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ORIGINAL ARTICLE

Analysis of multiple sweeteners and their degradation products in lassi by HPLC and HPTLC plates

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Abstract A solid phase extraction method using C_{18} cartridges was standardized for the isolation of multiple sweeteners (aspartame, acesulfame-K and saccharin) and their degradation products (diketopiperazine, L-phenylalanine, acetoacetamide and 2-sulfobenzoic acid) from *lassi*. Analytical conditions for HPLC were standardized over C_{18} column using UV detector for the simultaneous separation and estimation of multiple sweeteners and their degradation products in *lassi* sample isolates. A simple cartridge free method was developed for the isolation of sucralose from *lassi*. Method was also standardized for qualitative detection and quantitative estimation of sucralose over amino and silica gel plates of HPTLC.

Keywords Lassi · Saccharin · Acesulfame-k · Aspartame · Sucralose · HPLC · HPTLC

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Introduction

The use of artificial sweeteners like saccharin, acesulfame-k, aspartame and sucralose has been permitted in *halwa, khoya burfi, rasogolla, gulabjamun* and other milk products (PFA 2004). Since, the use of artificial sweeteners in dairy products is new, development of methods for qualitative as well as quantitative analysis of sweeteners and their degradation products in dairy products is necessary. Approval of new sweeteners, combined with the key role played by sweetener blends in the production of foods and beverages, have led us to analyze multiple sweeteners (aspartame, acesulfame-K, saccharin and sucralose) in *lassi*, a popular indigenous dairy product containing sugar. Use of artificial sweeteners in combination reduced the level of sweeteners required as compared to that used singly. Keeping in view the significance of use of multiple sweeteners, the present work was carried out for simultaneous isolation and analysis of sweeteners and their degradation products in *lassi* by HPLC and HPTLC.

Materials and methods

Sweeteners and their degradation products: Saccharin and its degradation product 2-sulfobenzoic acid, aspartame degradation products viz., 2, 5-diketopiperazine and L-phenylalanine, acesulfame-k and its degradation product acetoacetamide (Sigma-Aldrich, Lovfs, Missouri, USA), aspartame (NutraSweet Sweetener Company, 1762, Lovers Lane, Augusta, Georgia, USA), sucralose (Tate and Lyle Sucralose, Inc., Decatur, Illinios, USA).

Chemicals: Phosphoric acid, sulphuric acid, cetyltrimethylammonium bromide solution, di-potassium hydrogen phosphate and potassium dihydrogen phosphate (AR grade, Qualigens Fine Chemicals, India); silica gel 60 F_{254} (Kieselgel 60 F_{254}) HPTLC aluminium sheets (5 × 7.5 cm) and NH_2 F_{254S} HPTLC glass plates (10 × 10 cm) (E. Merck India Ltd.); acetonitrile, dichloromethane, methanol and

water (HPLC grade, Qualigens Fine Chemicals, India); Carrez solution No.1: 3.6 g of potassium ferrocyanide dissolved in 100 ml water; Carrez solution No.2: 7.2 g of zinc sulphate dissolved in 100 ml water; mobile phase A: 0.02 M phosphate buffer (pH 5.0): acetonitrile (97:3); mobile phase B: 0.02 M phosphate buffer (pH 3.5): acetonitrile (80:20).

Standard solutions of aspartame, acesulfame-K, saccharin and their degradation products: Ten mg each of the sweeteners and their degradation products were dissolved separately in 10 ml mixture of mobile phases A and B (1:1), to get standard solutions each with a concentration of 1 mg/ml. One hundred µl of each stock of standard solution of sweeteners and degradation products were pipetted out into separate 10 ml volumetric flasks and the volume was made up to mark with mixture of mobile phases A and B (1:1) to get standard solutions each with concentration of 10 ng/µl. This process was repeated with 125 µl of the stock solution to get concentration of 12.5 ng/µl. One hundred µl each of the stock standard solutions of aspartame, acesulfame-K, saccharin, 2, 5 diketopiperazine, L-phenylalanine, acetoacetamide and 2–sulfobenzoic acid were pipetted into a 10 ml volumetric flask. The volume was made up to the mark with mixture of mobile phase A and B (1:1), to get a concentration of 10 ng/µl of each component as in the mixed solution.

Equipments: Cream separator (Kamdhenu, KD-60E, Benny, India), vacuum filtration assembly (Millipore Millipore, Model XI1004704, USA), solid phase extraction C_{18} cartridge (Supelco, Bellfonte, PA, USA), ultra-sonifier (SONICS, Vibra Cell, Model VCx750, Newton, CT, USA), solid phase extraction vacuum manifold (Visiprep™ DL, Supelco, Bellfonte, Pennsylvania, USA), BOD Incubator (Narang Scientific Works Pvt. Limited, India), filter paper (Whatman No.1, England), electronic balance (Mettler AT-200, Switzerland), mixer (Braun, India), UV chamber (LABCO, India), hot air oven (Tempo, India), muffle furnace (Narang Scientific Works Pvt. Ltd, India), electric heater (Vikrant, Jain Enterprises, India), HPLC system (Shimadzu LC10A), Detector: UV, SPD-M10AV, Solvent filter: 0.45 µm, 47 mm diameter- durapore (Millipore), syringe: blunt type (Hamilton), loop injector: 20 µl. Bio-rad quantity one software for quantification over HPTLC plates.

Preparation of lassi: Buffalo milk samples were collected from Experimental Dairy of the institute, and sugar was procured from local market. A mixed culture (NCDC-167) was obtained from National Collection of Dairy Cultures (NCDC), Microbiology Division of the institute. Control *lassi* with sucrose (15%) and artificial sweetener used *lassi* samples with best optimized quaternary [aspartame × acesulfame-K × sucralose] × saccharin sweetener blend 70:30, 0.036% (i.e. aspartame 122.5 ppm, acesulfame-K 122.5 ppm, saccharin 75 ppm and sucralose 35 ppm) was prepared and it had the same sweetness and consistency / viscosity (cp) as the sucrose sweetened *lassi* (control).

Sample preparation and HPLC analysis: The sample preparation procedure (Fig. 1) used for the isolation of sweeteners (aspartame, acesulfame-K and saccharin) and their degradation products (diketopiperazine, L-phenylalanine, acetoacetamide and 2-sulfobenzoic acid) was essentially based upon the method of BSEN (1999). The elution with 10 ml of mobile phase A followed by 10 ml of mobile phase B gave maximum recovery of aspartame, acesulfame-K and saccharin (Arora et al. 2008).

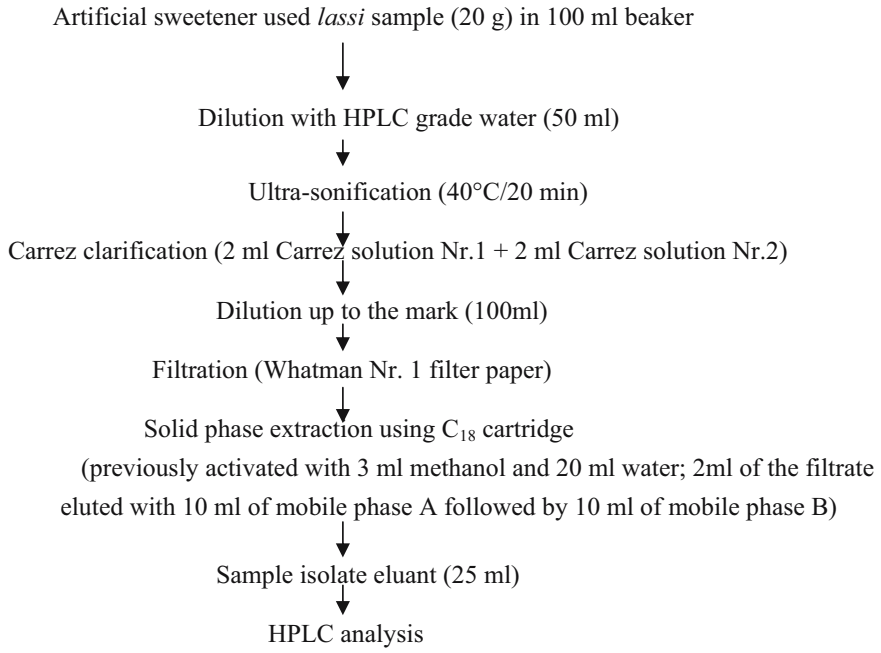

Fig. 1 Flowchart for isolation of sweeteners

Reverse phase HPLC analysis of reference standards of aspartame, acesulfame-K, saccharin and their available degradation products, 2, 5 diketopiperazine, L-phenylalanine, acetoacetamide and 2–sulfobenzoic acid and sample isolates from *lassi* were performed over Shimpak C_{18}, S-5 µm, 120A, 250 × 4.6 mm ID column. HPLC analysis was performed using binary gradient programming with mobile phase A and mobile phase B for 30 min using flow rate of 1 ml/min at UV 200 nm and 220 nm (Fig. 2). Gradient conditions were 0% mobile phase B for first 8 min, 0–100% B linear gradient from 8–13 min, 100% B from 13–25 min, 100–0% B linear gradient from 25–27 min and 0% B from 27–30 min. The actual pressure varied during programming was 80-160–80 kgmf.

Five-point calibration curves were plotted for aspartame, acesulfame-K, saccharin and their respective degradation products. Curves were prepared by injecting 5 µl (50 ng), 10 µl (100 ng), 15 µl (150 ng), 20 µl (200 ng) of standard solution (10 ng/ µl) and 20 µl (250 ng) of standard solution (12.5 ng/ µl) (since sample loop used was of 20 µl capacity) of sweeteners and degradation products. For sucralose, the curves were plotted by injecting 4, 8, 12, 16 and 2 µl of its standard solution (5 µg/µl). Calibration curves were then drawn by plotting concentration against peak area and correlation coefficients (R^2) determined. Linear regression equation was determined for each sweetener and degradation products standards to see the linearity of the system.

Recovery experiments of aspartame, acesulfame-K, saccharin and their respective degradation products were performed at 800, 800, 500 and 625 ppm (each) respectively in *lassi*. The detection limits of the HPLC system were also determined.

Isolation of sucralose and analysis over HPTLC plates Isolation of sucralose from *lassi* (Fig. 3) for further qualitative analysis was done by the method of Spangenberg et al. (2003) and for quantitative analysis over HPTLC plates was carried out by the method of Arora et al. (2009).

Qualitative detection over amino HPTLC plates: Ten microlitres of standard sucralose solution in methanol (1 µg/10 µl) and 10 µl of the sample filtrate were applied to a NH_2 F_{254S} HPTLC plate, which was previously activated in an oven at 100°C for 30 min. The plate was developed in a vertical chamber consisting of acetonitrile: water (4:1). The developed plate was removed from the chamber, dried and was subsequently heated at 190°C for 20 min in a muffle furnace. The fluorescent spots obtained were visualized under UV light at 365 nm. Treatment of the developed plate with 5 g/100 g methanolic cetyltrimethylammonium bromide solution enhanced the fluorescence. The detection limit of the NH_2 F_{254S} HPTLC system for sucralose was determined by reducing the concentration till the spot disappeared.

Quantitative analysis over silica gel HPTLC plates: Ten µl of the standard sucralose solution in methanol (1 µg/10 µl) and 10 µl of the sample filtrate were applied to silica gel 60 F_{254} (Kieselgel 60 F_{254}) HPTLC aluminium sheets, which were previously activated in an oven at 100°C for 30 min. The plate was developed using solvent system, dichloromethane: methanol (4:1). The developed plates were then sprayed with methanolic sulphuric acid (15 ml/100 ml) and dried. The plates were subsequently heated at 100°C for 10 min. The plates obtained were scanned and quantified by comparing with the standard sucralose spot density using Bio-rad quantity one software. The standard sucralose solution was obtained by dissolving 10 mg of sucralose in 100 ml of HPLC grade methanol to give a concentration of 1 µg/ 10 µl; 2.5, 5.0, 7.5, 10 and 12.5 µl of the standard solution were applied on the HPTLC silica gel plates at a distance

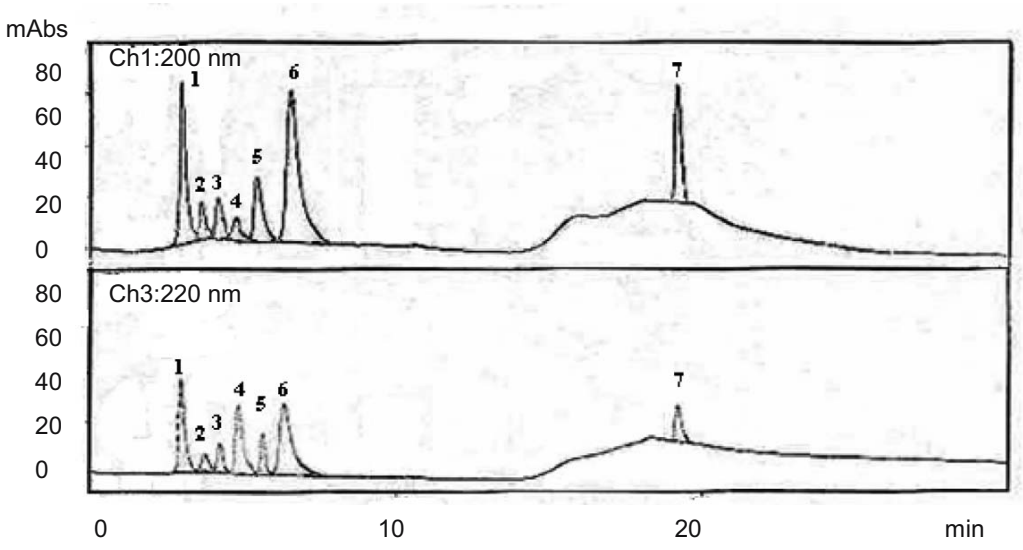

Fig. 2 HPLC chromatogram of sweeteners and their degradation products standards
(1) Diketopiperazine [2.953 min] (2) Acetoacetamide [3.192 min] (3) 2-Sulfobenzoic acid [3.420 min] (4) Acesulfame-K [3.789 min] (5) L-phenylalanine [3.990 min] (6) Saccharin [4.440 min] (7) Aspartame [19.190 min]

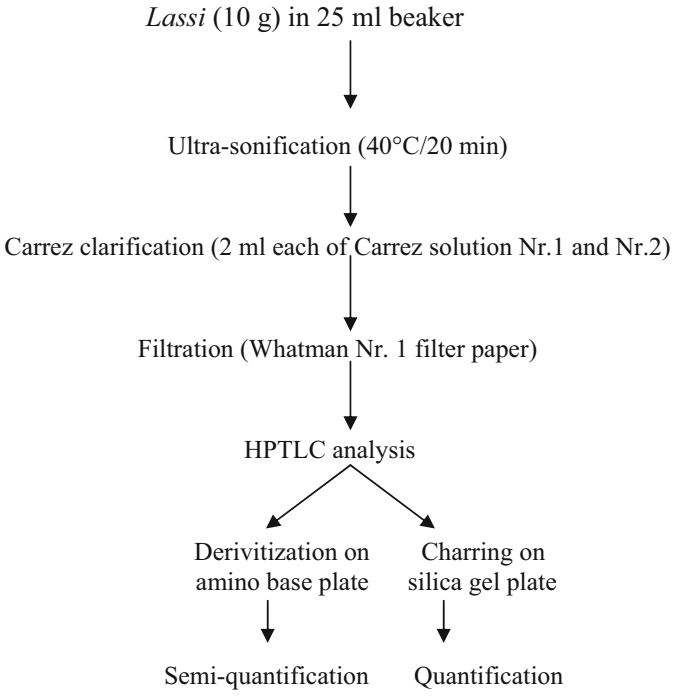

Fig. 3 Isolation of sucralose from *lassi*

of 1 cm, previously activated at 100°C for 30 min in an oven. The plates were dried, developed, sprayed, scanned and quantified to get the standard curve for sucralose. The detection limit of the silica gel HPTLC system for sucralose was determined by reducing the concentration till the spot disappeared. Recovery experiments were performed at 300 ppm level of sucralose in *lassi*.

Statistical analysis: Statistical analysis was carried out according to Snedecor and Cochran (1989). All analyses were done in triplicates.

Results and discussion

Sample preparation and HPLC analysis of multiple sweeteners and their degradation products: The sample preparation procedure (Fig. 1) was standardized for the isolation of sweeteners (aspartame, acesulfame-K and saccharin) and their degradation products (diketopiperazine, L-phenylalanine, acetoacetamide and 2-sulfobenzoic acid) in *lassi*. The method involved ultrasonification, Carrez clarification (protein precipitation), filtration and clean-up of fat/sample matrix through C_{18} cartridges.

The method of elution using mobile phase A and mobile phase B was standardized. Two ml of the clarified filtrate were added to the previously activated C_{18} cartridge and the sweeteners and their degradation products were eluted with 10 ml of mobile phase A followed by 10 ml of mobile phase B. This pattern of elution gave maximum recovery of all the three sweeteners and their degradation products. It was observed that single mobile phase A (20 ml) could not elute aspartame. Hence, elution with 10 ml of mobile A followed by 10 ml of mobile phase B was needed for efficient recovery of sweeteners and their degradation products.

All sweeteners and their degradation products showed λ max at 200 nm except for acesulfame-K, which absorbed maximum at 220 nm. Hence, the wavelength, 200 nm was selected for the analysis of all the sweeteners and their degradation products for the subsequent analysis of *lassi* sample isolates. However, sample isolates containing acesulfame-K were also scanned at 220 nm due to its sensitivity at this wavelength. The mobile phase gradient commenced at pH 5.0. This was required to ensure elution and separation of six components: (1) diketopiperazine (2) acetoacetamide (3) 2-sulfobenzoic acid (4) acesulfame-K (5) L-phenylalanine and (6) saccharin. The pH was lowered to 3.5 for the elution of component (7) aspartame. The large shift in baseline was due to this binary gradient programming (Fig. 2).

The chromatogram showed a narrow separation of diketopiperazine and acetoacetamide, acetoacetamide and 2-sulfobenzoic acid and acesulfame-K and L-phenylalanine. Flow rates of 0.8 and 0.5 ml/min were not successful. Hence, flow rate of 1 ml/min was selected for the maximum separation of the seven components. Set of experiments with mobile phase A [0.0125 M phosphate buffer, pH 3.5: acetonitrile (98:2, 95:5, 90:10, 85:15, 80:20, 70:30, 50:50)] and mobile phase B [0.0125 M phosphate buffer, pH 3.5: acetonitrile (90:10)] were also tried at flow rate of 1.0, 0.8 and 0.5 ml/min but these did not improve the separation. Therefore, the analytical conditions as described in materials and methods were used for the analysis of sample isolates of *lassi*. The regression equations and correlation

coefficient (R^2) for the enlisted seven components were determined. Correlation coefficient (R^2) of 0.99 for all the sweeteners and their degradation products showed the linearity of the system. The detection limits for the various enlisted sweeteners (Table 1) and their degradation products were in the range of 10–40 ng at 200 nm. The expected concentration in the sample isolates was in the range of 24–256 ng/20 µl injection volume (Arora et al. 2008). Hence, the sample isolates of *lassi* could be successfully analyzed over HPLC under the standardized analytical conditions as described earlier. Further the detection limits of acesulfame-K (40 ng), saccharin (10 ng), and aspartame (30 ng) were in accordance with those obtained by Lawrence and Charbonneau (1988) over C_{18} column at 200 nm (acesulfame-K: 20 ng; saccharin: 5 ng and aspartame: 24 ng). The detection limits of acetoacetamide, 2-sulfobenzoic acid, diketopiperazine and L-phenylalanine were 20, 10, 10 and 20 ng, respectively. However, no information regarding the detection limits of these degradation products of the sweeteners is available in the literature for comparison. Results of recovery experiments performed for. aspartame, acesulfame-K and saccharin and their degradation products are presented in Table 2. Recoveries of all the sweeteners and their degradation products were in range of 88–99%.

Table 1 Detection limits of sweeteners and degradation products

Sweetener/Degradation product	Detection limits (ng) at 200 nm
Aspartame	30
Acesulfame-K	40, 15**
Saccharin	10
Diketopiperazine	10
L-phenylalanine	20
Acetoacetamide	20
2-Sulfobenzoic acid	10

**Detection limit at 220 nm

The detection limit of sucralose was observed to be 20 µg (Arora et al. 2009) which was found to be too high to detect the expected concentrations (11.2–128 ng/20 µl) of the sweetener in the sample isolates of *lassi* if prepared by the isolation procedure involving solid phase extraction clean-up. Hence, an alternative isolation as well as analytical procedure using HPTLC plates (Arora et al. 2009) was followed for the estimation of sucralose in *lassi*.

Isolation procedure for sucralose: A simple procedure (Fig. 3) was used for the isolation of sucralose from *lassi*. The procedure involved ultrasonification, Carrez clarification and filtration followed by clean-up of fat/ sample matrix over HPTLC plates. This eliminated the use of C_{18} cartridges. In case of *lassi*, 10 g sample was subjected to ultrasonification, precipitation of proteins by Carrez solution and filtration. This resulted in concentrated sample isolate (1000 ng/10 µl) as compared to the sample isolates prepared by the isolation procedure as discussed involving solid phase extraction clean-up (Arora et al. 2009).

Qualitative detection over amino HPTLC plates: The qualitative detection of the standard sucralose was performed by separating the sucralose extract on amino TLC plate and derivatizing it reagent free by heating the developed plate at 190°C for 20 min. At this temperature, sucralose reacts with the amino groups of the HPTLC layer and forms a brilliant fluorescent spot (a fluorescent derivative of unknown structure) through Maillard reaction by losing water and forming high molecular mass derivative (Spangenberg et al. 2003). The fluorescent spots obtained were visualized under UV light at 365 nm. The detection limit of sucralose was 25 ng. Spangenberg et al. (2003) reported similar observations for the detection limit of sucralose for visual semi-quantification under UV light at 365 nm.

Quantification procedure over silica gel HPTLC plates: The quantitative analysis of standard sucralose was performed over silica gel HPTLC plates. The separation was performed in vertical developing chamber and was accomplished within 15 min. Subsequently, the dried plate was sprayed with 15 ml/100 ml sulphuric acid, heated at

Table 2 Recoveries of sweeteners and degradation products in spiked samples of *lassi*

Sweetener/Degradation Product (level ppm)	Recovery, %			Mean ± SEM
	I	II	III	
Aspartame (800)	90.6	88.2	89.6	89.4 ± 0.72
Acesulfame-K (800)	96.6	95.6	98.6	96.9 ± 0.89
Saccharin (500)	92.7	93.8	94.4	93.6 ± 0.49
Diketopiperazine (625)	95.7	94.7	95.2	95.2 ± 0.28
L-phenylalanine (625)	94.8	95.7	92.9	94.4 ± 0.83
Acetoacetamide (625)	93.4	96.2	95.2	95.0 ± 0.81
2-Sulfobenzoic acid (625)	94.6	96.8	95.9	95.8 ± 0.63
Sucralose (300)*	96.0	97.2	99.2	97.5 ± 0.91

(n = 3) * Recoveries using silica gel HPTLC plates

120°C for 10 min in an oven. At this temperature, sucralose appeared as charred spots having an R_f value of 0.40.

The regression equation and correlation coefficient obtained for sucralose were Y= 600.25X + 446.91 and 0.9999 respectively, showing linearity of the system. The detection limit over silica gel HPTLC plates for sucralose was 250 ng. The HPTLC method developed is simple, fast, requires less clean-up and highly sensitive as compared to HPLC method (Arora et al. 2009) and the previously reported HPLC methods reporting detection limits of 1600 ng and 2400 ng over UV and RI detectors, respectively (Lawrence and Charbonneau 1988).

Recovery experiments were performed for sucralose over silica gel HPTLC plates as delineated in Table 2. Recoveries of sucralose were in the range of 96–99%, which clearly demonstrated the efficiency of the method.

Conclusion

An isolation procedure involving pre-solid phase extraction and solid phase extraction using C_{18} cartridges was standardized for the isolation of multiple sweeteners (aspartame, acesulfame-K and saccharin) and their degradation products (diketopiperazine, L-phenylalanine, acetoacetamide and 2-sulfobenzoic acid) from *lassi* followed by HPLC analysis of the sample isolates. Recovery of the method was 88–99%. HPLC analytical conditions were standardized over C_{18} column using UV detector for the simultaneous separation and estimation of multiple sweeteners (aspartame, acesulfame-K and saccharin) and their degradation products (diketopiperazine, L-phenylalanine, acetoacetamide and 2-sulfobenzoic acid) in *lassi* sample isolates in a single run using binary gradient programming. Detection limits of sweeteners and their degradation products were in the range of 10–40 ng at 200 nm. A simple method was standardized for the isolation of sucralose from *lassi* with a recovery of 96–99%. A method was standardized for qualitative detection (semi-quantification) of sucralose in *lassi* over amino HPTLC plates. A simple, fast, sensitive, requiring less clean-up method for the quantification of sucralose over silica gel HPTLC plates was also developed.

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