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HPLC QUANTIFICATION OF THE CHEMICAL CONSTITUENTS FROM INDIGENOUS FRUITS AND VEGETABLES OF INDIAN HIMALAYAN REGION

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ABSTRACT

The purpose of the present work was to determine the phytochemical profiles by HPLC of the indigenous fruits and vegetables. The phenolic contents showed diverse variation in the selected fruits and vegetables. Development of genuine and dependable analytical methods with profile marker phytoconstituents in an extract containing a mixture of several components is a challenging task. A simple, rapid, precise and reliable HPLC method was developed for the quantification of phytochemicals from the extracts of selected minor fruits and vegetables. The *Taraxacum officinale* genus comprised a mixture of different bioactive compounds belonging to different chemical types, such as flavonoids, sesquiterpenes, triterpenes, phenolic acids, sterols. *Malva neglecta* contains different compounds including several phenolic acids, flavonoids and some non-phenolic compounds. Caffeoylquinic acids (3-, 4-, and 5-O-caffeoylquinic acids and 3,5-dicaffeoylquinic acid) are mainly present in *Cydonia oblonga* pulps. Three different hydroxycinnamic acid derivatives (neochlorogenic acid, p-coumaroylquinic acid and chlorogenic acid) were detected and quantified in *Prunus avium*.

1.Introduction

Indigenous fruits and vegetables are widely used by various traditions and cultures to fulfil energy source. They are potent sources of phytochemical components like polyphenols, antioxidants, minerals, vitamins, etc. Due to the special agro-climatic conditions in the temperate Himalayas of India, Kashmir has a variety of fruits and vegetables. The indigenous crops of the area are used by people to meet their basic needs. The indigenous fruits selected were Cherry (*Prunus avium)* and Quince (*Cydonia oblonga*) and the selected vegetables were Handh (*Taraxacum officinale*) & Sustchal (*Malva neglecta*).

Handh has potential health benefits due to the presence of phenolics, flavonoids, coumarins, terpenoids, sesquiterpene lactones, carotenoids, chlorophylls, dietary fibre and alkaloids (Colle *et al*., 2012; Gonzalez-Castejon, Visioli, & Rodriguez-Casado, 2012). Among flavonoids, apigenin-7-Oglycoside, luteolin-7-O-glycoside and naringenin-7-O-glycoside were identified and furthered analysed for their antimicrobial and antioxidant activities (Kenny *et al*., 2015).

Malva neglecta is referred to as Khebaiz or Khobb eiza in Arabic and belongs to Malvaceae family and is wildly grown in the Northern Border Province, Saudi Arabia. It has been traditionally used for insect bites, bladder infection, burns, inflammation, ulcers and wounds, as astringent, demulcent, diuretic, expectorant and laxative. Some of the

phytoconstituents reported in the literature are Quinic acid, Aconitic acid, Chlorogenic acid, Caffeic acid, Coumaric acid, Rutin, Hyperoside, Myricetin, Fisetin, Coumarin, Quercetin, Naringenin, Luteolin, Kaempferol, Apigenin, Rhamnetin and Chysin (Nesrin *et al*., 2017; Guder *et al*., 2012; Karak 2019; Essafi Benkhadir *et al*., 2012; Fattouch *et al*., 2007).

A large number of polyphenolic constituents were identified in quince fruit; in particular, flavan-3-ols such as Epicatechin, Procyanidin B2, eight Hydroxycinnamates, derivatives of Caffeoylquinic acid (CQA) and Coumaroylquinic acid, Kaempferol and Quercetin derivatives (Nagahora *et al*., 2013; Wojdylo *et al*., 2013; Hamauzu *et al*., 2005). The analysis of the phenolic profile indicated that derivatives of Quercetin and Kaempferol were minor components of quince fruit, while Procyanidins and Chlorogenic acid (CGA) derivatives constituted the majority of the polyphenolic fraction (Bystricka *et al*., 2017; Crozier *et al*., 2009; Strek *et al*., 2007; Joana *et al*., 2019). The other main component of quince are proanthocyanidins, compounds widely known for their antioxidant and protective effects on cardiovascular and cancer-related diseases, in part due to their ability to modulate pro-inflammatory and carcinogenic signals. (Joana *et al*., 2019; Serra *et al*., 2011). Procyanidins from quince fruit have been studied for their chemoprevention activity, and preliminary investigation indicated an effect against enzymes and receptors, suggesting a role in cancer prevention (Serra *et al*., 2011).

Sweet cherries are an excellent source of phytochemicals, namely phenolic compounds (Bastos *et al*., 2015). The concentration of polyphenols can vary between different sweet cherry cultivars and in different parts thereof (Tural and Koca 2008). Studies have shown that the antioxidant capacity in the stem is greater than the antioxidant capacity in the pulp, leading people to believe that this is due to the higher concentration of phenols found here as well. (Wang *et al*., 2017).The levels of phenolic compounds can be affected by several factors, and this is associated to both climatic and agronomic conditions (Toydemir *et al*., 2013; Liu 2013). Another study in acid cherry (*Prunus cerasus* L.) also allowed the phenolic composition to be determined using a high performance liquid chromatography coupled with fluorescence detection (HPLC-FLD) (Monteiro Egydio *et al*., 2013). A study of several sweet cherry cultivars using high-performance liquid chromatography equipment coupled to ultraviolet (HPLC–UV) and diode array (HPLC–DAD) (Toydemir *et al*., 2013) is also reported.

Currently, some studies support the idea that a healthy diet can prevent the development of certain diseases. (Liu 2013). In addition, research works have shown the high relationship between the consumption of fruits in prevention of various chronic health problems (Monteiro Egydio *et al*., 2013). Not only fruit pulp, but also peels and seeds from dietary plants and traditional medicinal herbs, play an important role in health because of their nutritional, antioxidant properties and wealth due to bioactive compounds. It is also interesting to know the presence and concentration of polar compounds (such as organic acids), because these compounds affect sensory and sensory properties. In addition, organic acids can be used as a chemical indicator of maturity, bacterial activity or storage conditions, because these compounds have good stability during processing and storage. (Sandin-Espana *et al*., 2016; Vagelas and Sugar, 2020).

Currently, there are few studies on phenolic compounds and organic acids of the selected minor fruits and vegetables. Due to the few available research studies about the characterization of bioactive compounds in selected minor fruits and vegetables, the aim of the present work was to comprehensive and tentative identify phenolic compounds and organic acids in the edible part of selected minor fruits and vegetables. This study presents the selected minor fruits and vegetables as potential sources of bioactive compounds with applications in the food, pharmaceutical and cosmetic industries.

2. Materials and Methods

2.1. Chemicals and Reagents

HPLC grade formic acid, Methanol, Water & Acetonitrile were purchased from Himedia (LBS, Marg, Mumbai, India).

Solvents were filtered using a Solvent Filtration Apparatus 58,061 (Supelco, Bellefonte, PA, USA). Double- deionised water was obtained with a Milli-Q-system from Millipore (Bangalore India). The standard compounds Quinic acid, Malic acid, tr-Aconitic acid, Gallic acid, Chlorogenic acid, Protocatechuic acid, Tannic acid, tr-Caffeic acid, Vanillin, P- Coumaric acid, Rosmarinic acid, Rutin, Hesperidin, Hyperoside, 4-OH-Benzoic acid, Salicylic acid, Myricetin, Fisetin, Coumarin, Quercetin, Naringenin, Hesperidin, Luteolin, Kaempferol, Apigenin, Rhamnetin, Chyrsin, 3-O-Caffeoyl quinic acid, 4-O-Caffeoyl quinic acid, 5-O-Caffeoyl quinic acid, 3,5-di Caffeoyl quinic acid, Quercetin 3- galactoside, Kaempferol glycoside, Kaempferol 3-glycoside, Kaempferol 3-Rutinoside, NeoChlorogenic acid, P- Coumaryl quinic acid, Cyanidin 3-glucoside, Cyanidin 3- Rutinoside, Peonidin 3-glucoside, Pelargonidin 3- Rutinoside, Peonidin 3- Rutinoside, Catechin, Epicatechin, 9- Cis Violaxanthin, Neochrome, All- trans-Neoxanthin, All-trans-Violaxanthin, 9-cis-Neoxanthin, Luteoxanthin, cis-Violaxanthin, Antheraxanthin, 9-cis-Violaxanthin, 13- cis-Lutein, All trans Lutein, All trans Zeaxanthin, 9 cis- Lutein, were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Extraction

A solid- liquid extraction was used to extract the polar fraction. Briefly, 0.5 g of freeze-dried sample powder was dissolved in 15 mL of a solution of methanol/ water (80: 20 v/v). The mixture was placed in a water bath for 15 min at room temperature and then it was centrifuged for 15 min at 5000 rpm, the supernatant was removed, and extraction was repeated once more. The supernatants were collected, evaporated and reconstituted in 2 mL of methanol/ water (80: 20, v/v). The final extracts were filtered with regenerated cellulose filters 0.2 µm, (Millipore, Bedford, MA, USA) and stored at -18 °C until the analyses.

2.3. UPLC DAD Analyses

The extracts obtained by solid-liquid extraction were analysed by using UPLC coupled to DAD in order to identify phenolic compounds. An Agilent 1290 Infinity II- LC system (Agilent Technologies, Waldbronn Germany) equipped with a vacuum degasser, autosampler, quaternary pump, and DAD was used for the chromatographic determination. All the methods were validated and optimized.

For *Taraxacum officinale*, the separation was done using an Agilent Poroshell Eclipse plus C30 column (4.6 × 250 mm, 5 µm) from Agilent Technologies operating at a flow rate of 1 mL/min and 40 ° C, thorough out the gradient. The mobile phases used were water with MeOH/ACN in the ratio of 79:14:7 (Phase A) and Methylene chloride (Phase B), and the solvent gradient changed according to the following conditions: 95% A in the beginning and maintained for 9 min, decreased to 85% A in 23 min, 83% A in 33 min, 71% A in 35 min, 70% A in 45 min, 66% A in 66 min and returned to original ratio in 71 min. The injection volume was 20 µL and chromatograms were recorded from 190-600 nm with 450 nm as maximum wavelength.

For *Malva neglecta*, the chromatographic separation was performed on a C18 reversed-phase Inertsil ODS-4 (150 mm × 4.6 mm, 3µm) analytical column. The column temperature was fixed at 40°C. The elution gradient consisted of mobile phase A (water, 5mM ammonium formate and 0.1% formic acid) and mobile phase B (methanol, 5mM ammonium formate and 0.1% formic acid). Use the gradient program t (min) of solvent B in the following proportions,% B: (0.40), (20.90), (23.99.90), (24.40), (29.40). The solvent flow rate was maintained at 0.5 ml / min and the injection volume was stable at 4 µl.

For *Cydonia oblonga*, chromatographic separation was carried out on a LiChroCART column (250 × 4 mm, RP-18, 5-µm particle size, Merck, Darmstadt, Germany) using two solvents: 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. The flow rate was 0.9 mL/min, and the injection volume was 80 µL. Detection was carried out at 270, 320, and 350 nm.

In case of *Prunus avium*, the HPLC equipment was used with a diode array detector (DAD). The system consists of a binary pump, a degasser and an autosampler. The chromatographic column used is Beckman Ultrasphere ODS (Roissy CDG, France): 4.6 mm x 250 mm, 5 µm, equipped with a guard column of 4.6 mm x 10 mm. The mobile phase consists of two solvents: solvent A, water/formic acid (95:5; v/v) and solvent B, acetonitrile/solvent A (60:40; v/v). Phenolic compounds were eluted under the following conditions: 1 mL/min flow rate and the temperature was set at 25 °C, isocratic conditions from 0 to 10 min with 0% B, gradient conditions from 0% to 5% B in 30 min, from 5% to 15% B in 18 min, from 15% to 25% B in 14 min, from 25% to 50% B in 31 min, from 50% to 100% B in 3 min, followed by washing and reconditioning the column.

3. Results and discussions

The separation of all the compounds in complex extract is one of the challenging tasks in analytical HPLC.

3.1. Taraxacum Officinale (Handh)

According to current knowledge, the genus Taraxacum includes a mixture of different biologically active compounds belonging to different chemical types, such as flavonoids, sesquiterpenes, triterpenes, phenolic acids, sterols, etc. Among them, flavonoids and phenolic acids were the more abundant metabolites, which always had similar structure skeleton. It has been well established that a C30 column can provide a much better resolution than a C18 column in separating carotenoids and their geometrical isomers (Chen et al., 2004; Dugo et al., 2008). Initially a gradient solvent system of methanol/acetonitrile/water (84:14:2, v/v/v) and methylene chloride developed by Inbaraj et al., (2008) was used to separate the various carotenoids in T. formosanum by a YMC C30 column (250 mm × 4.6 mm I.D., particle size 5 µm). However, several carotenoid peaks overlapped, which may be due to the high concentration of solvent in the mobile phase. Therefore, it is necessary to reduce the strength of the solvent by increasing the polarity of the mobile phase. After various studies, a gradient mobile phase of methanol/acetonitrile/water (79:14:7, v/v/v) (A) and methylene chloride (B) was developed to separate 25 carotenoids and their geometrical isomers within 66 min (Fig. 1), with the retention times ranging from 11.54 to 64.22 min, retention factor from 2.51 to 18.57, separation factor from 1.01 to 1.44 and peak purity from 88.3 to 99.8% (Table 1). Most carotenoid peaks were adequately resolved (Fig. 1), implying both solvent strength and selectivity of mobile phase to sample components were properly controlled.

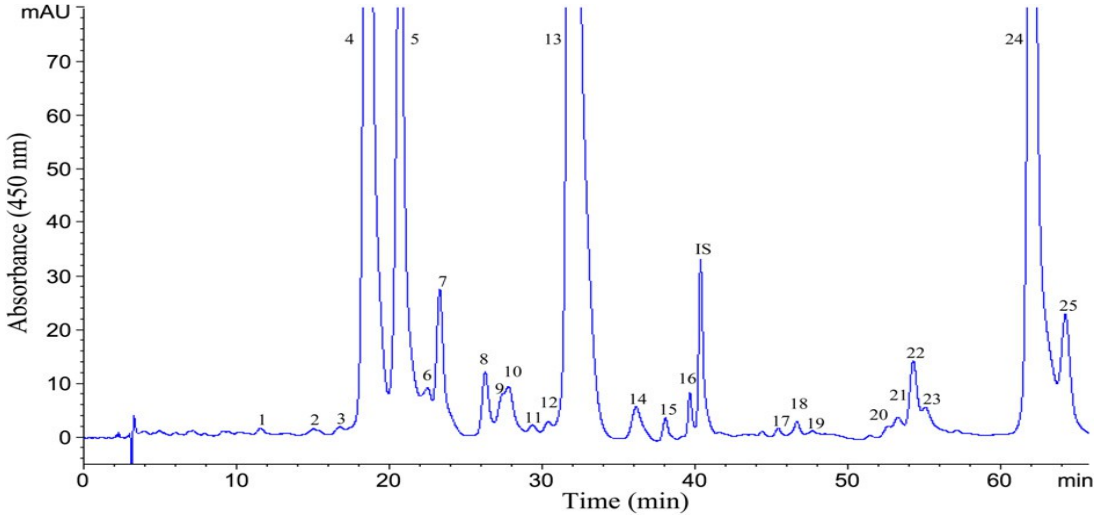

Figure 1. HPLC chromatogram of Standards of *T. officinale* (column: C30, mobile phase: (A) methanol–acetonitrile–water (79:14:7, v/v/v), (B) methylene chloride, flow rate: 1 mL/min, detection wavelength: 450 nm).

In Fig. 1, peak 1 was identified as 9- or 9'-cis- violaxanthin. Peak 2 was identified as neochrome. Both peaks of 3 and 4 were identified as all –trans forms of neoxanthin and violaxanthin, respectively. Peak 5 was identified as 9 or 9'- cis-neoxanthin. Both peaks 6 and 10 were identified as luteoxanthin based on absorption spectra and mass spectra characteristic and compared with that reported by Chen et al., (2004) and Dugo et al., (2008). Following the same approach, peaks 7 and 9 were identified as cis-violaxanthin and 9 or 9'-cis violaxanthin, respectively (Melendez-Martinez et al., 2008; Chen et al., 2004). Peak 8 was identified as antheraxanthin. Peaks 13, 14, 17 and 24 were positively identified as all-trans forms of lutein, zeaxanthin, β-cryptoxanthin and β-carotene, respectively. In several similar studies the major carotenoids, including neoxanthin, violaxanthin, β-carotene, zeaxanthin, β- cryptoxanthin and antherxanthin were also detected in Taraxacum officinale.

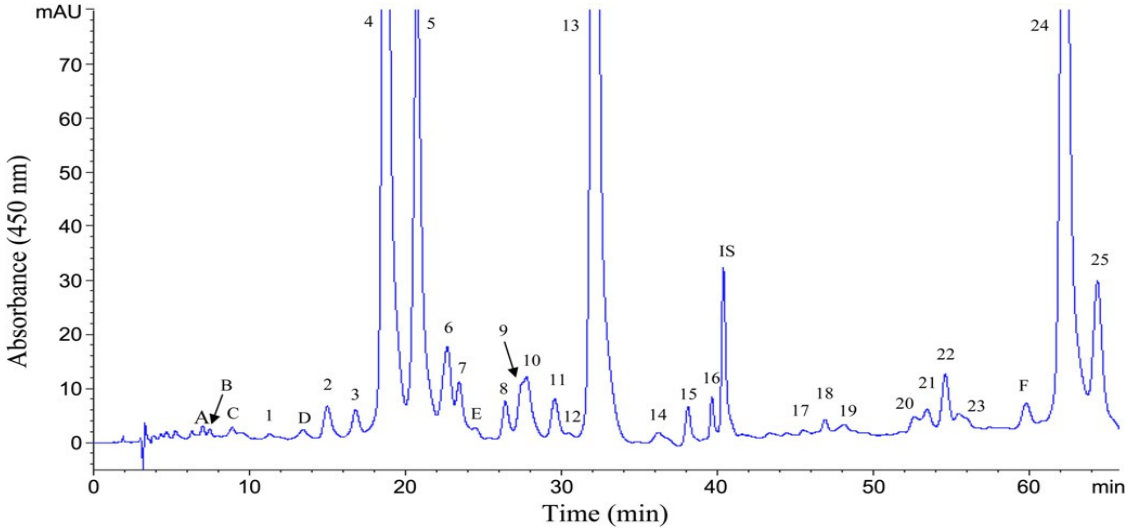

Figure 2. HPLC chromatogram of extract of *T. officinale*. Peak numbers with alphabetical letters (A-F) indicate additional compounds identified in extract fraction isolated by column chromatography, while 1-25 denote the same standard compound.

Fig. 2 shows the HPLC chromatogram of various carotenoids in carotenoid fraction isolated from Taraxacum extract. Following the same identification and quantitation criteria, a total of 31 carotenoids were separated and identified (Faria et al., 2009; Zepka et al., 2009). The peaks marked as A-F were identified as Auroxanthin, 13- cis-neoxanthin, Violaxanthin and 9-cis-βcarotene.

3.2. Malva Neglecta (Sustchal)

In the quantitative analysis of phenolic compounds various studies exist in literature reporting the use of HPLC (Mousavi et al., 2015; Fahimi et al., 2016). Figure 3 depicts the chromatogram of the standards of *Malva neglecta*.

Therefore, an accurate quantitative method was developed on HPLC for the analyses of twenty-seven compounds. The methanol extracts of M. neglecta were screened to quantify their phenolic composition by using an Agilent 1290 Infinity II- LC system.

This accurate method allowed us to qualify and quantify 27 different compounds including several phenolic acids, flavonoids and some non-phenolic compounds. To summarize, this is the first study to screen 27 compounds in M. neglecta. It is rich in terms of phenolic acids.

The detected compounds were Quinic acid, Malic acid, tr- Aconitic acid, Gallic acid, Chlorogenic acid, Protocatechuic acid, Tannic acid, tr- caffeic acid, Vanillin, p- Coumaric acid, Rosmarinic acid, Rutin, Hesperidin, Hyperoside, 4- OH Benzoic acid, Salicylic acid, Myricetin, Fisetin, Coumarin, Quercetin, Naringenin, Hesperedin, Luteolin, Kampferol, Apigenin, Rhamnetin and Chysin (Fig. 4).

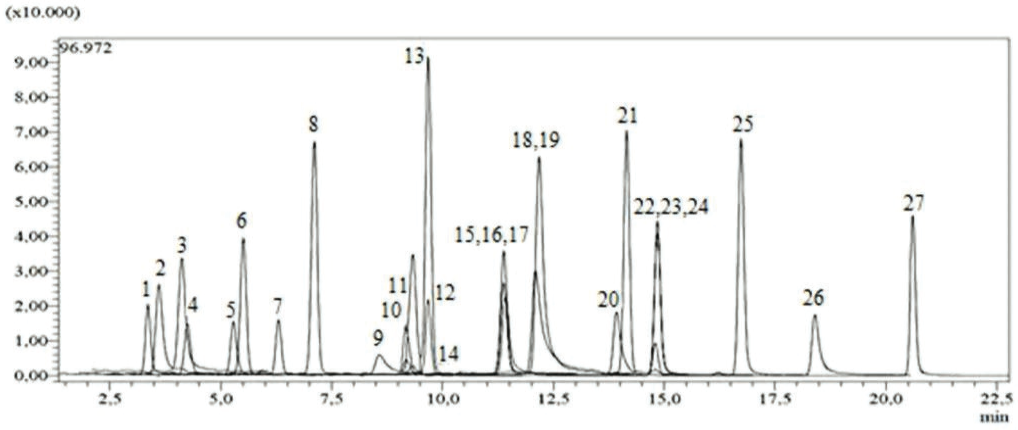

Figure 3. HPLC chromatogram of Standards of *Malva neglecta*.

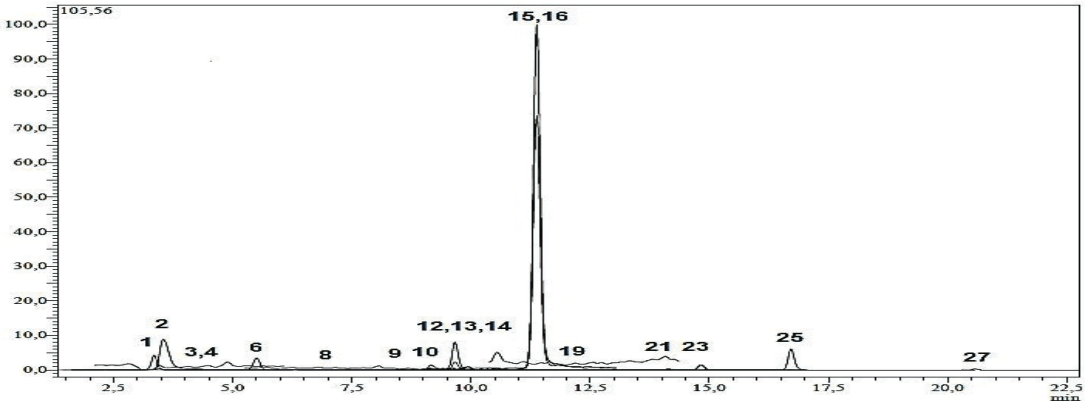

Figure 4. HPLC profile of *Malva Neglecta* methanol Extract. 1: Quinic acid, 2: Malic acid, 3: tr-Aconitic acid, 4: Gallic acid, 5: Chlorogenic acid, 6: Protocatechuic acid, 7: Tannic acid, 8: tr- caffeic acid, 9: Vanillin, 10: p-Coumaric acid, 11: Rosmarinic acid, 12: Rutin, 13: Hesperidin, 14: Hyperoside, 15: 4-OH Benzoic acid, 16: Salicylic acid, 17: Myricetin, 18: Fisetin,19: Coumarin, 20: Quercetin, 21: Naringenin, 22: Hesperetin, 23: Luteolin, 24: Kaempferol, 25: Apigenin, 26: Rhamnetin, 27: Chrysin.

3.3. Cydonia oblonga (Quince)

All quince pulps presented the same chemical profile, composed by at least five identified phenolic compounds: 3-, 4-, and 5-O-caffeoylquinic acids, 3, 5-dicaffeoylquinic acid, and rutin. Fig. 5 depicts the chromatogram of the standards of *Cydonia oblonga*.

The first group (39-46 min) was formed by five major peaks (peaks 5 to 9) and the second group (51-56 min) was formed by three peaks (Fig. 6).

All of the peaks showed identical spectral profile, with two maxima at 257 and 353-355 nm, which indicated that they could be flavonols or flavonol derivatives. HPLC analyses provided interesting information on the two mentioned groups of flavonoids. Quince fruit composition was characterised by the presence of 4- caffeoylshikimic acid, 4-caffeoyl quinic acid, quercetin-3, 7-diglucoside, kaempferol-3-O- rhamnoside and kaempferol-7-O-glucoside.

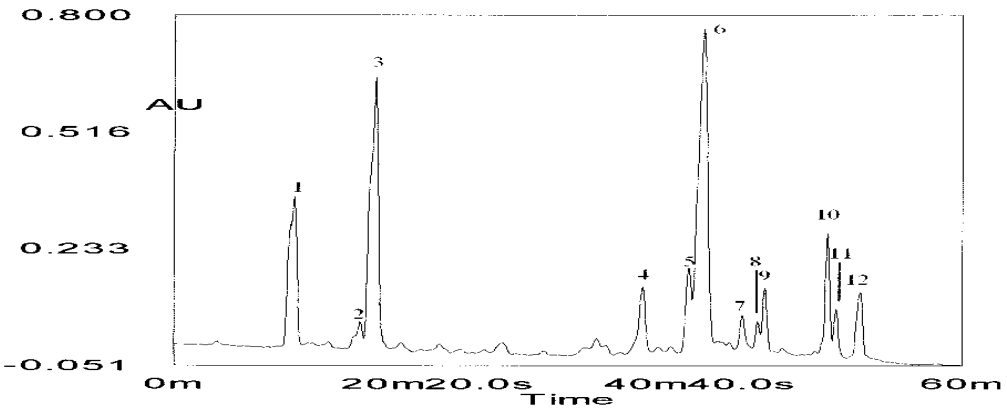

Figure 5. HPLC profile of a *Cydonia oblonga* Standards. Detection at 350 nm. Peaks: (1) 3-*O*-caffeoylquinic acid; (2) 4-*O*-caffeoylquinic acid*;* (3) 5-*O*caffeoylquinic acids; (4) 3,5-dicaffeoylquinic acid; (5) Quercetin 3-galactoside; (6) Rutin; (7) Kaempferol glycoside; (8) Kaempferol 3-glucoside; (9) Kaempferol 3-rutinoside; (10) and (11) Quercetin glycosides acylated with *p*-coumaric acid, and (12) Kaempferol glycoside acylated with *p*-coumaric acid.

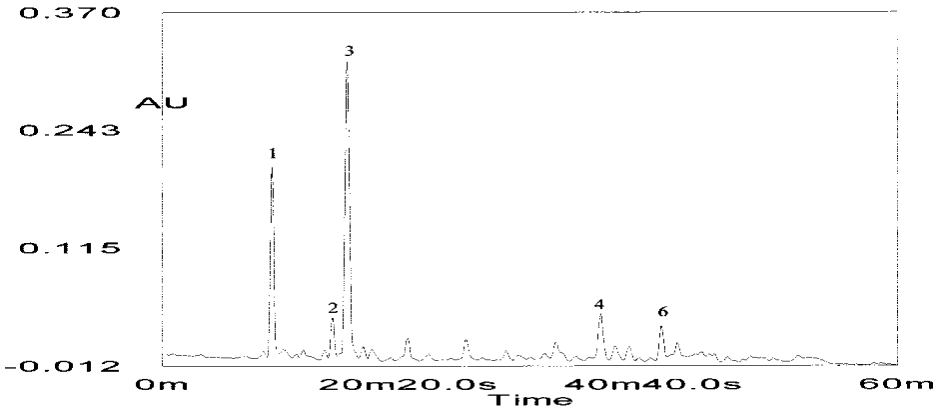

Figure 6. HPLC profile of *Cydonia oblonga* Extract. Detection at 350 nm. Peaks: (1) 3-*O*-caffeoylquinic acid; (2) 4-*O*-caffeoylquinic acid*;* (3) 5-*O*caffeoylquinic acids; (4) 3, 5-dicaffeoylquinic acid, and (6) Rutin.

3.4. Prunus avium (Cherry)

Figure 7 depicts the chromatogram of the standards of *Prunus avium.*

A total of 11 phenolic compounds, hydroxycinnamic acid derivatives (3), anthocyanins (5), flavan-3-ols (2) and flavonol (1), were identified and quantified in sweet cherry cultivars. Three different hydroxycinnamic acid derivatives (neochlorogenic acid, p-coumaroylquinic acid and chlorogenic acid) were detected and quantified in the cherry (Fig. 8). Among the hydroxycinnamic acids, neochlorogenic acid showed the highest concentration of the total hydroxycinnamic acid content. Goncalves et al., (2004) reported that neochlorogenic acid was the major hydroxycinnamic acid, varying from 22 to 190 mg ⁄ 100 g of FW and represented 19% to 71% of phenolics (Goncalves et al., 2004). Chlorogenic acid was the least abundant hydroxycinnamic acid. When we compare the total hydroxycinnamic acid derivative content of sweet cherries analysed with the previous reports, we see that our results were higher than the values obtained by Usenik et al., (2008) while lower than the values obtained by Goncalves et al., (2004). It was noted that cyanidin 3-rutinoside was the most dominant anthocyanin and it accounted for the largest proportion of the total anthocyanin contents.

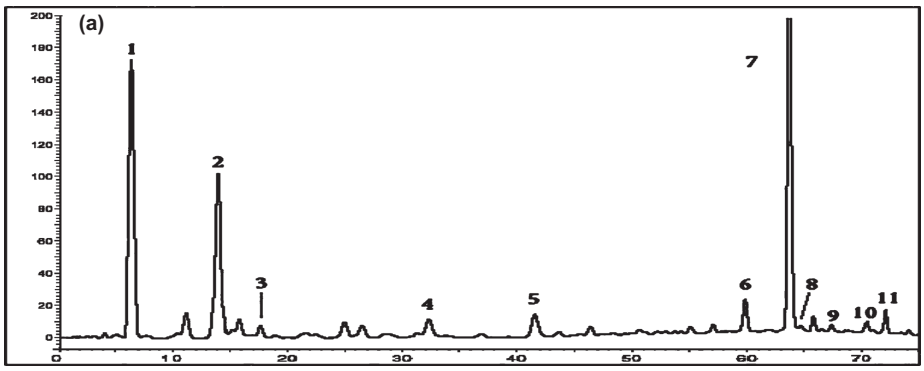

Figure 7. HPLC profile of Standards of *Prunus avium*. (1) Neochlorogenic acid; (2) *p*-coumaroylquinic acid; (3) Chlorogenic acid; (4) Cyanidin 3-glucoside; (5) Cyanidin 3-rutinoside; (6) Peonidin 3-glucoside; (7) Pelargonidin 3-rutinoside; (8) Peonidin 3-rutinoside; (9) Catechin; (10) Epicatechin; (11) Rutin

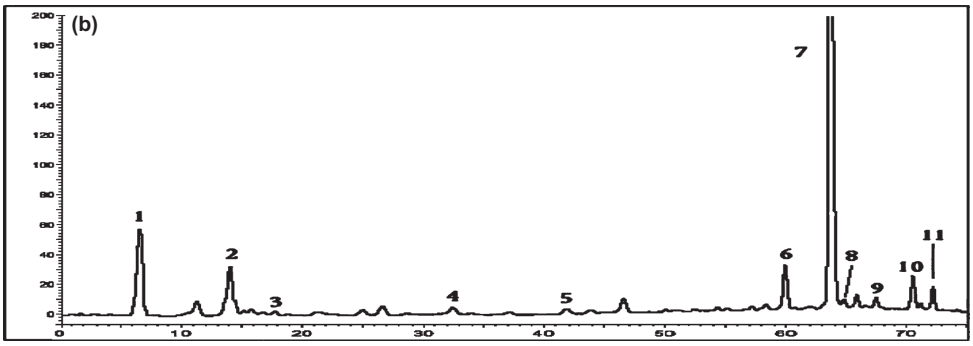

Figure 8. HPLC chromatograms of *Prunus avium* Extract recorded at 280 nm.

4. Conclusions

The HPLC gradient mobile phase was developed to separate different types of phytochemicals in the selected indigenous fruits and vegetables. The choice of mobile phase and buffer (organic matter and pH) composition plays an important role in the selectivity of separation. The final optimization can be done by changing the temperature, gradient slope and flow rate, and the type and concentration of the mobile phase modifier.

5. References

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Conflicts of Interest:

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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