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Short communication

Analysis of cyanogenic glycosides by micellar capillary electrophoresis

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Abstract

The separation of amygdalin, prunasin and their isomers neoamygdalin and sambunigrin could be achieved with micellar capillary electrophoresis (MEKC). The two isomers were obtained in alkaline conditions and were produced in less than 15 min at pH 11.0. The developed methods showed a good selectivity in the separation of the isomers only in the presence of SDS micelles. The working pH was optimized to allow best resolution and quantitative analysis of these compounds. With a linear calibration over an injection time from 1 to 20 s, the detection limit was found to be in the range of 5 μM ($S/N=3$; 20 s injection time). Two pH buffer systems (pH 5.2 and pH 9.1) were chosen to confirm the peak attributions of the compounds in the apple and peach seeds samples. Sambunigrin was found in both apple and peach seeds but could not be quantified because of missing standards. Prunasin and amygdalin were not found in the apple sample, while they were quantified in the peach seeds in concentrations of 50 μg/g and 90 μg/g (dry weight), respectively.

Keywords: Cyanogenic glycosides

1. Introduction

Cyanogenic glycosides are derivatives of α-hydroxynitriles, widely diffused in the plant kingdom. Some common cyanoglycosides are linamarin (α-hydroxybutyronitrile-β-D-glucopyranoside) present in cassava plants [1], in white clover [2] and in flaxseed [3], prunasin (D-mandelonitrile-β-D-glucopyranoside) and amygdalin (D-mandelonitrile-β-gentiobioside) found in passion fruits [4], black cherries [5] or in apricots [6,7] and bitter almond kernels [8]. These compounds are able to release cyanide in concentrations that can be very toxic for humans and herbivores [8] like many of the vegetables containing cyanoglucosides that are used both in feed and in food industry.

Since many years, considerable efforts have been devoted to the development of a reliable method for cyanogenic compounds determination. This is generally accomplished by enzymatic or chemical hydrolysis of these substances to cyanide and its subsequent analysis [1,6,7] by enzyme membrane

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reactors [7], titration with a standard $AgNO_3$ solution. The released cyanide can also be oxidized to a cyanogen halide by chloramine T (or *N*-chlorosuccinimmide) and the following reaction with pyridine and coupling with pyrazolone gives a colored complex (König reaction) that can be can be easily determined. One of the major disadvantages of these approaches is that they do not allow the identification and quantification of the specific compound responsible for cyanide release. Direct analysis of cyanoglycosides were performed by some chromatographic methods like reversed-phase (RP) liquid chromatography with UV [5] or refractive-index detection [3] or cyanide quantification after enzymatic post-column cleavage of the cyanoglycosides [9]. Pulsed amperometric detection, which has been proved very reliable for the determination of carbohydrates [10] could also be used if a post-column addition of a strongly alkaline solution is carried out. Gas chromatography–mass spectrometry (GC–MS) has been employed, for instance, in the identification and quantification of amygdalin and prunasin in passion fruits [4].

Amygdalin (a) and prunasin (p), as well as their isomers neoamygdalin (n) and sambunigrin (s) have been reported to co-occur in various seeds. Chassagne et al. [4] have identified sambunigrin and prunasin, together with amygdalin, in passion fruits. Moreover, some species of Acacia have been reported to contain sambunigrin and/or prunasin [11]; a selective method for the simultaneous identification of isomeric cyanoglycosides is therefore needed to fully characterize the extracts. This can be accomplished by GC–MS analysis of the trifluoroacetic derivatives [4,11], by nuclear magnetic resonance (NMR) [11], or by high-performance liquid chromatography (HPLC) with RP- [9] or cyclodextrin (CD)-based stationary phases [4].

Despite the good performances of capillary electrophoresis (CE) in the determination of saccharides and plant secondary metabolites related compounds [12,13], to our knowledge this technique has not been used yet for the analysis of cyanoglycosides. Among the different modes of CE, micellar capillary electrophoresis (MEKC) has proved to be very selective for the analysis of neutral compounds and their isomers by addition of chiral phases in the separation buffer [14]. In this work is presented an original method for the identification of amygdalin and prunasin along with their isomers neoamygdalin and sambunigrin, by MEKC without using any chiral phase addition. The methods were used to identify and quantify these cyanoglycosides in seeds of apples and peaches.

2. Experimental

2.1. Apparatus

Separations were performed with a Beckman P/ACE 2050 Series HPCE with Beckman System Gold Chromatography Software ver. 7.11. The fused-silica CE column [107 cm (length to the detector 100 cm)×50 μm I.D.×375 μm O.D.] was obtained from Laser 2000, Wessling, Germany.

2.2. Separation and washing cycles

The separation runs were done at constant temperature (30°C) and voltage (25 kV) with a UV–Vis filter detection at 214 nm. Hydrodynamic sample injection for 3 to 20 s was the sample introduction mode for all experiments. For the spiking of the real samples, the standards of the four cyanoglycosides were pre-injected for 3 s.

The separation solutions were prepared from and 200 m*M* sodium dodecyl sulfate (SDS), 0.1 *M* acetate and 0.1 *M* acetic acid as well as from 0.1 *M* hydrogen carbonate and 0.1 *M* carbonate stock solutions; pH was adjusted with concentrated NaOH.

A 2-min washing cycle (high-pressure modus) with 0.1 *M* NaOH was followed by a 2-min conditioning of the capillary with the run buffer before the sample injection; each measurement was ended with a 2-min 0.1 *M* NaOH washing cycle.

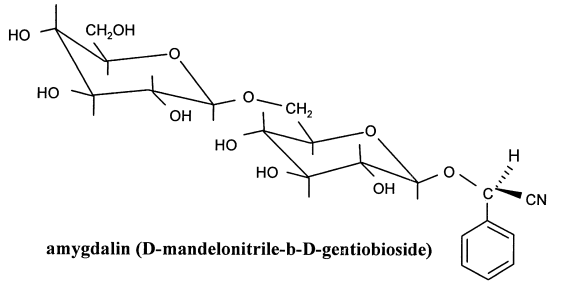
2.3. Extraction procedure

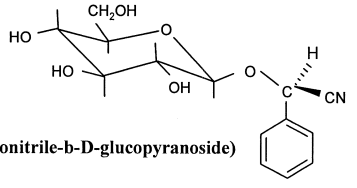
Apples (Cox Red) and peaches were bought on the local market, sliced and the seeds were collected. A 1-g amount of the ground dry seed was extracted for 30 min at 30°C in 25 ml of methanol–water (70:30, v/v) in an ultrasonic water bath [3]. The suspension was filtered with filter paper, the methanol was evaporated under vacuum at 40°C, and the

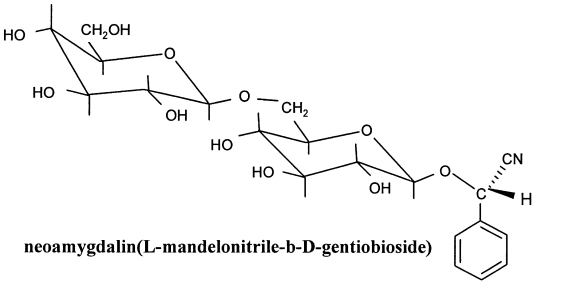
water was removed by freeze–drying. Before the injection, the dried sample was dissolved in 0.5 ml of distilled water, and filtered (0.2 μm).

2.4. Chemicals

Amygdalin and prunasin were obtained from Sigma–Adrich, Steinheim, Germany. The structures of the studied compounds is shown in Fig. 1. The isomerization was performed by simply bringing to pH 11 or 12 an aqueous solution of the two cyanoglycosides [4] for at least 15 min. The isomer-







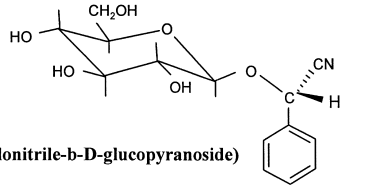


Fig. 1. Structures of analyzed cyanoglycosides and their isomers.

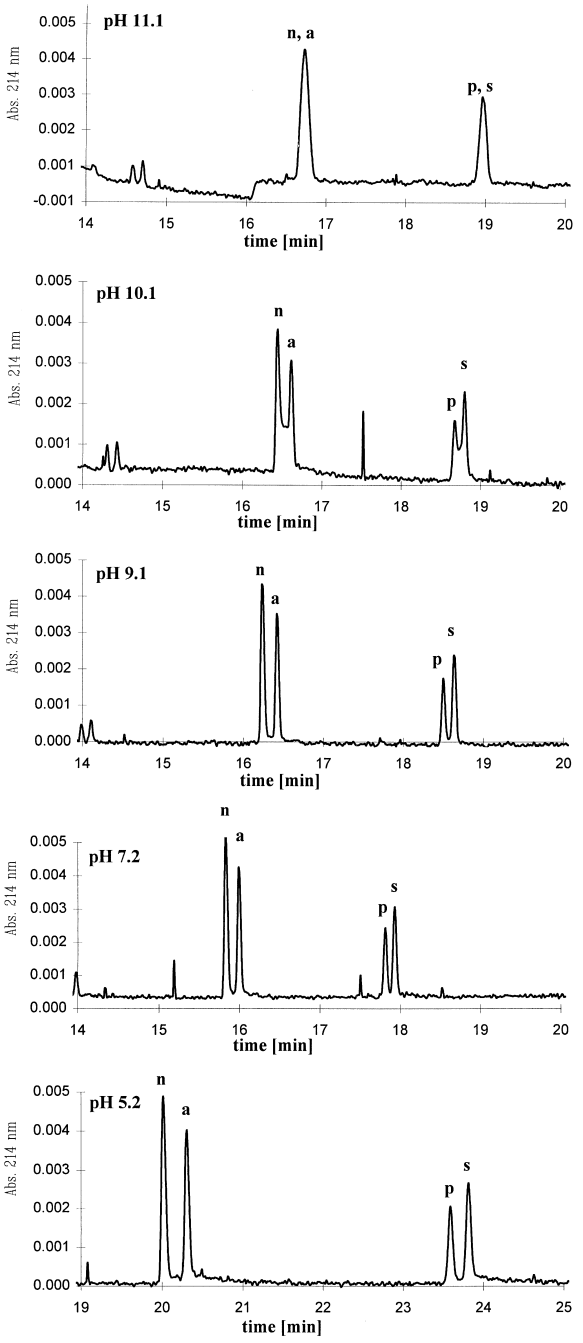


Fig. 2. Electropherograms of the separation of amygdalin (a), prunasin (p) and their isomers neoamygdalin (n) and sambunigrin (s) at different pH; the concentration of amygdalin and prunasin before isomerization was 0.5 m*M* (pH 5.2 and 7.2: 25 m*M* acetate buffer–100 m*M* SDS; pH 9.1, 10.1, 11.1: 25 m*M* carbonate buffer–100 m*M* SDS).

ized solutions were then neutralized and stored at 4°C.

3. Results and discussion

3.1. Separation of amygdalin, prunasin and their isomers by MEKC

Because the cited cyanogenic compounds are neutral we verified the performance of MEKC for their separation. Sambunigrin and neoamygdalin were not commercially available and we produced them by treatment of aqueous solutions of prunasin and amygdalin, respectively, with NaOH (pH 11 or 12) [4], yielding in each case a mixture of the two isomers. By simply changing the pH of a 100 m*M* SDS solution we already obtained a baseline separation of the isomers in slightly acidic, neutral and slightly alkaline solution without using any chiral additive. The isomers coeluted at pH higher than pH 11 (Fig. 2). The same elution order between the isomers was found using RP-HPLC [9] leading one to believe that similar interaction processes can be responsible for the isomeric selectivity in MEKC and RP-HPLC. With MEKC all four compounds were resolved while in RP-HPLC amygdalin was not resolved from sambunigrin and prunasin from neoamygdalin. The electropherograms obtained by MEKC of an aqueous mixture of amygdalin and prunasin before and after isomerization at pH 11 are reported in Fig. 3A and B. The isomerization was already completed after 15 min at room temperature (Fig. 3A); a small amount of sambunigrin and neoamygdalin was also observed in the three-day-old standard solution of amygdalin and prunasin in distilled water (Fig. 3B).

3.2. Quantification of amygdalin, prunasin and their isomers

The identification and quantification of these cyanoglucosides from real samples has to be performed at least in two different experimental con-

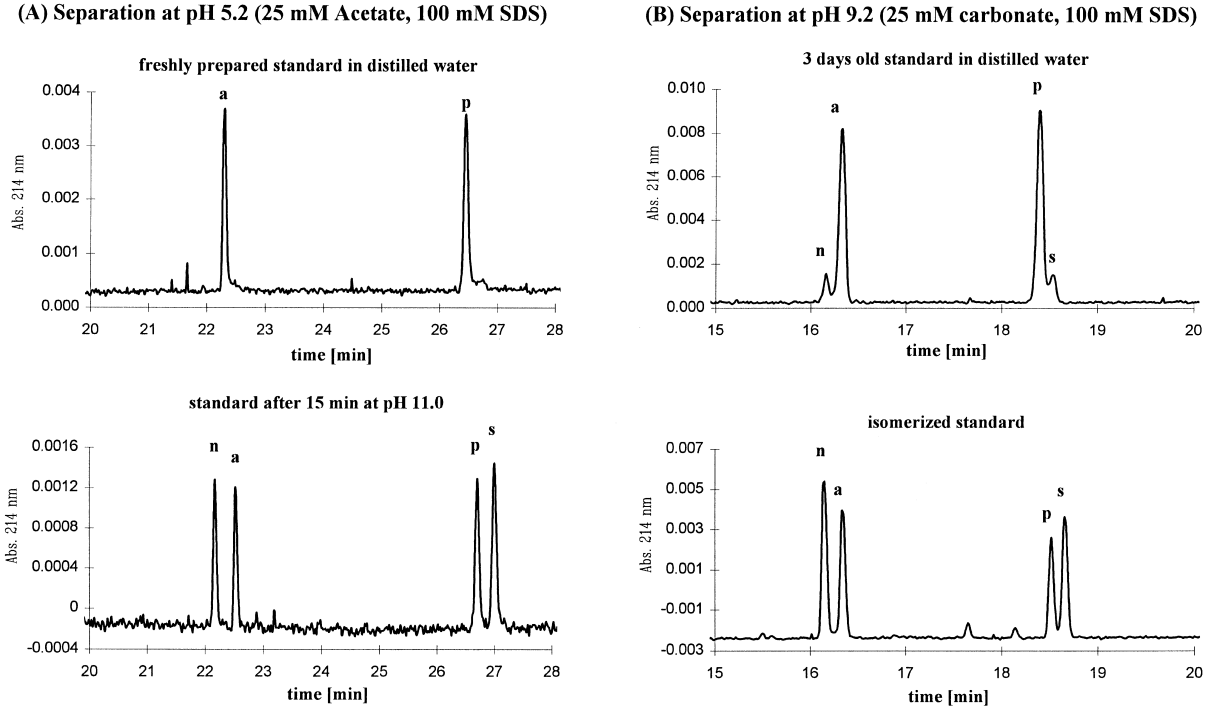


Fig. 3. Separations of the standard solutions of amygdalin (a), prunasin (p) and their isomers neoamygdalin (n) and sambunigrin (s) at pH 5.2 (A) and 9.2 (B) before and after isomerization (separation conditions as in Fig. 2).

ditions in order to verify the peak attributions. For this reason we chose two MEKC separation buffers at two different pH, i.e., acidic and alkaline conditions in which the compounds of interest were separated. The calibration plots were performed at pH 5.2 and 9.2 giving a linear range of the UV response to the concentration (0.05 to 0.5 m*M*) of amygdalin and prunasin always with $r^2>0.999$. Sambunigrin and neoamygdalin could not be quantified because of the missing standards. With a linear calibration over an injection time from 1 to 20 s ($r^2>0.998$), the detection limit was found to be in the range of 5 μM ($S/N=3$; 20 s injection time).

Fig. 4 shows the electropherograms of the apple and peach seeds extracts at the two chosen pH levels with and without spiking with the four cyanoglucosides for ''peak tracking''. A first screening of these samples was done at pH 9.2 and the suspected cyanoglucosides in the mixture were marked with a question mark. The second separation buffer at pH 5.2 confirmed the presence of only sambunigrin in the processed apple seeds (only qualitative identification of sambunigrin could be done). In the peach extract the presence of amygdalin, prunasin and sambunigrin could be verified and the quantification showed a concentration of 0.36 m*M* (90 μg/g dry

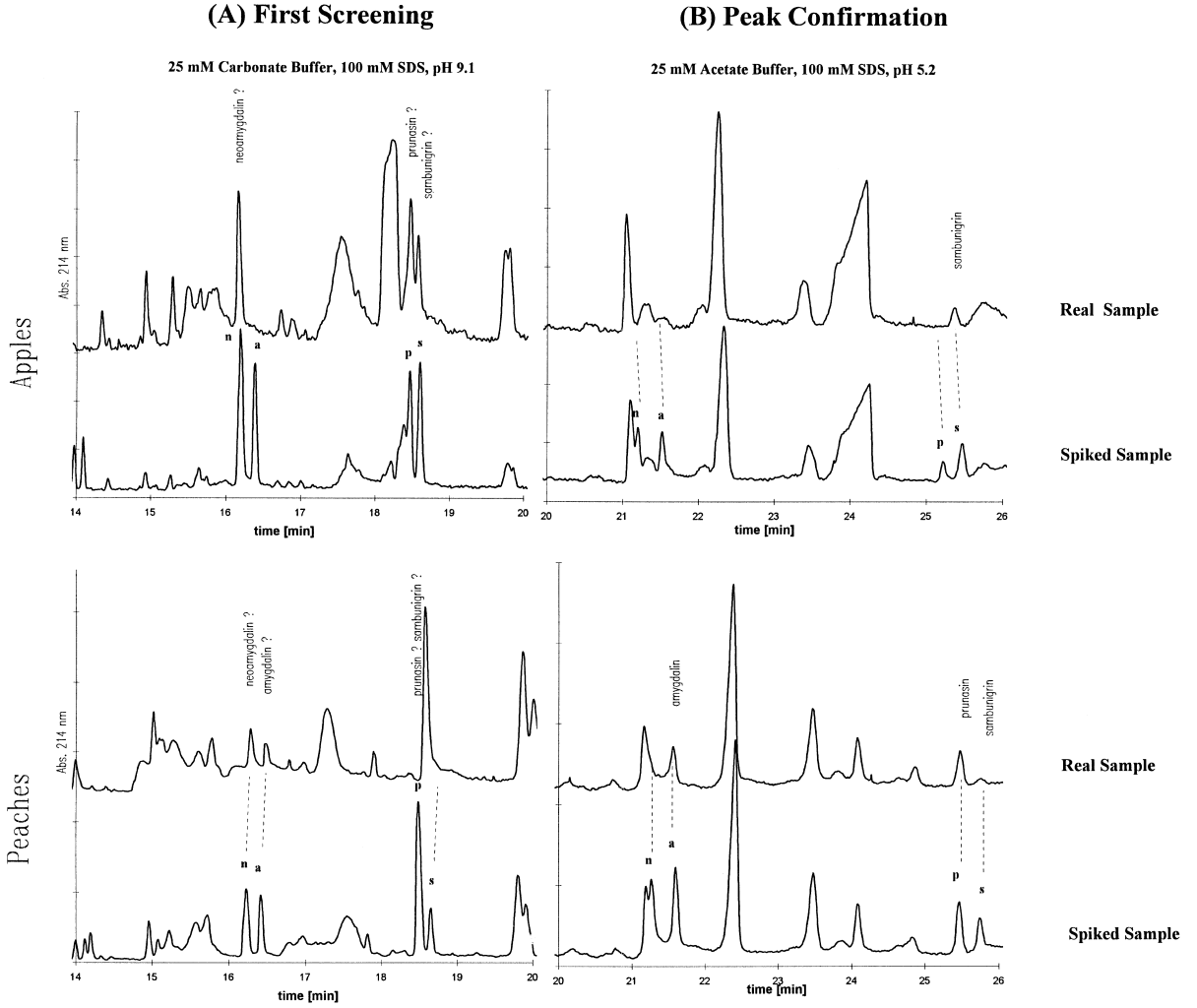


Fig. 4. Electropherograms of the apple (A) and peach seeds (B) extracts with two different buffer systems.

weight) for amygdalin and 0.33 m*M* (50 μg/g dry weight) for prunasin. This shows the usefulness of cross-checking the identity of peaks in complex matrix increasing the probability of correct identification

4. Conclusion

MEKC using UV detection at 214 nm allowed a good and rapid separation of some relevant cyanogenic glycosides. Only the presence of the anionic micelles (SDS) allowed a baseline resolution of amygdalin and prunasin from their *S*-isomers; the addition of a chiral agent in the separation buffer was not necessary. Identification and quantification of these compounds from real matrices was performed and showed the importance of cross-checking the results with two separation methods.

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