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Short communication

Liquid chromatographic determination of aniline in table-top sweeteners based on pre-column derivatization with 1,2-naphthoquinone-4-sulfonate

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Abstract

A liquid chromatographic method for the determination of aniline in cyclamate sweeteners based on a pre-column derivatization with 1,2-naphthoquinone-4-sulfonate (NQS) is proposed. Aniline traces were extracted from the cyclamate samples using dichloromethane. After solvent evaporation, the dry residue was derivatized with NQS at pH 9.5 and 85°C for 1 min. The aniline derivative, which was extracted from the reacting mixture, was redissolved in the eluent solution and injected into the chromatographic system. The separation of aniline derivative from other amine impurities was carried out in a C_{18} column using a 2% acetic acid–methanol (40:60, v/v) mobile phase. Results from the analysis of aniline in the sweetener samples with the proposed method were compared with those from the standard method. A good concordance between the two methods was observed.

Keywords: Derivatization; Sweeteners; Aniline; 1,2-Naphthoquinone-4-sulfonate; Cyclamate

1. Introduction

This paper describes a chromatographic determination of aniline in cyclamate samples. Cyclamate salts are widely used as non-caloric sweeteners in diet food and beverages, pharmaceutical products and table-top sweeteners. Aniline is a common impurity present in these samples which has been classified as priority organic pollutant; besides, other amines (e.g., cyclohexylamine and dicyclohexylamine) are often present in the cyclamate samples. The European Pharmacopoeia limits the maximum permissible concentration of aniline in table-top sweeteners to 1 ppm [1]. Some analytical methods for its control and determination, in industrial and dye wastes and pesticides fields, have been proposed, which include gas chromatography (GC) [1–3], liquid chromatography [4,5], colorimetry [6] and flow injection analysis [7].

In this study a new HPLC method for the analysis of aniline in cyclamate samples based on the pre-column derivatization with NQS and subsequent separation of its corresponding derivative is proposed. This method takes advantage of the simplicity and analytical characteristics of the HPLC techniques over the existing GC methods [1] for determining aniline in this type of sample. In these cases, owing to the low concentrations of aniline, a previous

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extraction and a derivatization procedures were required in order to improve the sensitivity and selectivity. As described in the literature [8–13], the derivatization of NQS with primary and secondary amino groups is developed, in general, in basic medium (pH 8.5–10.5) at temperatures from 25 to 70°C and reaction times from 5 to 40 min. NQS has been used successfully in liquid chromatography for the analysis of amphetamines [8–12] and amino acid-related compounds [13,14].

2. Experimental

2.1. Reagents, solutions and samples

Solutions were prepared with Millipore water.

Chromatographic eluent was prepared from acetic acid (Panreac, analytical grade) and methanol (Panreac, HPLC grade). The reagent solution consisted of 3×10^{-2} *M* sodium 1,2-naphthoquinone-4-sulfonate (Carlo Erba, analytical grade) in 0.1 *M* hydrochloric acid (Merck, analytical grade). The buffer solution was 0.05 *M* sodium borate+0.085 *M* sodium hydroxide (both from Merck, analytical grade).

Chloroform (Sigma, HPLC grade) was used for the extraction of the aniline derivative, while toluene (Carlo Erba, analytical reagent) and dichloromethane (Merck, HPLC grade) were used for the extraction of aniline from the sweetener tablets. Other solutions for the extraction were: 10 *M* sodium hydroxide solution (Merck, analytical reagent), 1 *M* acetic acid solution.

Aniline hydrochloride was supplied by Sigma, analytical grade. The internal standard solution for the HPLC method was 10^{-5} *M* cyclooctylamine (Fluka, analytical grade). The internal standard for the standard method was a 10^{-6} *M* tetradecane (Fluka, analytical grade) in dichloromethane [1].

Several low-calorie sweetener tablets containing sodium cyclamate were analysed: Acofarinas (Acofarma, Terrassa, Spain), Assugrin (Hermes Sweeteners, Switzerland), Natreen (Q.F. Bayer, Barcelona, Spain) and Nutter (Laboratorios Ordesa, Sant Boi de Llobregat, Spain). The amount of sodium cyclamate in these samples ranged between 40 and 50 mg per tablet.

2.2. Apparatus

The mobile phase was pumped with an LKB Bromma 2150 HPLC pump. The sample was injected by a Spark Holland Promis automatic injection system. The analytical column was a 150×4.6 mm I.D. Spherisorb ODS 2 column with 5-μm particle size. Chromatograms were monitored at 278 and 305 nm using a Kontron Instruments HPLC 430 detector furnished with a flow cell of 10 mm path length and 8 μl dead volume. Data were acquired with a Perkin-Elmer (PE) Nelson 900 interface coupled to a personal computer and stored for further analysis. A gas chromatograph Hewlett-Packard 5890 series II plus equipped with an FID detector was used for the analysis of aniline according to the standard method. In this case, the analytical column was a 3.8 m×3.2 mm I.D. WAW 80/100 Silicosteel Tecknokroma with a 10% Carbowax 20 *M*+2% KOH stationary phase.

2.3. HPLC procedure

An amount of 1 g of sweetener sample was dissolved in 5 ml of water in an stoppered glass vial. Subsequently, 0.5 ml of internal standard solution (10^{-5} *M* cyclooctylamine) and 0.5 ml of 10 *M* sodium hydroxide solution were added. As the resulting solution was made basic, aniline was shaken with 6 ml of dichloromethane for 1 min. The two phases were separated by centrifugation at 2500 rpm for 2 min. A volume of 4.5 ml of organic phase was recovered and the solvent was allowed to evaporate. The resulting dry residue, which contained the aniline, was derivatized according to the procedure described below. Standards for calibration were prepared in a similar way. In this case, volumes of 5 ml of standard aniline solutions with concentrations ranging from 2×10^{-6} to 3×10^{-7} *M* were used in the extraction procedure.

The aniline derivatization was carried out in a vial by mixing 250 μl of reagent solution (3×10^{-2} *M* NQS in 0.1 *M* HCl)+250 μl of buffer solution (0.05 *M* sodium borate+0.085 *M* sodium hydroxide)+250 μl of aniline solution. The reaction was developed at 85°C and pH 9.5 for 1 min.

The aniline derivative was then extracted with 375 μl of chloroform for 0.5 min at room temperature. The separation of the two phases was facilitated by

centrifugation at 2500 rpm for 2 min. A total of 325 μl of the organic phase recovered was allowed to evaporate and the dry residue was redissolved in 130 μl of mobile phase solution. The solution obtained in this way was injected into the chromatographic system. The injection volume was 25 μl.

The separation of the aniline derivative from derivatives of other amines present as impurities in the cyclamate samples was performed with a 2% acetic acid–methanol (40:60, v:v) mobile phase. The flow-rate was kept constant at 0.8 ml/min during the separation.

2.4. Standard procedure

The standard procedure [1] for the determination of aniline was as follows. An amount of 2 g of sweetener sample was dissolved in 18 ml of water; 0.5 ml of 10 *M* sodium hydroxide solution was added to make basic the medium. Aniline was extracted with 30 ml of toluene for 2 min and the mixture was then centrifuged at 4000 rpm for 3 min in order to separate the two phases. Twenty ml of the toluene solution recovered were treated with 4 ml of 1 *M* acetic acid solution; in acid medium, aniline was extracted into the aqueous phase. Finally, 3.6 ml of acetic acid solution containing aniline was first basified with 0.5 ml 10 *M* sodium hydroxide and extracted with 200 μl of dichloromethane (with 10^{-6} *M* tetradecane as internal standard) by shaking both phases vigorously. The volume of organic solution injected into the gas chromatograph was 1.5 μl.

Aniline standard solutions were subjected to the same treatment as samples.

For the gas chromatographic separation the flow-rate of helium through the column was 30 ml/min, and temperatures of the injector, column and detector were set to 150, 140 and 250°C, respectively.

3. Results and discussion

3.1. Study of HPLC conditions

Various NQS solutions with concentrations ranging from 0.01 to 0.1 *M* were prepared. In all cases, the concentration of hydrochloric acid was 0.1 *M*. Results obtained showed that peak areas corresponding to the aniline derivative were approximately constant in this range, so that an intermediate NQS concentration (0.03 *M*) was chosen.

The influence of pH on the reaction was studied in the range 6.5–11.5 by using different buffer solutions composed of sodium dihydrogenphosphate, sodium borate and sodium hydroxide. The chromatographic signal was almost constant throughout the whole range, which suggested that the reaction was completely developed. The buffer solution finally chosen was a 0.05 *M* sodium borate+0.08 *M* sodium hydroxide, which provided a derivatization pH of 9.5.

Temperature and reaction time were interrelated variables, which had to be simultaneously optimised. In this study, the temperature was varied from 26 to 85°C, and the reaction time from 1 to 60 min. Results obtained are represented as a response surface, as shown in Fig. 1. The experimental conditions selected were those which led to the maximum development of the reaction in a minimum time. These conditions corresponded to 1 min at 85°C. Higher reaction times and temperatures were not advisable because they may cause a degradation of the aniline derivative [13,14].

Methanol and acetonitrile percentages (45–60 and 20–40%, v/v, respectively) in the aqueous mobile phase were independently investigated; results with 60% of methanol were similar to those with 35% of acetonitrile. Acetic acid as organic modifier was studied from 0 to 3%. A mobile phase consisting of 2% acetic acid aqueous solution/methanol (40:60, v/v) provided suitable separation of the aniline derivative from other peaks (e.g., excess of NQS, degradation products, and other amine derivatives).

3.2. Extraction of the aniline derivative

The extraction of the aniline derivative was applied to remove the interfering peaks from polar compounds (e.g., the excess of NQS and degradation products of NQS) and to improve the sensitivity by concentrating the aniline derivative. Various organic solvents were studied to carry out the extraction of the aniline derivative. Among them, chloroform, dichloromethane, hexane, five diethyl ether and hexane–diethyl ether mixtures were assayed. The

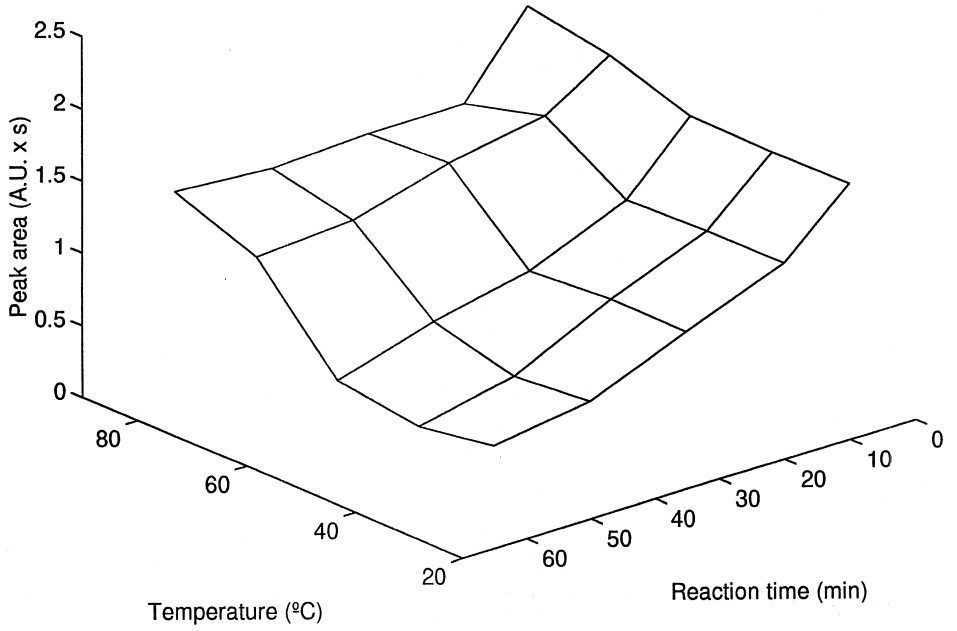

Fig. 1. Effect of temperature and reaction time on the peak area of the aniline derivative. Derivatization conditions: reagent solution, 0.03 *M* NQS+0.1 *M* HCl; buffer solution, 0.05 *M* $NaBO_2$+0.083 *M* NaOH (pH 9.5); 4×10^{-4} *M* aniline; volume of reagent, buffer and aniline solutions, 100 μl each; acidification, 40 μl 0.25 *M* HCl. Chromatographic conditions: injection volume, 100 μl; mobile phase, 2% acetic acid–methanol (40:60, v/v); flow-rate, 0.8 ml/min.

extraction was quantitative for dichloromethane and chloroform, while hexane, diethyl ether and their mixtures led to poorer recoveries (ranging from 60 to 90%).

As an example, Fig. 2 shows the chromatograms for aqueous and organic fractions after the extraction process using chloroform and hexane as solvents. The aqueous phases showed, in both cases, several peaks corresponding to the excess of NQS and degradation products (all these peaks appeared at retention times shorter than 4 min). Furthermore, the peak of the aniline derivative was absent in the aqueous phase for chloroform study, which suggested that the extraction was, thus, quantitative. Conversely, when hexane was used, a significant amount of aniline derivative was still present in the aqueous phase. As regards the organic phase, both chromatograms showed only one peak, which indicated that, the NQS excess and its degradation products remained in the aqueous phase. The peak height of aniline derivative for hexane was lower than for chloroform as hexane did not recover it quantitatively.

The effect of the extraction time on the recovery of the aniline derivative was studied in the range 0.5–8 min. In all cases, the extraction was quantitative, so even short extraction times were sufficient to ensure a total extraction. The time finally selected was 1 min. The ratio of volumes for the aqueous and organic phases was also optimised. It was found that a chloroform volume six times lower than the aqueous volume led to a quantitative recovery of the aniline derivative.

The aniline derivative was found unstable in the reaction medium (it degraded after 10 h) but stable for 7 days in the mobile phase.

3.3. Figures of merit

For the assessment of the figures of merit of the method proposed, parameters such as peak height, peak area and those relative to the internal standard

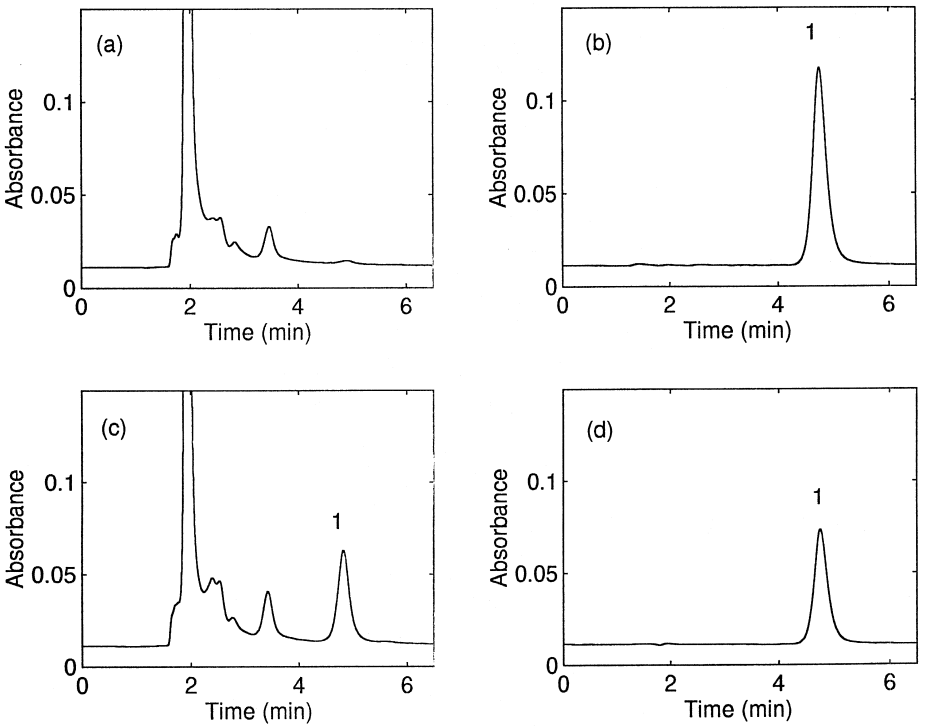

Fig. 2. Chromatograms of the aqueous and organic phases obtained after the extraction of the aniline derivative with chloroform (a,b) and hexane (c,d). (a,c) The aqueous phases and (b,d) the organic phases. Derivatization conditions: reagent solution, 0.03 *M* NQS+0.1 *M* HCl; buffer solution, 0.05 *M* $NaBO_2$+0.083 *M* NaOH (pH 9.5); 4×10^{-4} *M* aniline; volume of reagent, buffer and aniline solutions, 100 μl each; acidification, 40 μl 0.25 *M* HCl. Chromatographic conditions: injection volume, 100 μl; mobile phase, 2% acetic acid–methanol (40:60, v/v); flow-rate, 0.8 ml/min. Extraction conditions: organic solvent volume, 600 μl; extraction time, 1 min. Peak assignment: (1) aniline derivative.

(I.S.) were compared in order to determine which one provided better precision. The optimum parameter was the relative peak area (i.e., $A_{\text{aniline}}/A_{\text{I.S.}}$). At 278 nm the response was linear up to 3×10^{-3} *M*, the straight line equation was $A_{\text{aniline}}/A_{\text{I.S.}} = 0.982C_{\text{aniline}}/C_{\text{I.S.}}+0.013$, with a correlation coefficient $r=0.9995$, and the detection limit was 5.4×10^{-7} *M* (5.0 ng aniline when injecting 100 μl). The run-to-run precision (repeatability) studied at a 5×10^{-4} *M* aniline concentration was 2.9% for the injection of six standard solutions. The day-to-day precision (reproducibility) studied at a 5×10^{-4} *M* aniline concentration was 4.5% for six non-consecutive days; in this case, fresh reagent, buffer, and eluent solution were prepared daily. The repeatability and reproducibility for the retention time gave relative standard deviations of 1.2 and 1.4%, respectively.

3.4. Determination of aniline in commercial sweeteners

Owing to the toxicological significance of aniline, this compound present at low μg/g levels as impurity in various commercial sweetener samples was determined using the proposed method. The extraction of the aniline traces was carried out in basic medium (approximately at pH 14) using dichloromethane as organic solvent, as detailed in Section 2. Apart from aniline, cyclohexylamine and dicyclohexylamine were commonly present in the cyclamate samples and also derivatizable with NQS. The

concentrations of such compounds were 10–20 times higher than that of aniline. In all cases, the excess of reagent was sufficient to derivatize quantitatively the aniline. This HPLC procedure was able to separate their peaks from the aniline derivative in order to avoid interferences. As an example, Fig. 3 shows the chromatogram corresponding to a sweetener sample, in which the peak of the aniline derivative can be observed. Other peaks in the chromatogram corresponded to the internal standard and other amine derivatives. Analyses were carried out in triplicate. Results using the present method were compared with those from the standard method based on gas chromatography (see Table 1 for average values and standard deviations). From this table, a good concordance between the two methods was found. A *t*-test of paired measurements at a significant level $\alpha=0.05$ showed that there were no significant differences between both methods, as $t_{calc}=2.049$ was lower than the corresponding critical value (for three degrees of freedom t_{crit} was 3.182).

Table 1
Determination of aniline in sweeteners using the method proposed (HPLC method) and the standard method

Brand name	HPLC method (μg aniline/g cyclamate)	Standard method (μg aniline/g cyclamate)
Natreen	0.14±0.02	0.14±0.01
Acofarinas	0.14±0.01	0.12±0.01
Nutter	0.16±0.02	0.12±0.01
Assugrin	0.15±0.02	0.14±0.01

4. Conclusions

Aniline can be successfully determined in cyclamate samples using the present HPLC method with

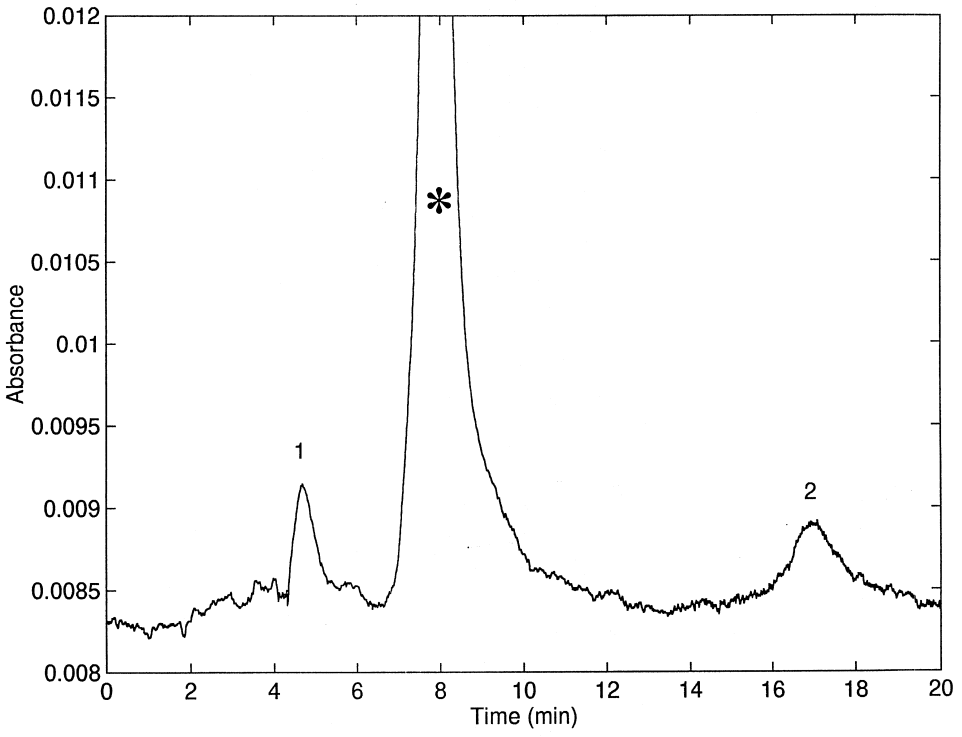

Fig. 3. Chromatogram at 278 nm of an extract of a commercial sweetener product. Conditions for the recovery of the aniline for the sample: sample amount, 1 g; aqueous solution, 6 ml; dichloromethane, 6 ml; pH 14; extraction time, 1 min. See Fig. 2 for other conditions. Peak assignment: (1) aniline derivative; (2) internal standard; (*) amine derivatives.

pre-column derivatization of the analyte with 1,2-naphthoquinone-4-sulfonate. The excess of reagent and degradation compounds, which may interfere with the chromatographic peak of aniline derivative, can be easily circumvented since they remain un-extracted in the aqueous solution. Results are comparable in terms of precision and accuracy to the standard method. The sensitivity and detection limits make the method suitable for analyzing aniline at low concentrations.

Acknowledgements

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