Analytical Methods

Cite this: *Anal. Methods*, 2011, **3**, 2836

www.rsc.org/methods

PAPER

Fast analysis of flavonoids in apple juice on new generation halo column by SPE-HPLC

Imran Ali,*[a] Salma M. Z. Al-kindy,[b] Fakhreldin O. Suliman[b] and Syed Dilshad Alam[a]

Received 12th August 2011, Accepted 8th September 2011
DOI: 10.1039/c1ay05496a

A fast SPE-HPLC method is described for the analysis of flavonoids in apple juice. The mobile phase used was water-acetonitrile (60 : 40, v/v) at 0.80 mL min^{-1} flow rate with UV detection at 252 nm. The column used was new generation Halo C_{18} (100 × 46 mm; shell particles; 2.7 μm). The capacity factors were in the range of 0.67 to 20.56, respectively. The separation and resolution factors ranged from 1.18–2.21 and 1.00–10.00, respectively. The values for LOD and LOQ for flavonoids ranged from 0.40–5.5 ng and 2.25–26.0 ng, respectively. The linearity was observed in the concentration ranges of 0.01 to 0.10 mg mL^{-1} for all flavonoids. The concentrations of quercetin, apigenin, kaemferol and chrysin in apple juice were 4.00, 0.50, 0.40 and 0.25 mg kg^{-1}, respectively. Therefore, the reported method can be used for the quality control of apple juice in food industries. Besides, this method can also be used for the analyses of flavonoids in other fruits and vegetables.

Introduction

The health benefits of several phenolic compounds mainly flavonoids have been a subject of great interest. The varied biological properties of flavonoids such as anti-oxidants and anti-allergic and anti-inflammatory *etc.* have stimulated interest in these compounds. The important benefits include improvements in cardiovascular health (decreased blood pressure, inhibit platelet aggregation and prevent thrombus formation), eye diseases, allergic disorders, arthritis, cancers, asthma *etc.*[1–4] The most important flavonoids are quercetin, fisetin, apigenin, kaemferol, 3,6-dihydroxyflavone, chrysin, galangine, and 3-hydroxyflavone due to their wide range of beneficial biological properties (Fig. 1). The flavonoids are found in many fruits and vegetables with apple being a good source of many flavonoids. That is why it is well said that an apple a day keeps the doctor away. Apples belong to Rosaceae family and genus Malus. The most cultivated apples are of *Malus pumila* genus *i.e., M. domestica*. Apples are composed of water (85%), carbohydrates (11%), dietary fibers (2%), fat (0.6%), organic acids (0.5%) and protein (0.3%). The determination of flavonoids in apples is of great importance to ascertain the quality of apples and the health concerns. Several HPLC methods have been reported in the literature to separate and quantify flavonoids in apples and processed apple products[5–10] but only a few methods describe apple juice analyses during processing. These methods are not economic and applicable in the present scenario. In these methods, HPLC chromatogram experimental times varied from 30 to 60 min, which seems to be quite high. The solvent systems used were costly having a high amount of acetonitrile. Moreover, the limits of detection have not been reported; except one method.[7] Solid phase extractions reported in these articles seem to be time and chemical-consuming methods. No detection limit has been given and, hence, difficult to compare their working efficiencies. Moreover, no time has been given for solid phase extraction

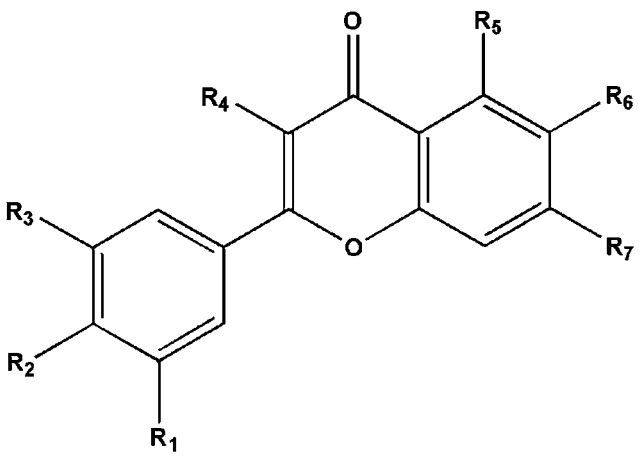

Fig. 1 Chemical structures of flavonoids; fisetin: ($R_2 = R_3 = R_4 = R_7 =$ OH), quercetin: ($R_1 = R_2 = R_4 = R_5 = R_7 =$ OH), apigenin: ($R_2 = R_5 = R_7 =$ OH), kaempferol: ($R_2 = R_4 = R_5 = R_7 =$ OH), 3,6-dihydroxyflavone: ($R_4 = R_6 =$ OH), chrysin: ($R_5 = R_7 =$ OH), galangin: ($R_4 = R_5 = R_7 =$ OH), 3-hydroxyflavone: ($R_4 =$ OH) and others: (R = H).

[a]*Department of Chemistry, Jamia Millia Islamia (Central University), New Delhi, 110025, India. E-mail: drimran_ali@yahoo.com; drimran.chiral@gmail.com; Web: http://jmi.ac.in/Fnat/imran_ch.htm; Fax: +0091-11-26985507; Tel: +091-9211458226*
[b]*Department of Chemistry, College of Science, Sulatn Qaboos University, P.O. Box 36, Muscat, 123, Oman*

methods. Therefore, a fast and economic HPLC method is required for apple juice quality control during its processing.

Nowadays, speed and economy are the most important aspects in HPLC analyses including flavonoids as thousands of batches are analyzed every month in juice processing industries for quality control. Worldwide, the strong economic pressure of increasing price of many food stuffs necessitates the enhancement of analyses throughput in order to reduce manufacturing cost. Recently, a special type of porous silica gel based column of super fast speed has been introduced into the market, which is called new generation Halo column. The column contains superficially porous particles (shell particles; 2.7 μm) providing super fast speed and reducing run time by 70% or more. Recently, Ali *et al.*[11] reviewed the applications of Halo columns and found them suitable for superfast analyses by using simple HPLC machine; without the need of costly UPLC. There is a great need to develop various HPLC methods for analyses of different compounds using Halo columns. Similarly, analysis of flavonoids using Halo column is of utmost importance; especially in quality control where thousands of experimental batches are carried out every month. In view of these facts, attempts have been made to develop and validate a fast, inexpensive, reproducible, accurate, effective and selective method for the qualitative and quantitative analyses of flavonoids by using new generation Halo column. The developed method was validated and utilized for the qualitative and quantitative analyses of flavonoids in fresh apple juice. To the best of our knowledge, this is the first manuscript that reports a rapid and simultaneous determination of flavonoids. The results of these findings are presented herein.

Experimental

Chemical and reagents

3-Hydroxyflavone, 3,6-dihydroxyflavone, galangin, quercetin and apigenin standards were purchased from Aldrich Chem. Co., USA. The other flavonoids such as chrysin and kaempferol were obtained from Fluka Chem. Co., USA while fisetin was supplied by Sigma Chem. Co., USA. Acetonitrile and methanol of HPLC grade were purchased from Fisher Scientific, Fairlawn, New Jersey, USA. Disodium hydrogen phosphate and *o*-phosphoric acid of A.R. grade were supplied by Merck, India, Mumbai. Purified water was prepared by using a Millipore Milli-Q, Bedford, USA, water purification system.

HPLC instruments

HPLC system used was of ECOM (Prague, Czech Republic) consisting of solvent delivery pump (model Alpha 10), manual injector, absorbance detector (Sapphire 600 UV-Vis.), chromatography I/F module data integrator (Indtech. Instrument, Mumbai, India) and Winchrome software. The column used was new generation Halo [Halo C_{18} (100 × 46 mm)] of Advance Material Technology, USA. Solid Phase Extraction (SPE) unit was purchased from Varian USA. C_{18} cartridges were obtained from Waters, Milky Way, USA. pH meter of Control Dynamics (model APX 175 E/C), centrifuge of Remi (model C-30BL) and membrane filter of Millipore, Ireland, were used.

Preparation of standard solutions

Stock solutions (0.10 mg mL^{-1}) of each flavonoid and their mixtures were prepared in methanol and were protected from sun light by covering with aluminium foil. A series of standard solutions of the flavonoids ranging from 0.01 to 0.10 mg mL^{-1} were prepared by appropriate dilution of the stock solutions with methanol.

Extraction of apples juice

Five *M. domestica* apples (500 g) were taken and washed with Millipore water (3.0 L) three times and air dried. The juice was extracted by means of a domestic juice maker and 200 mL juice was obtained. The juice was centrifuged at 10 000 rpm (11180 g) for 10 min. The juice was extracted by solid phase extraction immediately as described below.

Solid phase extraction of apple juice

To determine the errors, the standard solutions of flavonoids were loaded on to C_{18} cartridges and analyzed by HPLC. A mixture of 40.0 μL (0.10 mg L^{-1}) of all eight flavonoids was mixed with phosphate buffer (50.0 mL, 0.25 mM, pH 8.0) and shaken for 15 min and kept for a further 15 min. Sep-Pak C_{18} cartridges (1.0 mL Waters, Milky Way, USA) were pre-conditioned with 2.0 mL methanol followed by 5.0 mL Millipore water. 50.0 mL of phosphate buffer was passed through the cartridge at a 0.1 mL min^{-1} flow rate followed by washing with 5.0 mL Millipore water at the same flow rate. The cartridge was dried by passing hot air and the flavonoids were eluted by 25.0 mL methanol at 0.1 mL min^{-1} flow rate. The eluted solution was concentrated under vacuum to 1.0 mL and was used for HPLC analysis. The optimization of SPE was achieved by investigating different concentrations and pHs of phosphate buffer, flow rate of flavonoids sample and eluting solvents. The percentage recoveries were calculated and the error was determined for each flavonoid. The optimized solid phase extraction (SPE) method was applied for the extraction of flavonoids in apple juice. 10 mL Apple juice was mixed with phosphate buffer (40.0 mL, 0.25 mM, pH 8.0) and shaken for 15 min and kept for further 15 min. SPE method was used as described above and the final extraction volume was 1.0 mL for HPLC analyses. It is important to mention here that the extraction of juice from apple and its SPE consumed only 15 and 20 min, separately and respectively.

HPLC conditions

All the experiments were carried out by HPLC system as described above. The aliquots of 2.0 μL of standard solutions of each flavonoids and their mixture (0.10 mg mL^{-1} in methanol) were injected onto HPLC column. The mobile phase used was water-acetonitrile (60 : 40, v/v) in isocratic mode (0.8 mL min^{-1}). The mobile phase was prepared, filtered and degassed daily before use. All the experiments were carried out at 27 ± 1 °C temperature with detection at 252 nm. The chromatographic parameters such as retention (k), separation (α) and resolution (Rs) factors were calculated. The order of elution was ascertained by running individual flavonoids. The qualitative and quantitative analyses were carried out by using retention times and peak

areas, respectively. The chromatographic method was optimized and validated by carrying out extensive experimentation. The optimized and validated HPLC method was applied for analysis of the flavonoids in fresh apple juice.

Validation of HPLC method

The validation of HPLC method was carried out by using LOD, LOQ, linearity, specificity, accuracy, precision and ruggedness.

LOD and LOQ

The limit of detection (LOD) and the limit of quantitation (LOQ) were determined as 3 and 5 times the baseline noise, respectively, following the United States Pharmacopoeia.[12] The results of the statistical analyses of the experimental data such as relative standard deviation, coefficients of determination (r^2) and confidence limits were calculated by Microsoft Excel software program. Good linearity of the calibration graphs and the negligible scatter of experimental points are clearly evident by the values of the coefficients of determination (r^2) and relative standard deviations.[13]

Linearity

Equal volumes (2.0 μL) of the standard solutions and apple juice preparation as described above were injected onto HPLC column and the chromatograms recorded. The calibration standards of each concentration (0.01–0.10 mg mL^{-1}) were analyzed five times. The calibration curves of all flavonoids were constructed by using the observed peak areas *versus* nominal concentrations of the analytes.

Specificity

The specificity of the method was investigated by observing any interference in the chromatographic parameters due to the presence of some impurities in apple juice.

Accuracy

The different concentrations of flavonoids were used to determine the accuracy of HPLC method. Three concentrations, *i.e.*, 0.01, 0.05 and 0.10 mg mL^{-1} were used and chromatographic runs carried out five times ($n = 5$). The accuracy was determined by interpolation of five replicate peak areas of these molecules.

Precision

To calculate precision data, three different concentrations (0.01, 0.05 and 0.10 mg mL^{-1}) of each flavonoid were used. Five sets of the chromatographic runs were carried out for all the three concentrations.

Ruggedness

The ruggedness of the method was determined by the versatility of the experimental factors that affected the peak areas.

Results and discussion

Extraction from apple juice

The flavonoids were extracted as discussed above and the calculated recoveries were 96.8 to 98.0%, respectively (Table 1). The different concentrations and pHs of phosphate buffer, flow rate and eluting solvents were tried but the best one is reported herein. The good percentage recoveries (96.8 to 98.0%) were due to the experimental pH of the buffer, in which these flavonoids get adsorbed on C_{18} cartridge resulting into good adsorption. Consequently, the adsorbed flavonoids were desorbed from cartridge by methanol. The relative standard deviation, coefficient of determination (r^2) and confidence limit were 2.51, 0.9999 and 99.7 for the extracted flavonoids. On HPLC analyses quercetin, apigenin, kaempferol and chrysin were found in SPE extracted liquid.

HPLC method development

The separation and identification of flavonoids was carried out on the column and mobile phase as described earlier. The capacity (k), separation (α) and resolution (Rs) factors for these compounds in standard solutions and apple juice are calculated and given in Table 2. The chromatograms of standard (fisetin, quercetin, apigenin, kaempferol, 3,6-dihydroxyflavone, chrysin, galangin and 3-hydroxyflavone) and apple juice (quercetin, apigenin, kaempferol and chrysin) flavonoids are shown in Fig. 2 and 3, respectively. Fig. 2 clearly indicates a base line separation of standard flavonoids within 25 min. The chromatograms of individual flavonoids were also recorded under the identical chromatographic conditions and the order of elution was determined. The order of elution of flavonoids was fisetin > quercetin > apigenin > kaempferol > 3,6-dihydroxyflavone >

Table 1 Regression analyses data for the extraction of flavonoids from standard solutions[a]

	Extraction			
Flavonoids	Recoveries (%)	%RSD	Coefficient of determination (r^2)	Confidence limits (%)
Fisetin	96.8	2.51	0.9999	99.7
Quercetin	97.0	2.51	0.9999	99.7
Apigenin	97.5	2.51	0.9999	99.0
Kaempferol	98.0	2.51	0.9999	99.8
3,6-Dihydroxyflavone	96.0	2.51	0.9999	99.7
Chrysin	96.8	2.51	0.9999	99.7
3-Hydroxyflavone	96.3	2.51	0.9999	99.7

[a] $n = 5$.

Table 2 Chromatographic and precision data of flavonoids[a]

Flavonoids	α	*Rs*	RSD	Coefficient of determination (r^2)	Confidence limits (%)
Standard Solutions					
Fisetin (0.67)-quercetin (1.48)	2.21	1.60	2.35–2.38	0.9998–0.9999	99.70–99.80
Quercetin (1.48)-apigenin (2.42)	1.64	2.35	2.33–2.37	0.9998–0.9999	99.71–99.81
Apigenin (2.42)-kaempferol (2.86)	1.18	1.03	2.32–2.36	0.9998–0.9999	99.71–99.81
Kaempferol (2.86)-3,6-dihydroxyflavone (7.26)	2.54	2.60	2.34–2.37	0.9996–0.9999	99.70–99.80
3,6-Dihydroxyflavone (7.26)-chrysin (9.54)	1.31	2.68	2.33–2.36	0.9997–0.9999	99.72–99.81
Chrysin (9.54)-galangin (12.08)	1.27	2.99	2.32–2.36	0.9998–0.9999	99.71–99.81
Galangin (12.08)-3-hydroxyflavone (20.56)	1.70	10.00	2.33–2.37	0.9996–0.9999	99.72–99.81
Apple Extract					
Quercetin (1.48)-apigenin (2.42)	1.64	2.22	2.36–2.39	0.9998–0.9999	99.68–99.79
Apigenin (2.42)- kaempferol (2.56)	1.06	1.00	2.35–2.38	0.9998–0.9999	99.69–99.79
Kaempferol (2.56)-chrysin (9.55)	3.73	7.87	2.35–2.38	0.9997–0.9999	99.68–99.79

[a] *k*: Capacity given in parenthesis, α = Separation and *Rs*: Resolution factors. Column: Halo C_{18} (100 × 46 mm) [Advance Material Technology, USA]. *Sample Volume*: 2.0 μL. *HPLC Conditions*: Isocratic; *Mobile Phase*: Water-acetonitrile (60 : 40, v/v); *Flow Rate*: 0.80 mL min^{-1}. *Temperature*: 27 ± 1 °C. *Detection*: 252 nm. $n = 5$. *SD*: Standard deviation of *Rs* values.

chrysin > galangin > 3-hydroxyflavone. The basic unit of all flavonoids is the same and these differ only in their cyclic substituent. This HPLC separation trend cannot be explained on the basis of their molecular size. It is due to their various retentions owing to their different stereo-configuration and steric effect. The capacity factors were in the range of 0.67 to 20.56, respectively. The separation and resolution factors were from 1.18–2.21 and 1.00–10–00, respectively. RSD, coefficient of determination (r^2) and confidence limits (%) for capacity, separation and resolution factors were in the range of 2.32–2.39, 0.9996–0.9999 and 99.68–99.80.

HPLC method optimization

To optimize the chromatographic conditions, various combinations of acetonitrile and water were tried. Besides, water and methanol mixtures and various buffers were also used to achieve the best and fastest separation. As a result of exhaustive experimentation the best solvent system was optimized and reported herein. The effect of different concentrations of acetonitrile was also studied. It was observed that high concentrations of acetonitrile resulted in poor separation and resolution factors. Moreover, the values of these parameters were zero at 90% acetonitrile, which might be due to poor retention of the flavonoids by the used column. On the other hand, low concentrations of acetonitrile also resulted in poor separation and resolution factors. It was interesting to observe that the peaks were broad at a low amount of acetonitrile. There was no resolution at zero concentration of acetonitrile (100% water). Moreover, there was no detection of quercetin, 3,6-dihydroxyflavone, galangine and 3-hydroxyflavone.

Mechanism of separation

Halo column is of unique features made of a solid core with outer layer of porous silica gel (shell particles; 2.7 μm). The column is more efficient than columns packed with 5.0 or 3.5 μm particles, which can be run at high mobile phase linear velocity. The fast analysis on this column is due to its porous structure. The thin porous shell is responsible for excellent mass transfer (kinetics) of solutes, which allows flavonoids to enter rapidly and exit the porous structure for interaction with the stationary phase. Due to this, high mobile phase velocities can be used for very fast separations. In spite of small particle size, Halo column can be

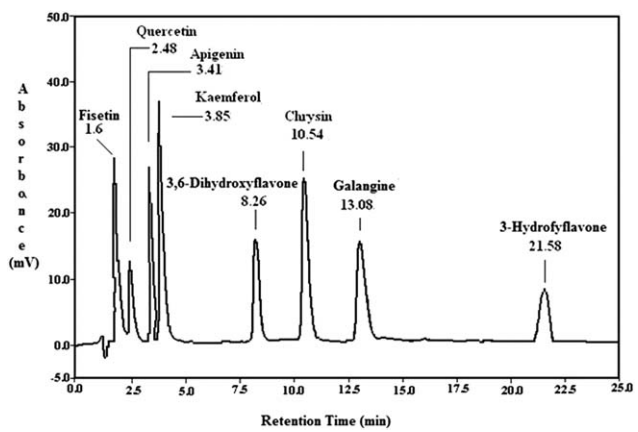

Fig. 2 Chromatograms of flavonoids in standard solutions. Experimental conditions as given in text.

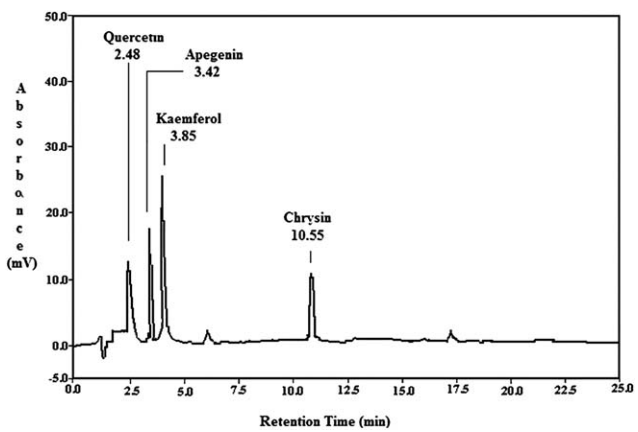

Fig. 3 Chromatograms of flavonoids in apple juice. Experimental conditions as given in text.

used on normal HPLC machine. Moreover, there is no need of costly UPLC system, which is required for normal small particle size columns (3.5–5.0 μm). The separation mechanisms on this column are the same as in the case of normal C_{18} silica gel, *i.e.*, hydrogen bonding, Van der Waals forces, dipole-induced-dipole attractions and steric effect. The above cited elution order of flavonoids may be due to their various retentions on the column because of different structures.

Validation of HPLC method

HPLC method was validated with respect to various parameters including LOD, LOQ, linearity, specificity, accuracy, precision and ruggedness.[14]

LOD and LOQ

The limits of detection (LOD) and quantitation (LOQ) were calculated from the calibration graphs of all flavonoids as three and five times of the noise level for LOD and LOQ, respectively.[15] The values for LOD and LOQ for flavonoids ranged from 0.40–5.5 and 2.25–26.0 ng, respectively. The resultant RSDs for these were in the range of 2.51–3.33%.

Linearity

The linearity was tested by least squares linear regression analysis of the calibration curve. The linearity of calibration curves (peak area *vs.* concentration) for flavonoids standards as well as in apple juice were checked over the concentration ranges of 0.01–0.10 mg mL^{-1}. The plotted curves were linear over these concentration ranges ($n = 5$) for all flavonoids. The peak areas of flavonoids were plotted against their respective concentrations. The linear regression analysis was performed on the resultant curves. The coefficient of determination (r^2) were found to be 0.9996–0.9999 for all flavonoids ($n = 5$). The values of RSDs and confidence limits were in the range of 2.32–2.36% and 99.79–99.81, respectively, across the concentration ranges studied.

Specificity

The method is quite specific as can be seen from Fig. 2 and 3. The retention times of all flavonoids are almost similar in both standard solutions and apple juice. In spite of the presence of some impurities in extracted apple juice sample (Fig. 3) the peak retention times, symmetry, LOD and LOQ were the same. These findings indicate a good specificity of the reported method.

Accuracy

The accuracy of the method was tested by injecting pure standards of the different flavonoids at various concentrations. The accuracy was determined by interpolation of replicate ($n = 5$) peak areas of three accuracy standards (0.01, 0.05 and 0.10 mg mL^{-1}) of different concentrations, from a calibration curve; described earlier. In each case, the percentage error was calculated and found in the range of 0.5 to 1.0%. This range indicated a good accuracy of the developed method.

Precision

The precision data was calculated by taking three concentrations of all flavonoids, *i.e.*, 0.01, 0.05 and 0.10 mg mL^{-1}. Five chromatographic runs were carried out for all the three

Table 3 A comparison of SPE-HPLC methods for analyses of flavonoids in apple juice[a]

HPLC separation time (min.)	Extraction time (min.)	Detection limits	Economy	Refs.
55	SPE (time not given)	NG	Expensive due to high consumption of time, chemicals, electricity and labour	5
<30	SPE (time not given)	NG	Fairly expensive due to moderate consumption of time, chemicals, electricity and labour	6
35	No extraction as samples collected from apple juices of the selected varieties	0.3–1.68 × 10^{-7} M L^{-1}	Fairly expensive due to moderate consumption of time, chemicals, electricity and labour	7
60	SPE (time not given)	NG	Expensive due to high consumption of time, chemical, electricity and labour	8
<30	SPE (time not given)	NG	Fairly expensive due to moderate consumption of time, chemicals, electricity and labour	9
<30	SPE (time not given)	NG	Fairly expensive due to moderate consumption of time, chemicals, electricity and labour	10
20	SPE; 20 min.	0.40–5.5 ng	Inexpensive	Present Work

[a] Briefly, present work is better than previously reported ones in terms of economy and low limits of detection. NG: Not given.

concentrations. RSD was calculated and ranged from 2.51 to 2.55%; indicating a good precision of HPLC method.

Ruggedness

As discussed above the determination of ruggedness was carried out by varying slightly the optimized HPLC conditions for analysis of the flavonoids. The small changes made include in mobile phase compositions, flow rates, amounts loaded and detection wavelengths. It was observed that there were no remarkable variations in HPLC results. No change in HPLC results by varying the above experimental conditions indicated the reported method as robust.

Application of the developed and validated HPLC method

The developed and validated HPLC method was applied to analyze flavonoids in apple juice. The qualitative and quantitative analyses of flavonoids were carried out by using the above mentioned HPLC conditions. The chromatograms of flavonoids in apple juice are shown in Fig. 3. The quantitative analyses of flavanoids in apple juice were carried out by comparing their peak areas with those of standards. For calculation of flavonoid concentrations in apple juice, five sets of HPLC experiments were carried out under identical conditions. The amounts of quercetin, apigenin, kaemferol and chrysin in apple juice were 4.00, 0.50, 0.40 and 0.25 mg kg^{-1}, respectively.

Conclusion

A fast, reproducible, accurate, inexpensive and effective HPLC method is described for the analyses of flavonoids by using new generation Halo column. Total experimentation including apple juice processing, SPE and HPLC took only 60 min time. The values for LOD and LOQ for flavonoids ranged from 0.40–5.5 ng and 2.25–26.0 ng, respectively. The linearity was observed in the concentration ranges of 0.01 to 0.10 mg mL^{-1} for all flavonoids. The method was used for the analyses of flavonoids in apple juice. The concentrations of quercetin, apigenin, kaemferol and chrysin in apple juice were 4.00, 0.50, 0.40 and 0.25 mg kg^{-1}, respectively. The results of the proposed method have been compared (Table 3) and was observed that the present method is better than the reported ones. Therefore, the reported method can be used for the quality control of apple juice in food industries. Besides, this method can also be used for the analyses of flavonoids in other fruits and vegetables.

Acknowledgements

One of the authors (IA) is thankful to the Head, Department of Chemistry, College of Science, Sultan Qaboos University for allowing to carry out this work. Thanks are also given to Advance Materials Technology, USA, for providing Halo column as a gift.

References

1 P. Lakhanpal and D. K. Rai, Quercetin: A versatile flavonoid, *Int. J. Med Update*, 2007, **2**, 22–37.
2 R. C. Wiley; C. R. Binkley. Applesauce and other canned apple products. In:*Processed apple products*. Downing, D. L. (Ed.). AVI (Van Nostrand Rheinhold), New York, USA. 2000.
3 A. A. Van der Sluis, M. Dekker, R. Verkerk and W. M. F. Jongen, An improved, rapid in vitro method to measure antioxidant activity; application on selected flavonoids and apple juice, *J. Agric. Food Chem.*, 2000, **48**, 4116–4122.
4 J. A. Vinson, J. Jang, Y. A. Dabbagh, M. M. Serry and S. Cai, Plant polyphenols exhibit lipoproteinbound antioxidant activity using an in vitro oxidation model for heart disease, *J. Agric. Food Chem.*, 1995, **43**, 2798–2799.
5 B. Suárez, A. Picinelli and J. J. Mangas, Solid-phase extraction and high-performance liquid chromatographic determination of polyphenols in apple musts and ciders, *J. Chromatogr., A*, 1996, **727**, 203–209.
6 A. A. Mohamed, A. de Jager and L. M. van Westing, Flavonoid and chlorogenic acid levels in apple fruit: Characterisation of variation, *Sci. Hortic.*, 2000, **83**, 249–263.
7 S. Karaman, E. Tutem, K. S. Baskan and R. Apak, Comparison of total antioxidant capacity and phenolic composition of some apple juices with combined HPLC-CUPRAC assay, *Food Chem.*, 2010, **120**, 1201–1209.
8 A. Schieber, P. Keller and R. Carle, Determination of phenolic acids and flavonoids of apple and pear by high-performance liquid chromatography, *J. Chromatogr., A*, 2001, **910**, 265–273.
9 A. A. Mohamed, A. de Jager, L. H. W. van der Plas and A. R. van der Krol, Flavonoid and chlorogenic acid changes in skin of 'Elstar' and 'Jonagold' apples during development and ripening, *Sci. Hortic.*, 2001, **90**, 69–83.
10 A. A. Mohamed, A. de Jager, M. Dekkerc and W. M. F. Jongenc, Formation of flavonoids and chlorogenic acid in apples as affected by crop load, *Sci. Hortic.*, 2001, **91**, 227–237.
11 I. Ali, V. D. Gaitonde and A. Grahn, Halo Columns: New generation technology for high speed liquid chromatography, *J. Chromatogr. Sci.*, 2010, **48**, 386–394.
12 *The United State Pharmacopeia*. United States Pharmacopeial Convention Rockville, MD, USA, 2000.
13 J. C. Miller; J. N. Miller. *Statistics for analytical chemistry*, Wiley, New York, USA, 1984.
14 *United States Pharmacopeia*. United States Pharmacopeial Convention Inc., Rockville, MD, USA, 2005.
15 *ICH draft guidelines on validation of analytical procedures: Definitions and Terminology*. Federal Register, Vol. 60, IFPMA, Switzerland, 1995.