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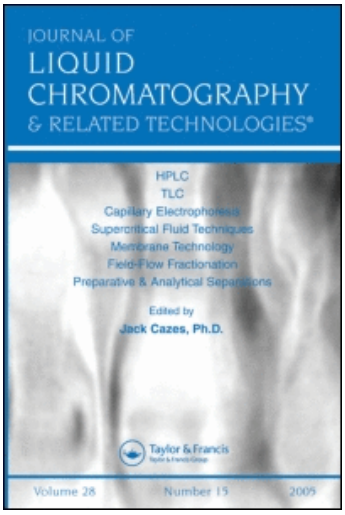

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 30 November 2001

To cite this Article Silva, B. M. , Andrade, P. B. , Seabra, R. M. and Ferreira, M. A.(2001) 'DETERMINATION OF SELECTED PHENOLIC COMPOUNDS IN QUINCE JAMS BY SOLID-PHASE EXTRACTION AND HPLC', Journal of Liquid Chromatography & Related Technologies, 24: 18, 2861 — 2872
To link to this Article: DOI: 10.1081/JLC-100106954
URL: http://dx.doi.org/10.1081/JLC-100106954

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J. LIQ. CHROM. & REL. TECHNOL., 24(18), 2861–2872 (2001)

DETERMINATION OF SELECTED PHENOLIC COMPOUNDS IN QUINCE JAMS BY SOLID-PHASE EXTRACTION AND HPLC

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ABSTRACT

With the purpose of improving an analytical method for the determination of selected phenolic compounds in quince jams, a comparative study of recoveries by two different non-polar sorbents, Isolute C18 non end-capped (NEC) and Isolute C18 end-capped (EC), was performed. Significant differences in the recovery percentages were found. The results suggest that Isolute C18 (NEC) is the most suitable for the recovery of the selected phenolics from quince jams, when 1% of methanol was added to the samples.

The reversed-phase High-Performance Liquid Chromatography/Diode Array Detector (HPLC/DAD) procedure is rapid, sensitive, reproducible, and accurate. The improved sample

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preparation was simple, involving only a C18 Solid-Phase Extraction (SPE) purification step.

The detection limit values for phenolic compounds were between 0.1 and 1.6 μg mL^{-1} and the method was precise. As a general rule, the recovery values were high, except for arbutin.

This technique can also be useful in the evaluation of commercial quince jams genuineness.

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INTRODUCTION

Quince jam is industrially manufactured, or at home, by boiling a mixture of sugar and quince puree (pulp of fruit of *Cydonia oblonga* Miller, var. *maliformis* or *piriformis*) until a convenient texture is obtained (usually to reach 65-72ºBrix). However, when quince production is scarce, industry manufacturers are tempted to adulterate quince jam by adding apple (*Malus communis* Lamk) and/or pear (*Pirus communis* Lin.) due to their low cost.

Phenolic compounds are widely distributed in nature and have been successfully used in the determination of the genuineness of some fruit products.(1-7) The main problem in the analysis of phenolics in quince jams is the latter's very high sugar content, which renders the extraction of these metabolites and sample preparation for HPLC analysis difficult. Liquid-liquid partitions produce inconvenient interphases, which do not permit the complete recovery of phenolic compounds. This problem was solved by using the non-ionic polymeric resin Amberlite XAD-2.(8-10)

In previous papers,(8-10) we reported the identification of selected phenolic compounds in quince products (pulps, jams, and jellies) using an Amberlite XAD-2 column chromatography and a simplified extractive technique with methanol, which also allowed the detection of adulterations by the addition of apple and/or pear. However, the extraction *via* Amberlite XAD-2 is a time-consuming technique, unsuitable for routine analysis in quality control determinations.

The aim of the present study was to improve the analytical technique, using C18 SPE columns, to avoid utilising the Amberlite XAD-2 chromatography, in the qualitative and quantitative analysis of selected phenolics in quince jams.

EXPERIMENTAL

Quince Jam Samples and Standards

Quince jam samples (A and B) were purchased in the Portuguese market.

The standards were from Sigma (St. Louis, MO, USA) and from Extrasynthése (Genay, France). 3- and 4-*O*-caffeoylquinic acids were not commer-

cially available, so they were prepared by transesterification of 5-*O*-caffeoylquinic acid using tetramethylammonium hydroxide.(11,12) HPLC grade methanol and formic acid were obtained from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

SPE Columns

The Isolute C18 non end-capped (NEC) and Isolute C18 end-capped (EC) SPE columns (50 μm particle size, 60 Å porosity; 10 g sorbent mass/70 mL reservoir volume) were purchased from International Sorbent Technology Ltd (Mid Glamorgan, UK). The chemical structure of the non-polar sorbent Isolute C18 (NEC) consists in C18 silane covalently bonded to the surface of silica. The sorbent Isolute C18 (NEC) is constituted by C18 silane and trimethyl silyl group, covalently bonded to the surface of silica.

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Determination of Sorption Capacity

In order to test the sorption properties of the different sorbents, an aqueous solution of arbutin (0.51 mg mL^{-1}), 5-*O*-caffeoylquinic acid (2.5 mg mL^{-1}), phloretin 2'-glucoside (0.55 mg mL^{-1}), quercetin 3-galactoside (0.30 mg mL^{-1}), rutin (0.20 mg mL^{-1}), and quercetin 3-rhamnoside (0.065 mg mL^{-1}) (pH 2 with HCl)(13) was prepared. Two aliquots (250 mL each) were passed through the different columns. 1% of methanol was added as a wetting agent to a third aliquot (250 mL), which was then applied to a Isolute C18 (NEC) column. Each column was preconditioned with 60 mL of methanol and 140 mL of water (pH 2 with HCl). The phenolic fraction remaining in the column was then eluted with methanol (ca. 50 mL) (until a negative reaction to NaOH 20%). The methanolic extract was evaporated to dryness under reduced pressure (40°C), redissolved in methanol (1 mL), and 20 μL were analysed by HPLC.

Extraction of Phenolic Compounds from Quince Jams

Each quince jam (ca. 3 g) was thoroughly mixed with water (pH 2 with HCl)(13) until complete extraction of the phenolic compounds (negative reaction to NaOH 20%), and filtered through cotton wool. 1% methanol was added to the filtrate, which was then passed through an Isolute C18 (NEC) column, preconditioned as described previously. Sugars and other polar compounds were eluted with the aqueous solvent. The retained phenolic fraction was then eluted with methanol (ca. 50 mL) (until negative reaction to NaOH 20%). The extract was

concentrated to dryness under reduced pressure (40ºC), redissolved in methanol (1 mL), and 20 µL were analysed by HPLC.

HPLC Analysis of Phenolic Compounds

Separation of the selected phenolics was achieved as reported previously,(8-10) with an analytical HPLC unit (Gilson), using a Spherisorb ODS2 (25.0 x 0.46 cm; 5 µm, particle size) column. The solvent system used was a gradient of water-formic acid (19:1) (A) and methanol (B), starting with 5% methanol and installing a gradient to obtain 15%B at 3 min, 25%B at 13 min, 30%B at 25 min, 35%B at 35 min, 45%B at 39 min, 45%B at 42 min, 50%B at 44 min, 55%B at 47 min, 70%B at 50 min, 75%B at 56 min, and 80%B at 60 min, at a solvent flow rate of 0.9 mL/min. Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the range 200-400 nm, and chromatograms were recorded at 280 and 350 nm. The data were processed on an Unipoint® system software (Gilson Medical Electronics, Villiers le Bel, France).

The compounds in each sample were identified by comparing their retention times and UV-Vis spectra in the 200-400 nm range with the library of spectra previously compiled by the authors. Peak purity was checked by means of the Gilson 160 SpectraViewer Software Contrast Facilities.

Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. 3- and 4-*O*-caffeoylquinic acids were quantified as 5-*O*-caffeoylquinic acid, phloretin 2'-xylosylglucoside as phloretin 2'-glucoside and quercetin 3-xyloside as quercetin 3-rhamnoside. The other compounds were quantified as themselves.

RESULTS AND DISCUSSION

Sorption Capacity Study

The results of sorption capacity for the selected phenolic compounds are summarised in Table 1.

Analytical Curves and Detection Limits

Under the assay conditions described, a linear relationship (Table 2) between the concentration of arbutin and phloretin 2'-glucoside and the UV absorbance at 280 nm was obtained, as it happened with 5-*O*-caffeoylquinic acid,

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Table 1. Phenolic Compounds Recoveries (%) by ISOLUTE C18 Columns

	Phenolic Compounds					
SPE Column	Arbutin	5-O-Cafeoylquinic Acid	Phloretin 2'-Glucoside	Quercetin 3-Galactoside	Rutin	Quercetin 3-Rhamnoside
EC	14.3	63.2	85.2	53.5	62.5	46.5
NEC	22.4	77.0	86.8	58.7	71.5	51.0
NEC*	18.5	95.3	105.2	100.4	100.1	88.5

EC– end-capped; NEC– non end-capped; *addition of 1% methanol.

quercetin 3-galactoside, rutin, and quercetin 3-rhamnoside and the UV absorbance at 350 nm. The correlation coefficient for the standard curves invariably exceeded 0.99. The calibration curves (Table 2) were obtained by triplicate determinations of each of the calibration standards, the peak area values (arbitrary units) were plotted as average values. The relative percent average deviations of triplicates were less than 2% in all cases. The detection limit values were calculated as the concentration corresponding to three times the standard deviation of the background noise.

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Validation of the Method

Results from quantification of the selected phenolic compounds applied to quince jam samples are shown in Table 3. Figure 1 shows the HPLC profile of a quince jam sample, obtained by C18 SPE extraction. The retention times (RT) obtained for phenolic compounds are presented in Table 4.

Table 2. Equations for Regression Lines and Correlation Coefficients, Concentration Range of Linearity and Detection Limits for Phenolic Compounds

Phenolic Compounds	Equation	Linearity ($\mu g\ mL^{-1}$)	Detection Limit ($\mu g\ mL^{-1}$)
Arbutin	y* = 4,83×106x r = 0.99861	134–1070	1.6
5-*O*-Caffeoylquinic acid	y = 2,59×107x r = 0.99735	50–5000	0.3
Phloretin 2'-glucoside	y* = 5,91×107x r = 0.99997	4–500	0.1
Quercetin 3-galactoside	y = 4,62×107x r = 0.99823	5–600	0.2
Rutin	y = 3,06×107x r = 0.99763	3–400	0.3
Quercetin 3-rhamnoside	y = 3,03×107x r = 0.99992	1–130	0.3

y – peak area at 350 nm; y* - peak area at 280 nm; x - μg of phenolic compound; r – correlation coefficient.

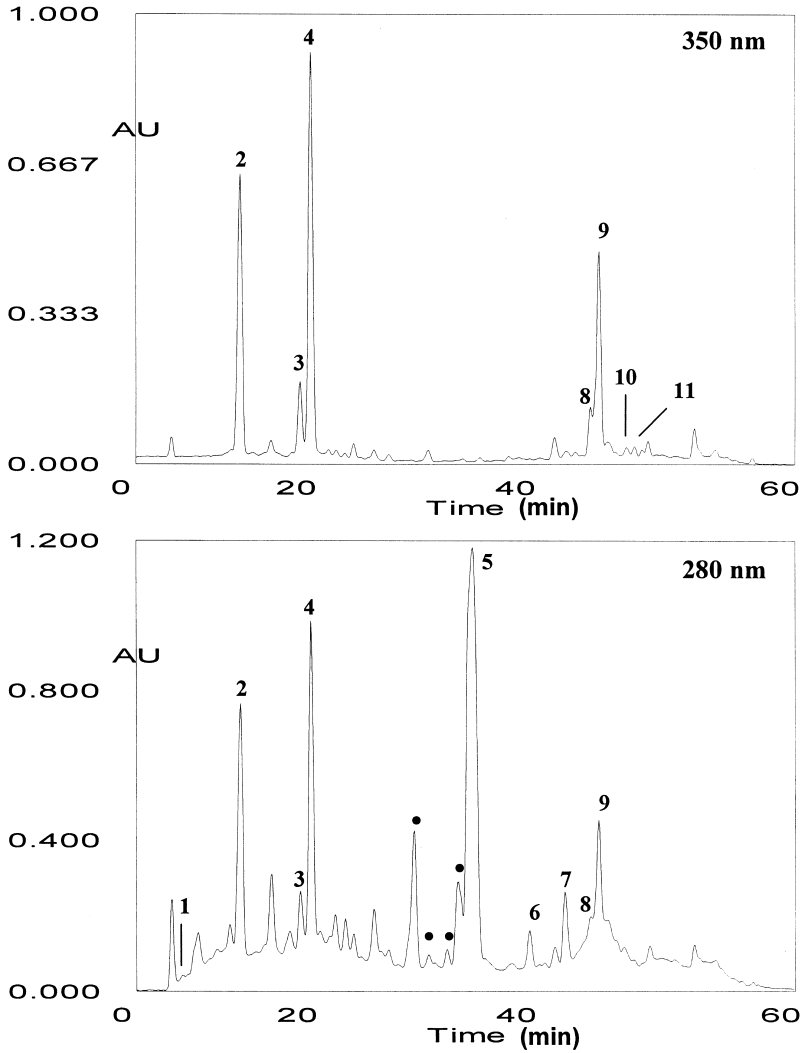

Figure 1. HPLC phenolic profile of a quince jam sample, obtained by C18 SPE extraction. (1) arbutin; (2) 3-*O*-caffeoylquinic acid; (3) 4-*O*-caffeoylquinic acid; (4) 5-*O*-caffeoylquinic acid; • unidentified phenolic compounds (under study); (5) sodium benzoate; (6) phloretin 2'-xylosylglucoside; (7) phloretin 2'-glucoside; (8) quercetin 3-galactoside; (9) rutin; (10) quercetin 3-xyloside; (11) quercetin 3-rhamnoside.

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Table 3. Phenolic Composition of Quince Jam Samples (mg Kg^{-1})[a] (Quantification by External Standard Techniques)

	Samples	
Phenolic Compounds	A	B
3-*O*-Caffeoylquinic acid	44.8 (2.49)	48.7 (0.56)
4-*O*-Caffeoylquinic acid	13.3 (0.12)	15.8 (0.40)
5-*O*-Caffeoylquinic acid	113.4 (4.61)	102.5 (1.86)
Phloretin 2'-xylosylglucoside	9.3 (0.23)	—
Phloretin 2'-glucoside	20.2 (0.38)	—
Quercetin 3-galactoside	5.1 (0.02)	12.4 (0.16)
Rutin	39.1 (0.57)	47.5 (1.17)
Quercetin 3-xyloside	1.1 (0.10)	0.5 (0.01)
Quercetin 3-rhamnoside	2.9 (0.03)	0.9 (0.04)

[a]Values are expressed as mean (standard deviation) of three determinations; A and B – commercial quince jams from different origins.

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The precision of the analytical method was evaluated by measuring the peak chromatographic area of phenolic compounds six times on the same sample. The standard deviations and the coefficients of variation (%) of these compounds are presented in Table 5.

In order to study the recovery of the procedure, one quince jam sample was added to known quantities of arbutin, 5-*O*-caffeoylquinic acid, phloretin 2'-glucoside, and rutin (Table 6). The sample was analysed in triplicate before and after the additions. Recovery values were between 2.9 and 12.6 % for arbutin, 82.2

Table 4. Retention Times of the Phenolic Compounds (n = 3)

Phenolic Compounds	Retention Time[a] (min)	SD (min)
Arbutin	4.22	0.03
3-*O*-Caffeoylquinic acid	9.55	0.07
4-*O*-Caffeoylquinic acid	15.10	0.05
5-*O*-Caffeoylquinic acid	16.10	0.10
Phloretin 2'-xylosylglucoside	36.38	0.04
Phloretin 2'-glucoside	39.70	0.09
Quercetin 3-galactoside	42.07	0.11
Rutin	42.83	0.08
Quercetin 3-xyloside	45.43	0.06
Quercetin 3-rhamnoside	46.13	0.13

[a]Mean of the retention times; SD – standard deviation.

Table 5. Evaluation of the Analytical Method Precision (n = 6) (Quantification by External Standard Techniques)

Phenolic Compounds	SD (mg Kg^{-1})	CV (%)
3-*O*-Caffeoylquinic acid	0.56	1.15
4-*O*-Caffeoylquinic acid	0.40	2.55
5-*O*-Caffeoylquinic acid	1.86	1.81
Phloretin 2'-xylosylglucoside	0.23	2.49
Phloretin 2'-glucoside	0.38	1.89
Quercetin 3-galactoside	0.16	1.30
Rutin	1.17	2.45
Quercetin 3-xyloside	0.01	2.72
Quercetin 3-rhamnoside	0.04	3.76

SD – standard deviation; CV – coefficient of variation.

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Table 6. Recoveries of Arbutin, 5-*O*-Caffeoylquinic Acid, Phloretin 2'-Glucoside and Rutin from a Spiked Quince Jam Sample (Quantification by External Standard Techniques)

Phenolic Compounds	Present (mg Kg^{-1})	Added (mg Kg^{-1})	Found[a] (mgKg^{-1})	SD (mg Kg^{-1})	CV (%)	Recovery (%)
		62.5	8.8	0.59	6.66	12.6
Arbutin	7.4	165.0	8.9	0.90	10.17	5.2
		331.1	9.9	0.48	4.84	2.9
		199.3	262.2	16.25	6.20	83.9
5-*O*-Caffeoylquinic	113.4	436.2	459.8	25.22	5.48	83.7
acid		866.7	805.9	24.72	3.07	82.2
		165.6	179.9	8.89	4.94	96.8
Phloretin	20.2	250.0	240.3	0.73	0.30	88.9
2'-glucoside		312.5	298.8	2.22	0.74	89.8
		66.2	100.2	0.08	0.08	95.2
Rutin	39.1	132.9	138.3	3.28	2.37	80.4
		234.9	212.2	3.66	1.72	77.4

[a]Mean value found for three assays for each studied concentration; SD – standard deviation; CV – coefficient of variation.

and 83.9 % for 5-*O*-caffeoylquinic acid, 88.9 and 96.8 % for phloretin 2'-glucoside, and between 77.4 and 95.2 % for rutin.

Sorption Capacity Study

SPE is a very simple technique, employing inexpensive, disposable extraction columns that are available in a multitude of column sizes and sorbents. Due to the fact that phenolics are acidic compounds, our first choice was a C18 SPE column, once these sorbents provide enhanced retention of this kind of compound. As can be seen in Table 1, the recovery of the selected phenolic compounds from the sorbent is higher using the Isolute C18 (NEC) column. This can be justified by the fact that this sorbent has secondary silanol interactions associated with surface silanol groups (non-polar, polar and cation exchange) compared to Isolute C18 (EC). As a general rule, adding 1% methanol to the sample, as A wetting agent, we could increase the percentage of recovery of the selected phenolics. This addition was required in order to maintain an active sorbent surface, once we were dealing with large volume samples (ca. 750 mL). This last procedure was applied to quince jam samples.

The flow rate was controlled and maintained in all determinations, to ensure reproducibility.

Analytical Curves and Detection Limits

In previous papers(8-10) we reported the presence of 3-, 4- and 5-*O*-caffeoylquinic acids, unidentified phenolic compounds (under study), quercetin 3-galactoside, rutin, quercetin 3-xyloside, and quercetin 3-rhamnoside in quince jams. When these products were adulterated by addition of pear and/or apple, they also contained arbutin (characteristic compound of pear), phloretin 2'-xylosylglucoside, and phloretin 2'-glucoside (chemical markers of apple). For these reasons, calibration curves, concentration ranges of linearity, and detection limits of all of these phenolic compounds were now determined (Table 2).

In order to minimise the quantification errors, quercetin-3-galactoside and rutin standards were always injected simultaneously.

Validation of the Method

The selected phenolics from two quince jam samples were analysed by the proposed technique (Table 3), in order to validate this procedure and assess its application to the routine phenolic analysis of quince jams.

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In previously published material(8-10) we have identified two quercetin glycosides as rutin and quercetin 3-galactoside, rutin having a lower RT than quercetin 3-galactoside, which agrees with several authors;(1,7) but now we have deduced that this correlation is not correct, and quercetin 3-galactoside, in reality, is the compound with lower RT. We have come to this conclusion by comparing the RT of these compounds with different blends of standards of rutin and quercetin 3-galactoside from Extrasynthese. We have tested two columns, Spherisorb ODS2 (25.0 x 0.46 cm; 5 μm, particle size) and Hypersil ODS (20.0 x 0.40 cm; 5 μm, particle size) for the separation of these standards. We have tried several gradients and flows. In all cases, quercetin 3-galactoside was the compound with lower RT. One would expect quercetin 3-rhamnosylglucoside (rutin) to have a smaller RT than the quercetin 3-galactoside. However, according to Casteele et al. (1982), the glycosylation of an OH group may (due to hydrogen linkages or steric hindrance) hinder some of the hydrophilic moieties of the molecule.(14)

The analytical method is precise, once the coefficients of variation of phenolics were between 1.15 and 3.76 % (n = 6) (Table 5).

Given the similarity of chemical structures between the several caffeoylquinic acids, phloretin, and quercetin glycosides and, therefore, their UV spectra and absortivity, the recoveries of the extractive method were only determined for arbutin, 5-*O*-caffeoylquinic acid, phloretin 2'-glucoside, and rutin (Table 6). This procedure demonstrated the effectiveness of the extraction and the accuracy of the proposed method, except for arbutin.

The phenolic profile obtained with the developed SPE procedure (Figure 1) is similar to that obtained in previous works with Amberlite XAD-2 and methanolic extractions.(8-10) In those studies, arbutin was only detected in the extracts obtained with a simplified technique using methanol as extractive solvent. The presence of arbutin in the SPE extracts can be explained by the combination of both strong primary (non-polar) and secondary (silanol) interactions, which allows some retention of polar compounds. However, when this extractive procedure was used, arbutin presented low recover rates, which could be due to the polarity of this compound, allowing its elution with sugars and other polar compounds. So, the simplified technique is also needed for the quantification of arbutin, in adulterated quince jams. This method is suitable for detection but not for quantification of arbutin.

One of the analysed samples presented, the dihydrochalcones phloretin 2'-xylosylglucoside and phloretin 2'-glucoside, considered the chemical markers of apple, suggests a problem with this fruit.

The extraction with Amberlite XAD-2 presents some disadvantages. It is a time-consuming technique, unsuitable for routine analysis in quality control determinations. Additionally, the chromatograms obtained were not so clean as those from SPE extraction, which shows that this new extraction technique is

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more suitable for phenolics analysis. Finally, Amberlite XAD-2 is rather expensive, although it can be reutilized for a large number of assays.

The main advantages of the SPE method herein developed, are that it presents highly purified extracts, good recoveries of analytes, high reproducibility, and requires small amounts of sample. The analytical procedure is simple and the costs of experiments low.

In conclusion, the proposed reversed-phase HPLC procedure for phenolic profile determination is simple, rapid, sensitive, reproducible, and accurate, and is suitable for routine analysis of phenolics in quince jams. The utilisation of C18 (NEC) SPE columns avoids the use of Amberlite XAD-2 resin, which is a rather time consuming technique. This procedure also allows the detection of adulterations by addition of apple and/or pear; however, it can't be used in the quantification of arbutin (marker of pear).

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ACKNOWLEDGMENT

B.M. Silva is grateful to Fundação para a Ciência e a Tecnologia for a grant (PRAXIS XXI/BD/21339/99).

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Received February 11, 2001 Manuscript 5519
Accepted June 10, 2001

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