54 after 1 h; oil bath temperature 108 °C
k	heating in deionized water in a washed Wheaton glass vial	87.2	44.0	41.1	ratio of *R*:*S* isomer 46:53 after 1 h; oil bath temperature 106 °C
l	heating in deionized water Supelco glass vials (as received)	44.2	40.5	36.3	ratio of *R*:*S* isomer 47:53 after 1 h; oil bath temperature 108 °C
m	heating in deionized water in a washed Supelco glass vials	80.0	51.9	47.2	ratio of *R*:*S* isomer 49:51 after 1 h; oil bath temperature 108 °C
n	heating in deionized water in a Restek vial (as received)	51.9	41.6	40.6	ratio of *R*:*S* isomer 47:53 after 1 h; oil bath temperature 109 °C
o	heating in deionized water in a washed Restek glass vial	76.4	53.7	49.9	ratio of *R*:*S* isomer 52:48 after 1 h; oil bath temperature 109 °C
p	heating in high-density polyethylene (HDPE)	98.4	98.6	97.8	ratio *R*:*S* isomer 82:18 even after 3 h; oil bath at 110 °C

[a]The percentage of *R*-amygdalin remaining is expressed as chromatographic peak area as a function of time (note that in as-received glassware, side products form during boiling). [b]The ratio of *R*:*S* given in the column is expressed without regard for the minor side product that forms on boiling.

epimerization. Two early, very detailed, epimerization studies, which are more than four decades old, exist in the German literature.[23,24] The authors examined some of the factors influencing amygdalin epimerization such as (i) solvent, water, methanol, ethanol, ethyl acetate, and an azeotrope of methanol/ethyl acetate; (ii) temperature; and (iii) pH. The mandelonitrile stereogenic center changes configuration as indicated (Figure 1):

$$R\text{-amygdalin} \underset{k_S}{\overset{k_R}{\rightleftarrows}} S\text{-amygdalin} \tag{1}$$

$$\frac{[S]_{eq}}{[R]_{eq}} = \frac{k_R}{k_S} = K \tag{2}$$

Making measurements at different times in different conditions, Nahrstedt determined that the k_R and k_S kinetic rate constants were greatly affected by pH, solvent, and temperature but that the K thermodynamic equilibrium constant was little affected.[24] Similar observations have been made on the epimerization of lactose, where the equilibrium constant changed by only ∼10% in the temperature range of 25−49 °C.[25] This means that the epimerization equilibrium is reached more or less rapidly depending on the experimental conditions, but eventually produces a mixture of 43% *R*-amygdalin plus 57% *S*-amygdalin. This implies that, by nature, the *S*-isomer will predominate in a mixture.

Nahrstedt established that the amygdalin equilibrium constant was $K = 1.3$ with kinetic constants $k_R = 3.4 \times 10^{-4}$ s^{-1} and $k_S = 2.6 \times 10^{-4}$ s^{-1} in distilled boiling water.[24] Figure 3A shows the amygdalin epimerization data. Unfortunately, the details on water purification procedure, its storage, and glassware history were not provided in this work. In Figure 3B, we show a striking comparison when the glassware is thoroughly cleaned, deionized water (18.2 ΩM) is utilized, and there is minimum contact with other glassware. When amygdalin is heated in deionized water, the initial reaction rate is very slow (cf. Figure 3A), and after a certain period, it tends to proceed more rapidly. This reaction profile does not follow Nahrstedt's first-order kinetics in the beginning but eventually reaches the same equilibration ratio of products. We speculate that the water used by the author was perhaps distilled and stored in glass (which already contained enough "leachates" to accelerate the reaction). If the same *R*-amygdalin starting material solution is heated in the presence of glass pieces, made by crushing Pasteur pipets, the reaction proceeds more quickly with a >60 min difference in equilibration time. The glassware effect was tested using different heating methods in different flasks (direct heating on a mantle vs oil bath heating), and all produced general results; that is, initial conversion is slow and, after a certain period, the epimerization proceeds more rapidly.

We did an extensive study on the nature of the glassware (see Materials and Methods for glassware sources). Table 1 lists the epimerization amounts obtained in different conditions taken from the literature or obtained by our own experiments using the RPLC method with a cyclodextrin chiral stationary phase.[26] The first observation is that a protic solvent such as water is required for epimerization to occur; for instance, freeze-dried amygdalin samples were stable as were dimethyl sulfoxide solutions.[27] The epimerization is also negligible in ethanol at 65 °C; however, Nahrstedt noted that heating amygdalin in ethanol (95 °C oil bath temperature) also converted 14% of the *R*-amygdalin into the *S*-isomer. However, it was not clear whether the ethanol employed was anhydrous or not. In our

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initial part of the experimental work, we noted that dry amygdalin remains stable with respect to epimerization.

With the availability of modern columns and instruments, it is now possible to revisit early epimerization studies. The most interesting aspect is that the nature of the container seems to have a significant effect on the epimerization of amygdalin. In Table 1, we show an extensive list of glassware (vials) that was tested for epimerization. It is likely that modern researchers will use vials/containers from one of these major manufacturers for storing/extracting amygdalin samples. *R*-Amygdalin was heated in pure deionized water to assess the glassware effect and evaluate the conversion amounts. Examination of Table 1 will show a trend in epimerization. First, if vials are used as received, the epimerization of *R*-amygdalin is very rapid (e.g., Wheaton/Restek/VWR/Supelco). All vials are considered clean for routine analytical work. However, after thorough washing of the vials in a base bath, detergent, hot water, and deionized water, the reaction rates become slower. Eventually, in most cases, the *S*-isomer dominates. One interesting observation in Table 1 is that epimerization is almost nonexistent after boiling a distilled water amygdalin solution in a platinum vessel. The authors used nuclear magnetic resonance (NMR) for their studies.[27] As seen in Table 1, boiling amygdalin in high-density polyethylene bottles does not promote epimerization like Pt vessels. Even after 3h of heating in plastic, 80% *R*-amygdalin remained intact this work. On the basis of the information included in Table 1, it is clear that glassware plays a role in the epimerization because Pt and plastic vessels significantly slow the epimerization process. It is likely that different forms of glassware leach soluble components in hot deionized water (e.g., silicates), which act as weak bases, thus promoting the epimerization of amygdalin.

Analysis of Commercial Amygdalin Samples. Commercial amygdalin is usually sold as "vitamin B17". One major vendor sells injectable solutions of amygdalin with a label D-mandelonitrile-β-D-glucosido-6-β-glucoside in amber glass vials. We directly injected the commercial amygdalin samples to see the chromatographic profile of the commercial sample on core–shell bonded (*R*,*S*)-hydroxypropyl β-cyclodextrin. Figure 4 shows the chromatograms of the ampule components. It is clear that the major components are indeed amygdalin and its isomer. In each injectable ampule, the *S*:*R* amygdalin ratio is 54:46, which is very close to the equilibrium value of 57:43 reported by Nahrsted and from our own observations.[24] It is interesting to note that the pH of the injectable solution is 6.1. At such a pH the epimerization rate is very slow.[24] We note an additional impurity, which appears to be a side product of amygdalin degradation (see Figure 4). The chromatographic fraction at 0.4 min was analyzed by ESI-mass spectrometry. The m/z ratio of the peaks is 498.25, which corresponds to the sodium adduct of the cyano group in amygdalin being replaced by the amide. Thus, the natural amygdalin being sold as injectable solution is contaminated by >50% of unnatural *S*-isomer and smaller amounts (~5%) of the amide formed by the hydration of the nitrile group in amygdalin.

The *R*-amygdalin content in "vitamin B17" was quantified using an external calibration curve. The typical concentration of *R*-amygdalin was found to be 87245 ppm with a relative standard deviation of 0.2%. The limit of detection, as calculated from 3 times the standard error in the intercept, was calculated to be 14.6 ppm. All amygdalin standards had to be prepared in ~0.1% acetic acid solution to prevent significant epimerization.

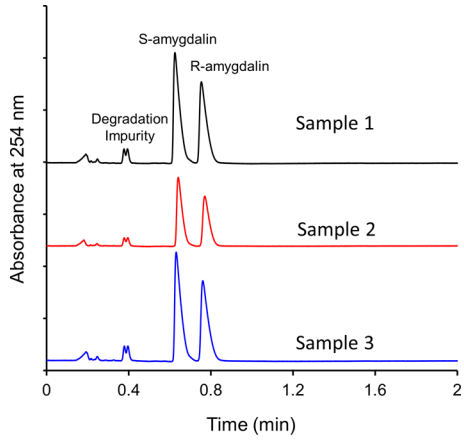

Figure 4. Ultrafast separation of commercial as-received injectable amygdalin solutions sold as vitamin B17 by a vendor. Chromatographic profiles of three separate vials are overlaid. Chromatographic conditions: 5 × 0.46 cm i.d. column containing 2.7 μm core–shell particles bonded with (*R*,*S*)-hydroxypropyl β-cyclodextrin; mobile phase, 10:90 MeOH/H_2O at 1.8 mL/min; injection volume, 0.03 μL; detection at 254 nm.

Standards prepared without acid epimerized by ~30% during the analysis.

Comments on Amygdalin Analysis and Storage. The important point when dealing with an amygdalin sample is to know the chemical nature of the compound considered. The naturally occurring amygdalin can convert into the undesired *S*-amygdalin during extraction, refluxing, and storage in various vials (all of which can be accelerated by pH, glass, and temperature). This trend is very clear in Table 1. Even worse, the amygdalin solution sold as an "alternative cure" to treat various illnesses is also sold in glass ampules. As stated earlier, most of the recent analytical methods have either neglected or missed the signal from *S*-amygdalin from the C_{18} columns. Although C_{18} or reversed phase cyano columns can practically separate the isomers as shown in the published results,[7,14] depending on the conditions, more recent studies do not seem to mention or notice the formation of the *S*-amygdalin.[4,10−12,15,16,18] Additionally, amygdalin is easily hydrolyzed by acids and bases. To reduce amygdalin hydrolysis by the digestive track, injectable solutions were sold (in alternative medicine, no FDA approval) in physiological solution and glass vials.[28] Depending on the nature of the glass making the vial, the storage time, and conservation temperature, it is most likely that epimerization changes the amygdalin content of such vials to 43% of the nominal label concentration with 57% of this concentration being *S*-amygdalin. Because both epimers can potentially release hydrogen cyanide, both are toxic.

In a recently published work, the ELISA method was proposed for amygdalin analysis. The problem is that the hapten–protein conjugate, needed to trigger the antibody formation by rabbits, was prepared in glassware at pH 9.4 using a carbonate–bicarbonate buffer for >6 h.[11] To see the effect of carbonate–bicarbonate buffer on amygdalin, we tested amygdalin epimerization in the same system at room temperature in a HPLC vial (which is certified to be clean from VWR, and at room temperature epimerization was negligible). The amygdalin–carbonate bicarbonate system was sampled after 10 min. Starting from 97% *R*-isomer, in just 30 min, 88% of the *R*-isomer remained. Within 2 h, only 68% of the *R*-amygdalin remained. After 3 h, 60% *R*-amygdalin

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remained, and the rest was converted into the *S*-isomer. Note that this isomerization is slower as compared to the ammonia method (complete epimerization is within 20 min). Hence, the ELISA-reported results are likely the global epimer concentration fitting the simultaneous HPLC analyses obtained with an achiral C_{18} column that was perhaps unable to separate the epimers and therefore gave a combined peak for the two forms.[11]

Amygdalin tablets and pills are still proposed commercially today as a natural dietary supplement under the wrong name of laetrile or the questionable name of "vitamin B17". There are scientifically unsupported blog comments suggesting health benefits and possible cancer remissions.[28] Dry products may contain pure amygdalin found in apricot seeds; however, vials of liquids (for intravenous injection) upon long-standing contain only 43% amygdalin and 57% of its epimerized form. Considering that 1 g of amygdalin can release 59 mg of HCN and that the lethal dose for human cyanide poisoning is about 2.5 mg/kg body weight, six tablets containing 500 mg of amygdalin each could theoretically kill an adult if the molecule is completely hydrolyzed, releasing 180 mg of HCN. Children have been reported to have been subjected to such poisoning.[17] From an analytical point of view, it is important to know that the epimerization of amygdalin can be fast in aqueous solutions depending on the temperature and pH. Furthermore, the containers of amygdalin aqueous solutions can accelerate epimerization as seen in Figure 4.

Because the separation of the two epimers is rapid and selective when cyclodextrin chiral columns or porous graphitic carbon columns are used in liquid chromatography, these approaches are recommended for all such assays. We suggest better practices for amygdalin storage. If glassware is being used for extraction, it should be thoroughly washed (as detailed under Materials and Methods) before use. Ideally, solution or extracts of amygdalin should be stored in inert containers such as high-density polyethylene (HDPE) and the solutions should be mildly acidic (e.g., 0.1% acetic acid). Some authors recommend using citric acid/ascorbic acid to prevent epimerization for long-term storage of amygdalin solutions.[29] We also suggest that the amygdalin solutions be refrigerated to slow the kinetics of epimerization. We also note that dry amygdalin is sold and stable in glass containers with respect to epimerization.

In this work, our aim was to set good guidelines for the analysis of amygdalin and potentially other molecules, which can easily epimerize during storage. In the case of amygdalin, the importance of using chromatographic columns with different selectivity than a typical C_{18} column is essential when a given analyte can epimerize easily during storage. Using the same unique selectivity columns on superficially porous particles, the analysis time can be reduced to sub-minute separations for the first time. We showed an important result that the epimerization of amygdalin is heavily glassware dependent; its treatment history and even "clean" vials from various vendors can show drastically different reaction rates of the conversion to the optical isomer (*S*-amygdalin). We explicitly show that even clean *as-received* glassware from various vendors can also significantly alter the rate of epimerization reactions.

AUTHOR INFORMATION

Corresponding Author

*(D.W.A.) Fax: (817) 272-0619. Phone: (817) 272-0632. E-mail: sec4dwa@uta.edu.

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