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RECOVERY, SEPARATION AND CHARACTERIZATION OF PHENOLIC COMPOUNDS AND FLAVONOIDS FROM MAPLE PRODUCTS

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement of the degree of Master of Science

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Montreal, Québec

March, 2000

SHORT TITLE

PHENOLIC COMPOUNDS AND FLAVONOIDS FROM MAPLE PRODUCTS

ABSTRACT

Comparative high-performance liquid chromatography (HPLC) and gas-liquid chromatography (GC) analyses of selected phenolic and flavonoid standards were developed using a wide range of detectors, including ultraviolet diode-array (UV-DAD) and electrochemical (EC) detectors for HPLC and flame ionization detector (FID) and mass spectrometry (MS) for GC. The results demonstrated that the limits of detection obtained with HPLC-EC analysis were 10 to 500-time higher for phenolic acid standards and 2 to 50-time higher for flavonoid standards than those obtained with the HPLC-UV analysis. HPLC-EC was more sensitive than GC/FID for all investigated standards, especially for vanillin and syringaldehyde. The results indicated that GC/FID/MS analysis of phenolic and flavonoid standards was more efficient than that of HPLC, providing a fast analysis with better resolution and baseline separation of all standards with minimum co-elution. The only co-elution encountered in GC/FID was with coniferol and *p*-coumaric acids. For HPLC analysis, (-)-epicatechin, caffeic and homovanillic acids were co-eluted at 28.04 min and sinapic and ferulic acids at 34.57 min. Phenolic compounds and flavonoids were extracted from maple sap and maple syrup with ethyl acetate and the recovered compounds were subjected to HPLC and GC analyses. Tentative identification of phenolic compounds and flavonoids in maple sap and maple syrup indicated the presence of protocatechuic acid, hydroxycinnamic acid derivatives, (+)-catechin, (-)-epicatechin, vanillin, coniferol, syringaldehyde, flavanols and dihydroflavonols related compounds. In addition, the identification by GC/MS of protocatechuic acid, vanillin, syringaldehyde, coniferol and *p*-coumaric acid was made by comparing mass spectrum characteristics of individual peak from total ion chromatogram (TIC) to that of standard compounds. The seasonal variation of selected phenolic compounds and flavonoids present in maple sap and maple syrup was also investigated; the results indicated a slight seasonal increase for most of the identified compounds, particularly for coniferol, protocatechuic and *p*-coumaric acids.

RÉSUMÉ

Une étude comparative de standards de composés phénoliques et de flavonoïdes en chromatographie liquide à haute performance (HPLC) et chromatographie liquide en phase gazeuse (GC) fut développée en utilisant plusieurs types de détecteurs: les détecteurs ultra-violet à diode-array (UV-DAD) et électrochimique (EC) pour l'HPLC, et les détecteurs à ionisation de flamme (FID) et de spectrométrie de masse (MS) pour le GC. Les résultats ont démontré que les limites de détection obtenues avec l'HPLC-EC étaient 10 à 500 fois plus élevées pour les acides phénoliques et 2 à 50 fois plus élevées pour les flavonoïdes que celles obtenues avec l'HPLC-UV. Par contre, le GC/FID démontre une sensibilité plus faible pour la plupart des composés, spécialement pour la vanilline et du syringaldehyde. Les résultats indiquent que les analyses en GC/FID pour les composés phénoliques et flavonoïdes sont plus performantes que celles obtenues par l'HPLC avec une meilleure séparation effectuée en moins de temps et un minimum de co-élution. La seule co-élution rencontrée en GC/FID est celle du coniférol et de l'acide *p*-coumarique à 10.76 min. Pour les analyses en HPLC, l'acide caféique, l'acide homovanillique et l' (-)-épicatéchine co-éluent à 28.04 min et les acides sinapinique et férulique à 34.57 min. L'extraction des composés phénoliques et des flavonoïdes de la sève et du sirop d'érable fut effectuée à l'aide de l'acétate d'éthyle. Le résidu obtenu après l'extraction fut soumis aux analyses d' HPLC et de GC. Un travail d'identification préliminaire effectué à l'aide de l'HPLC révèle la présence, dans les produits de l'érable, de l'acide protocatéchuique, de dérivés de l'acide hydroxycinnamique, de la (+)-catéchine, de l' (-)-épicatéchine, de la vanilline, de l'alcool coniféryl, du syringaldéhyde, de l'acide *p*-coumarique et des dérivés de flavanol et dihydroflavonol. De plus, l'identification des composés préalablement identifiés avec l'HPLC fut effectuée à l'aide du GC/FID. Des analyses effectuées en chromatographie gazeuse à l'aide du détecteur de spectrométrie de masse (GC/MS) utilisant les différents spectres d'ionisation de masse de chaque standard injecté, ont permis une identification plus approfondie. La présence de l'acide protocatéchuique, de la vanilline, du syringaldéhyde, de l'alcool coniféryl et de l'acide *p*-coumarique, identifiés par HPLC, fut confirmée. En dernier lieu, une évaluation de la variation des composés phénoliques et des flavonoïdes sélectionnés fut réalisée au cours de la saison d'écoulement. Une légère augmentation fut observée pour chacune des méthodes utilisées.

ACKNOWLEDGEMENTS

First I would like to express my thankfulness to my supervisor Dr. S. Kermasha for his scientific guidance, patience, support and confidence during this study. I would like to mention my gratitude toward is good sense of humor it was a pleasure working with you.

I would like to pursue my gratefulness to Xavier for his excellent advice and professionalism as well as Wigdan and Abzal for always being there to help whenever needed and keeping a wonderful spirit in the lab.

Furthermore, I would like to thank all the people in the lab that were there for me when I needed them and made my time the most memorable. Special thanks to Fabienne, Ndeye, Barbara, Tracy, Marie-Odile, Mohammed, Aurélie, Magali, Didier, Mathieu. As well as my dearest friends Karine, Stéphanie, Caroline and Martine.

My deepest gratitude goes to my wonderful family without them I wouldn't have been able to enjoy this experience the same way. Their support and encouragement were what kept me smiling during the hard times.

Finally, a very special and unique thanks goes to my boyfriend Luigi Marcangione. Thank you for understanding, believing and helping me during these last two years.

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LIST OF ABREVIATIONS

(+)C: (+)-Catechin

CA: Caffeic Acid

CAL: Coniferal

COL: Coniferol

CH: Chlorogenic Acid

CI: Chemical Ionization

DAD: Diode-Array Detection

DMF: Dimethylformaldehyde

(-)E: (-)-Epicatechin

EC: Electrochemical

ECD: Electrochemical detector

EI: Electron Impact

FA: Ferulic Acid

GA: Gallic Acid

GC: Gas Chromatography

GC/FID: Gas Chromatography with Flame Ionization Detection

GLC: Gas Liquid Chromatography

GC/MS: Gas Chromatography with Mass Spectrometry

K: Kaempferol

LOD: Limit of Detection

HBA: Hydroxybenzoic Acid

HCA: Hydroxycinnamic Acid

HD: Hesperidin

HPLC: High Performance Liquid Chromatography

HT: Hesperetin

HVAC: Homovanillic Acid

NICI: Negative Ion Chemical Ionization

p-CA: *p*-Coumaric Acid

PCC: Protocatechuic Acid

Q: Quercetin

QR: Quercitrin

R: Rutin

RP-HPLC: Reverse-Phase High-Performance Liquid Chromatography

SA: Sinapic Acid

SIM: Selective Ion Monitoring

SYC: Syringic Acid

SYL: Syringaldehyde

TMS: Trimethylsilylation

TIC: Total Ion Chromatogram

TFA: Trifluoroacetic Acid

UV: Ultraviolet

VA: Vanillin

VAC: Vanillic Acid

VIS: Visible

1. INTRODUCTION

The analysis of phenolic and flavonoid compounds, often present as traces, requires the use of efficient and extremely sensitive analytical techniques to allow their proper separation and characterization (Macheix *et al.*, 1990). Such techniques include high-performance liquid chromatography (HPLC) and gas-liquid chromatography (GLC).

Recent advances in technology have made it possible to accurately identify diverse and complex molecules. The identification of phenolic compounds and flavonoids was made by comparing their retention times and UV spectra with those of standards using the diode-array detector (Escarpa and Gonzales, 1998; Benavente-Garcia *et al.*, 2000). HPLC, with UV diode-array (UV-DAD) and electrochemical (EC) detectors enabled accurate identification of trace components. Previous work has shown that spectral and electrochemical (Kermasha *et al.*, 1995a,b) characteristics may be used to assign standard compounds to unknown sample components. HPLC coupled with EC detection has become a widely accepted and useful technique due to its high selectivity and sensitivity (Hensley *et al.*, 1999). Accurate identification work can therefore be achieved by matching retention time data, spectral and electrochemical characteristics of the corresponding sample peaks with those of standards. Furthermore, GC with mass spectrometry (MS) analysis has been used to confirm that unidentified peaks detected by HPLC were of a phenolic nature (Cinquanta *et al.*, 1997). Recovered phenolic compounds and flavonoids were characterized by reverse-phase HPLC using UV and mass spectral detection (Ryan *et al.*, 1999). In addition, phenolic compounds were characterized by HPLC and the structure of new compounds was elucidated by mass spectrometry (Brenes *et al.*, 1999). Moreover, GC with flame ionization detector (FID) and mass spectrometry (MS) is often used for quantification studies (Valdez *et al.*, 1999). Soleas *et al.* (1997a) reported that a wide range of biologically active phenolic compounds and flavonoids were analyzed by GC/MS; these authors suggested that the method should be suitable to determine polyphenols in fruits, vegetables and other food products.

Phenolic compounds and flavonoids embrace a wide range of substances present in all plant tissues and frequently form the most abundant secondary metabolites in fruits (Macheix *et al.*, 1990). They have a wide distribution among the plant kingdom and have been reported in many fruits and vegetables, herbs and spices, maple products, medicinal plants such as the Ginkgo biloba tree and beverages such as red wine and green and black tea (Macheix *et al.*, 1990; Kermasha *et al.*, 1995a,b; Auroma *et al.*, 1996; Rice-Evans *et al.*, 1996; Soleas *et al.*, 1997b and Packer *et al.*, 1998). Phenolic compounds and flavonoids are known to be multi-functional components, primarily by contributing to the sensory quality of fruits, such as color, astringency, bitterness and flavor. In addition, some compounds possess antimicrobial activity whereas others are shown to have pharmacological properties for therapeutic purposes.

Little is known about the identification and characterization of phenolic compounds and flavonoids from maple sap and maple syrup and some biochemical components within maple sap and maple syrup remain to be identified (Willits and Hills, 1996).

Previous work in our laboratory aimed at the optimization of methods for the extraction of phenolic compounds from fruits and maple products as well as the development of procedure for HPLC analysis (Kermasha *et al.*, 1995a,b).

This work is part of ongoing research aimed at the development of various analytical methods for the separation and characterization of phenolic compounds and flavonoids in fruits in the prospective of a potential use as nutraceutic and natural bio-ingredients.

The specific objectives of this work were:

(1) To optimize HPLC and GC methods for the separation and identification of phenolic compounds and flavonoids, using a wide range of standard compounds.

(2) To identify and characterize the phenolic compounds and flavonoids present in maple sap and maple syrup.

(3) To evaluate the changes in phenolic compounds and flavonoids profile in maple sap and maple syrup.

2. LITERATURE REVIEW

2.1. Phenolic Compounds and Flavonoids

Phenolic compounds and flavonoids are secondary metabolites found in numerous plant species (Friedman, 1997). Phenolics embrace a considerable range of substances, which possess an aromatic ring bearing one or more hydroxyl substituents (Macheix *et al.*, 1990). Flavonoids are a group of polyphenolic compounds, diverse in chemical structure and characteristics, found ubiquitously in plants (Cook and Samman, 1996). It is well known that diets rich in fruit and vegetables are protective against cardiovascular disease and certain forms of cancer (Block, 1992) and perhaps against other disease also. These protective effects have been attributed, in large part, to the antioxidants present including the antioxidant nutrients vitamin C and β-carotene, but also the minor carotenoids, and plant phenolics such as the phenolic compounds and flavonoids (Rice-Evans *et al.*, 1996).

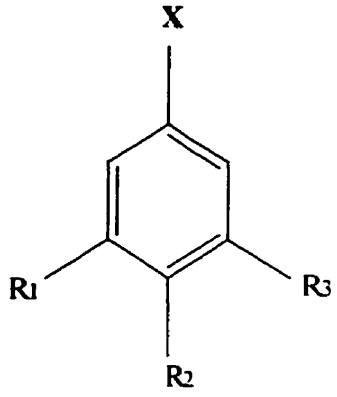
2.1.1. Classification of Phenolic Compounds and Flavonoids

The several thousand polyphenols which have been described in plants can be grouped in several classes most of which are found in fruits. Distinction between each classes is drawn first of all on the basis of the number of constitutive carbon atoms and then in the light of the structure of the basic skeleton (Macheix *et al.*, 1990).

Phenolic compounds and flavonoids belong to the huge family of phenolics, which are characterized by the presence of a phenol residue within their structure. The diversity between each compounds lies in multiple hydroxylation, methylation and glycosylation pattern at various positions around the molecule (Bors *et al.*, 1998).

Phenolic acids belong to two different classes, hydroxybenzoic acids (HBA) and hydroxycinnamic acids (HCA) which are derived from two nonphenolic molecules, benzoic and cinnamic acids, respectively (Fig. 1). Most of the time phenolic acids are found in combined forms in fruits either soluble, in the vacuole or insoluble, linked to cell wall components (Macheix and Fleuriet, 1998).

Hydroxybenzoic Acid (HBA)



X=COOH

Pcc= R1 = R3 = OH

Syc= R1 = R3 = OCH_3, R2 = OH

Vac= R1 = OCH_3, R3 = OH

Ga= R1 = R2 = R3 = OH

X=CHO

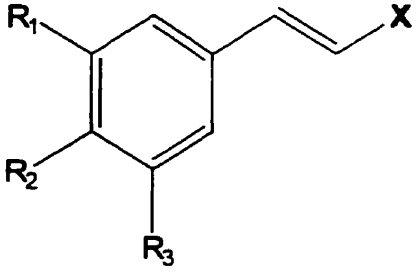
Va= R1 = OCH_3, R2 = OH

Syl= R1 = R3 = OCH_3 , R2 = OH

X=CH_2COOH

Hvac= R1 = OCH_3, R2 = OH

Hydroxycinnamic Acid (HCA)



X= COOH

p-Ca = R3 = OH

Ca = R1 = R2 = OH

Fa = R1 = OCH_3, R2 = OH

Sa = R1 = R3 = OCH_3, R2 = OH

X= COO-quinic acid

Ch= R1 = R2 = OH

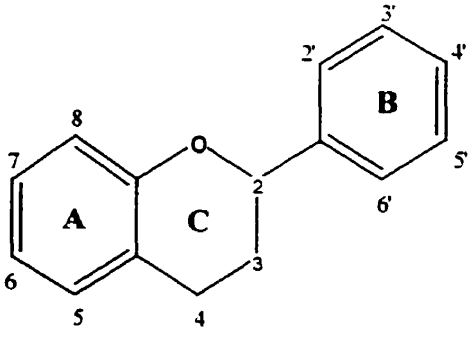
X= CHO

Cal = R2 = OH, R3 = OCH_3

X= CH_2OH

Col = R2 = OH, R3 = OCH_3

Flavonoid



Flavanol: R3= OH

(+)C= R3= R5=R7= R3'= R4'= OH

(-)E= R3= R5=R7= R3'= R4'= OH

Flavanone: R4 = Oxo

Ht= R3= R5= R7= R3'= OH; R4'= OCH_3

Hd= R3= R5= R3'= OH; R4'= OCH_3; R7= Rh-glc

Flavonol: R3= OH, R4= Oxo, R2 = R3

Q= R3= R5=R7= R3'= R4'= OH

K= R3= R5=R7= R4'= OH

R= R5=R7= R3'= R4'= OH; R3= Rut

Qr= R5=R7= R3'= R4'= OH; R3= Rh

Figure 1. Chemical structures of selected phenolic compounds and flavonoids.

The HBA and HCA are structurally similar except the HCA have an ethylenic chain attached to the aromatic ring. The presence of a double bond in the lateral chain of the HCA family leads to the possible existence of two isomeric forms: *cis* and *trans*. Native compounds are mainly of the *trans* form, but isomerisation can occur during extraction and purification and also under the effect of light (Macheix *et al.*, 1990).

HBA can occur as free acid after hydrolysis (acid/base/enzymatic) but frequently they are present as derivatives such as glycosides. HCA on the other hand, occur as free acid only after exceptional conditions such as brutal extraction, contamination by microorganisms and technological processing. Therefore, HCA are present very often as glucose esters, glucosides and can be linked to flavonoids or lignins (Macheix *et al.*, 1990).

Flavonoids are low molecular weight polyphenolic substances which possess the same C_{15} (C_6-C_3-C_6) basic skeleton. The generic structure of flavonoids and the numbering system used to distinguish the carbon positions around the molecule are presented in Figure 1. The carbon atoms in the C and A ring are numbered from 2 to 8 and those in the B ring from 2' to 6' (Hertog and Katan, 1998). The three phenolic rings are referred to as A. B. and C (or pyrane) rings. The biochemical activities of flavonoids and their metabolites depend on their chemical structure and the relative orientation of various moieties on the molecule (Cook and Samman, 1996).

2.1.2. Occurrence of Phenolic Compounds and Flavonoids

Phenolic compounds and flavonoids embrace a wide range of substances present in all plant tissues and frequently form the most abundant secondary metabolites in fruits (Macheix *et al.*, 1990). They have a wide distribution among the plant kingdom and have been reported in many fruits and vegetables, herbs and spices, maple products, medicinal plants such as the Ginkgo biloba tree and beverages such as red wine and green and black tea (Macheix *et al.*, 1990; Kermasha *et al.*, 1995a,b; Auroma *et al.*, 1996; Rice-Evans *et al.*, 1996; Soleas *et al.*, 1997; Packer *et al.*, 1998).

2.1.3. Hydroxybenzoic Acids and Derivatives

The hydroxybenzoic acids content of fruits is generally low, except in certain fruits of the Rosaceae family and in particular blackberry, in which protocatechuic and gallic acids contents may be very high. Syringic acid has only been reported in grapes, and it appears to be of very limited distribution in fruits. Protocatechuic acid is found in soft fruits in the form of glucosides. Finally, vanillic acids are also present in numerous fruits: grapes, cherry, strawberry and native forms are frequently simple combinations with glucose (Macheix *et al.*, 1990). It is not impossible that vanillic, and syringic acids derive at least partially from degradation of certain lignified zones of the fruit when these exist (stone, seed, and teguments) (Macheix and Fleuriet, 1998).

Lignin monomers, present in maple sap are known to be flavor precursor and the oxidation and alkaline hydrolysis of these phenolic compounds are presumed responsible for vanillin and syringaldehyde formation (Potter and Fagerson, 1992).

Most compounds identified in maple saps, concentrates, and syrups were related to lignin derivatives. Specific hydroxybenzoic acids and derivatives found were vanillic acid, syringic acid, homovanillic acid, vanillin, and syringaldehyde (Kermasha *et al.*, 1995a). These results were in agreement with those reported by Potter and Fagerson (1992) who identified the presence of vanillin, homovanillic, syringic, and vanillic acids in maple syrup.

2.1.4. Hydroxycinnamic Acids and Derivatives

The hydroxycinnamic acids present a higher diversity and quantity when compared to the hydroxybenzoic acids. In tomato fruit, *p*-coumaric and ferulic acids are present both as glucosides and as glucose esters whereas caffeic acid is only represented by caffeoylglucose. HCA derivatives with sugars and hydroxy acids are present simultaneously in numerous fruits: apples, tomatoes, and cherry. Comparing HCA contents in numerous fruits reveals enormous variations between species and cultivars, e.g., form approximately 2g/kg fresh weight in blueberries to only traces in

Cucurbitaceae (Hermann. 1989). The relative proportions of each HCA mainly represent a good characteristic of a fruit in the mature stage (Macheix and Fleuriet, 1998).

In 1995, Kermasha *et al.*, identified in maple sap, concentrate and syrup the following hydroxycinnamic acids and derivatives: *p*-coumaric acid, ferulic and sinapic acids, coniferol and coniferal. These results were in agreement, once again, with those reported by Potter and Fagerson (1992) who identified the presence of coniferal and coniferol in maple syrup.

2.1.5. Flavonoids

The flavonoids constitute a large class of compounds, ubiquitous in plants, containing a number of phenolic hydroxyl groups attached to ring structures, conferring an antioxidant activity. They often occur in glycosidic form, cleavage of the free polyphenol-taking place possibly in the gastrointestinal tract. Plant polyphenols are multifunctional and can act as reducing agents, hydrogen donating antioxidants, and singlet oxygen quenchers. In some cases metal chelation properties have been proposed (Benavente-Garcia *et al.*, 1997).

Flavonoids belong to the recently popular class of phytochemicals, which are plant products with potential benefit for human health. Since these compounds exist as secondary metabolites, they are an important part of human diet. They are also considered to be the active principles in many medicinal plants (Bors *et al.*, 1998).

2.1.5.1. Flavanol

Flavans are flavonoids characterized by a saturated 3-C chain (Fig. 1). They are of two types, flavan-3, 4-diols and flavanols. Flavanols (catechins) are some of the most widely occurring flavonoids. They have two asymmetric carbons (2, 3) and therefore four possible isomers. The (+)- and (-)- catechins have the number 2 and 3 hydrogen in *trans* configuration, while they are *cis* in the epicatechins (Spanos and Wrolstad, 1992). (+)-Catechin is generally more plentiful than (-)-epicatechin with the mean reaching 79% in berry skins whereas it is only 50% in the seeds (Bourzeix *et al.*, 1986).

2.1.5.2. Flavanone

This type of flavonoid is characterized by a C_6-C_3-C_6 structure in which the 3-C chain is saturated and with an oxygen atom in the 4-position (Fig. 1). Flavanones correspond to flavones whose double bond between carbons 2 and 3 is saturated, hence the name dihydroflavones which is sometimes used. They have an hydroxyl group in the 3-position and are also referred to as dihydroflavonols. Because of the saturation of the bond between C-2 and C-3, flavanones possess an asymmetrical carbon (in the 2-position). Flavanones are widely distributed in the plant kingdom but are very rarely present in fruits (Bohm, 1975; Bohm, 1982) except in *Citrus* (Horowitz, 1961; Kefford and Chandler, 1970).

2.1.5.3. Flavonol

Flavonols are flavonoids characterized by an unsaturated 3-C chain with a double bond between C-2 and C-3 and by the presence of an hydroxyl group in position 3 (Fig. 1). Glycosylation at position 3 can result in glycosylated flavonoids such as rutin and quercitrin. In fruits, almost only the glycosides of four aglycones are found: kaempferol, quercetin, myricetin and isorhamnetin. The two most common are quercetin and kaempferol. The combination of quercetin and kaempferol is by far the most frequent in most fruits (Macheix *et al.*, 1990).

2.1.5.4. Chalcone

Chalcones are characterized by a C_{15} skeleton with an open 3-C chain. Natural chalcones are all hydroxylated at nuclei A and B and there are numerous methoxylated derivatives. Although a large number of chalcones have been identified in flowers, wood, and bark, data on fruit chalcones are scarce (Bohm, 1975 and Bohm, 1982).

2.1.5.5. Flavonoid Glycosides

Glycosylation occurs preferentially on the hydroxyl group in the 3-position. In fruits as in other parts of plants, 3-*O*-monoglycosides are very predominant. Flavonol glycosides have been found acylated with *p*-coumaric, ferulic, caffeic, *p*-hydroxybenzoic, gallic, acetic, and malonic acids, but they have rarely been reported in fruits for which

data is still fragmentary. In contrast with the high number of flavonol glycosides known in the plant kingdom (approximately 250), only about 50 have been identified in fruits and most of these are quercetin glycosides (Harborne and Williams, 1975). The most frequent flavonol glycoside is the quercetin 3-glucoside (isoquercitrin), which was found in 80% of the fruits examined, and whose equivalent is the cyanidin 3-glucoside which is also the most common anthocyanin in fruits (Harborne, 1964).

2.2. Importance of Phenolic Compounds and Flavonoids in Foods and Food Products

Phenolic compounds and flavonoids are of great interest to man in different domains. They first contribute to sensory qualities such as color, astringency, bitterness and aroma. In addition, some compounds possess antimicrobial activity and finally others are shown to have pharmacological properties for therapeutic purposes.

These different aspects, which justify the increasing interest in phenolic compounds and flavonoids in fruits and many other plants, could support the interest of their identification and characterization in maple products.

2.2.1. Sensorial Properties

Color is an important feature of food and food products from the ecological point of view and for identification or determination of their commercial value. Indeed, as with all human foods, consumer choice is influenced by color, both in the case of direct use of food products and in that of processed products (fruit juices, fermented beverages, jellies, jams, syrups, preserves, etc.).

Bitterness can be elicited by many compounds, which may be present in certain fruits, such as heavy metals, amino acids, peptides, alkaloids, terpenes and different phenolics. For example, the bitterness of wine and cider is caused mainly by phenolic compounds and in particular by oligomeric proanthocyanidins (Lea and Arnold, 1978b and Arnold *et al.*, 1980). In addition, the sensation of bitterness may be modified by the physicochemical environment of the active molecules, and in particular by the °Brix/acid ratio of fruit juices (Tatum and Berry, 1987). It can even be completely removed with

buffer strength or in the presence of high concentrations of sugars (Lea and Arnold, 1978b).

Hydroxybenzoic and hydroxycinnamic acids are known as simple monocyclic acids there importance arises from their ability to undergo decarboxylation, either by thermal fragmentation or through the activities of microorganisms. Consequently, high flavor-active phenols are produced. In certain beers these flavor-active phenols may be appreciated but in others they may be regarded as distasteful (Macheix *et al.*, 1990).

The polyphenolic compounds of apples have been studied fairly well because they contribute to the color and flavor of apple juices (Timberlake and Bridle, 1971 and Lea and Timberlake, 1974). Flavonoid compounds occupy a prominent position among natural phenols, particularly due to their conspicuous presence in green plants as well as to their importance in the flavor and nutritional quality of foodstuffs (Pietrogrande and Kahie, 1994). Finally, the amount of phenolic compounds in virgin olive oil is an important factor when evaluating the quality of virgin olive oil because natural phenols improve its resistance to oxidation and its sharps bitter taste (Cinquanta *et al.*, 1997).

2.2.2. Antimicrobial Properties

Over the past 10 years, phenolic compounds have been known to play a role in resistance of plants to different stresses such as wounding, various chemical treatment or microbiological infection (Bell, 1981). Whatever the type of stress, one of the most common responses is the increase in the total phenolic content (Macheix *et al.*, 1990).

Phenolic compounds are involved in the synthesis of lignins, which are complex polymers, by providing monomeric precursors from hydroxycinnamic acids. In fruits, this lignification related to phenolic metabolism is enhanced after wounding or attack by parasites (Macheix *et al.*, 1990). Healing consists of the formation of polymers, such as lignin and suberin by cell close to the wound.

A number of flavonoids also possess antiviral activity (Beladi *et al.*, 1982). There have been many reports of antiviral and antibacterial activities of lignans (MacRae and

Towers, 1984). It has been demonstrated that quercitin has an antiviral effect *in vitro* and *in vivo*, in particular on poliovirus type 1, herpes simplex virus type 1, parainfluenza virus type 3, and respiratory synctial virus (Musci, 1986).

2.2.3. Pharmacological Interest of Phenolic Compounds and Flavonoids

Numerous works over the past 20 years have shown that flavonoids may represent an interesting new therapeutical approach because they can interfere with different steps involved in the development of vascular diseases. Procyanidins are physiologically active substances which in particular combat the formation of atheromas which cause myocardial infarction (Masquelier, 1982).

The plant contains a mixture of flavonoligans, silybin, silydianin, and silychristin which all possess antihepatotoxic activity. They are important since they are nonimmunosuppressant drugs, which can be used to treat liver diseases and prevent the action of several liver poisons (Sonnenbichler *et al.*, 1981).

In addition to a wide spectrum of pharmacological properties, phenolic compounds, and specifically quercetin, have been shown to inhibit the growth of cells derived from human and animal cancers, such as leukemia and Ehrlich ascites tumors (Soleas *et al.*, 1997b).

2.3. The Maple Products

The unique flavor of maple syrup has made it popular both in the confectionery industry and to consumers. In addition, emphasis on the consumption of natural foods has resulted in the use of maple syrup as an alternative sweetener (Anon, 1984).

About 70% of the World's production of concentrated maple sap (maple syrup) is collected in Canada and 90% of it originates from the Province of Quebec. In 1992, 16 billion liters of maple syrup, having a market value of 45 million Canadian dollars, were produced. Although most of the concentrated maple sap that is produced is consumed as syrup or used to aromatize jams, desserts and tobacco, 5-20% of the total production is classified as low-grade because of flavor, color, taste or appearance defects. One way of

disposing of this low-grade sap is to blend 1 liter of it into each 60 liters of concentrated maple sap of standard quality. This practice lowers the quality of maple products and, on a long-term basis it may jeopardized the maple product industry of Quebec. Finding good usage of the low-grade maple syrup without having to decrease the quality of pure maple syrup could be of great importance to the maple syrup industry (Morin *et al.*, 1995).

2.3.1. Maple Sap Production

The sap is collected from the tree (*Acer Saccharum*) in early spring when temperatures fluctuate from freezing at night (-5 to -10°C) to thawing during the day (5 to 10°C). The sap itself is a clear water-like substance which tastes only slightly sweet but which contains all the precursors required for the development of flavor and color which are characteristic of maple syrup (King and Morselli, 1983 and Willits and Hills, 1996).

2.3.1.1. Chemical Composition of Maple Sap

Many factors affect changes in sap biochemistry, both in the sugarbush and in the storage tank before evaporation. These partly determine the shades of amber coloration within and between syrup color grades, as well as the flavor. Not all the biochemical components of sugar maple sap have been identified. It is important to identify and understand the roles and interactions of sap precursors of maple syrup colors and flavors. This can help minimizing syrup off-flavors and control syrup grade production (Willits and Hills, 1996).

The initial maple sap represents a solution in which sucrose is the major component. In addition, minor quantities of reducing sugars (Jones and Alli, 1987), organic acids as well as minerals and nitrogenous compounds (Morselli and Whalen, 1986) and phenolic compounds (Kermasha *et al.*, 1995a) have been reported to be present in maple sap. It is the interaction of these compounds during the boiling process that accounts for the flavor associated with pure maple syrup.

Sucrose is the most prevalent sugar, comprising 98-99% of the dry matter of sap. This leads to a misunderstanding by the casual observer that sap is just sugar water. If that were true, sap would not sustain the life of the tree. It is that small percentage (2.0%

or less) of amino acids, organic acids, phenolic compounds, hormones, minerals and salts, and other components that allows sap to be the physiological liquid, with the right pH and buffering capacity, responsible for helping initiate growth within the tree (Willits and Hills, 1996).

2.3.1.2. Sap Chemical Change Due to Microorganisms

Maple sap is basically sterile in the vascular bundles, but becomes contaminated during collection, storage and production (Dumont *et al.*, 1993). The overall effect of this contamination may not be readily seen by the producer, except when the sap becomes turbid and milky looking. Sap is an ideal growth medium for microorganisms, growing faster in warm sap because it has a carbon source (sugar) as well as nitrogen, phosphorus and is mixed with oxygen. Accelerated microbial enzymatic activity upon the sap changes its biochemistry (Whalen and Morselli, 1985).

2.3.2. Maple Syrup Production

Once collected, the sap is concentrated to a Brix value of ~66.5°. This is accomplished by water evaporation or by employing reverse osmosis followed by evaporation. The unique flavor characteristics of maple syrup are developed during this evaporation (~93-110°C for 1.5 h) process (Willits and Hills, 1996).

2.3.2.1. Development of the Maple Syrup Flavor

The flavor of maple syrup develops like the color also during evaporation. In gas chromatograms of dichloromethane extracts more than 133 substances have been observed, 48 peaks were identified. From that 41 components were phenol derivatives which represented the bulk (about 70%) of the extract. In principle two types of flavor bearing constituents occur. Primarily, thermal sugar degradation; secondly, derivatives of lignin precursor: coniferyl, dihydroconiferyl, and dihydrosinapyl alcohol. In particular the derivatives vanillin and syringaldehyde that are known to be flavor-bearing constituents. It is remarkable that also in maple syrup (as of course in maple sap) these precursors are present in higher concentrations than the actual flavor bearing constituents. Meaning that maple syrup contains a flavor reserve, which theoretically could be activated by further

oxidation. The concentration of the lignin derivatives does in contrast to the sugar degradation compounds vary considerably depending on provenance and processing history (Potter and Fagerson. 1992; Belford and Lindsay, 1992; Dumont, 1995 and Kermasha *et al.*, 1995a).

Flavor compounds of maple syrup include volatile phenolic compounds, carbonyl compounds, and alkylpyrazines (Kallio, 1988; Belford *et al.*, 1991). The alkylpyrazines, are typical products of the advanced stage of the Maillard reaction, and have been subjected to numerous studies because of their implication on color and flavor of foods (Maga, 1982). Sucrose, glucose, and fructose (Jones and Alli, 1987) and amino acids (Morselli and Whalen, 1986; Kallio, 1988), present in maple sap, would be expected to be the principle precursors for the formation of pyrazines in maple syrup. The principal pyrazine in maple syrup was 2,6-dimethylpyrazine, representing 34-43% of the total identified pyrazines (Akochi-K *et al.*, 1994).

2.3.2.2. Maple Syrup Flavor Evolution

The source of vanillin and syringaldehyde in maple syrup has been suggested to be lignin or lignin fragment. Later, Potter and Fagerson (1992) reported on the identification of phenolic lignin monomers and related flavor compounds in dichloromethane extracts of maple syrup. In addition, a vanillin-glucoside was identified in maple sap as a precursor of vanillin in maple syrup (Belford *et al.*, 1992).

Kermasha *et al.* (1995a) identified ten major phenolic compounds in maple sap, concentrate and syrup including, vanillic acid, syringic acid, homovanillic acid, coniferol, vanillin, syringal, *p*-coumaric acid, sinapic acid, ferulic acid and coniferal were positively identified. On the other end, spectral characteristics of five major unknown peaks did not allow the identification of these compounds and consequently further investigations were needed.

2.3.2.3. Specific Syrup Characteristics

Québec regulations stipulate that maple syrup must have 66% of refractometric dry substance at 20°C and must consist of concentrated maple sap (Gouvernement du

Québec, 1983; Dumont *et al.*, 1993). While the minimum syrup density of 66.0° Brix is a legal requirement in most states, there are also several practical reasons for carefully controlling the finished density of maple syrup.

2.3.2.4. Chemistry of Maple Syrup

Pure maple syrup consists primarily of sugars (90-100% sucrose and 0-10% glucose). Other chemical components of maple syrup include amino acids, proteins, phenolic compounds, organic acids and trace levels of vitamins. However, a large amount of mineral material has been found dissolved in maple syrup with potassium and calcium being the most prevalent (Willits and Hills, 1996).

Stuckel and Low (1996) studied the chemical composition, pH and °Brix of 80 pure maple syrup samples produced in North America. The chemical composition of maple syrup is of importance because temptation exists to adulterate maple syrup via the addition of inexpensive sweeteners. It is also important for nutritional reasons (Morselli, 1975).

The major carbohydrate found was sucrose whereas glucose and fructose were present in much lower quantities. Differences in concentrations for these three carbohydrates may be due to the age of the maple syrup samples analyzed in the study since monosaccharide levels increase as storage time increases. High levels of glucose and fructose observed in some samples could be attributed to processing, processing method and/ or microbial load (Whalen and Morsellin, 1985).

The °Brix values obtained correlated with the combined carbohydrate content because approximately 99% of the total solids present in maple syrup are sugars and mainly sucrose (Stuckel and Low, 1996).

Sample pH ranged from 5.64 to 7.90 and had a mean value of 6.66. The variation in sample pH can be related to microbial contamination, the removal of organic acids with niter, or their conversion to flavor compounds during evaporation of the sap (Robinson *et al.*, 1989; Willits and Hills, 1996).

The major organic acids present in maple syrup are malic, citric, succinic and fumaric, whereas, the major minerals were calcium, magnesium and potassium. Finally, the study suggested that the mineral content of maple syrup may be used to establish the origin of the syrup since it was the only variable that showed statistical difference between geographical regions (Stuckel and Low, 1996).

2.4. Extraction and Recovery of Phenolic Compounds and Flavonoids

The extraction method must be suited to the plant material chosen and the types of phenolic compounds and flavonoids to be studied. Such method must enable complete extraction of phenolic compounds and flavonoids avoiding chemical modification which results in artifacts (Macheix *et al.*, 1990). Purification of the raw extract is essential in order to remove non-phenolic substances (sugars, organic acids, proteins and pigments) which can interfere during chromatographic separation and assays.

Mahler *et al.* (1988) used ethyl acetate to extract eight non-flavonoid phenols in Vidal blanc wines. The combined ethyl acetate extracts were dried over anhydrous sodium sulfate and decanted.

Kermasha *et al.* (1995a) determined the phenolic compound profiles in maple products by high performance liquid chromatography. The mean percentage recovery for all phenolic and furfural compounds using different methods of extraction, was obtained in a decreasing order as follows: ethyl acetate (87.6%) > Sep-Pak (82.2%) > lyophilization (62.9%) > ether (44.3%) > Supelclean (41.8%). Additional work on ethyl acetate extraction indicated a very good reproducibility and therefore the ethyl acetate method of extraction was chosen for the study.

Chedier *et al.* (1999) reported a comparative methodology for the isolation of flavonoid glycosides from *Clusia criuva* Cambess. It was actually possible to isolate flavonoid glycosides from ethyl acetate extract of that particular plant in a very short operation time.

2.5. Separation and Identification of Phenolic Compounds and Flavonoids

Separation of phenolic compounds and flavonoids is an essential stage prior to the application of identification and measurement techniques. Only modern methods of analysis can make it possible to accurately identify these diverse and complex molecule often present as trace (Macheix *et al.*, 1990).

The separation of phenolic compounds and flavonoids has greatly progressed over the past decade due to the use of high performance liquid chromatography. In particular, the development of reversed phase columns has greatly improved the separation performance of phenolic compounds and flavonoids as well as the use of diode-array (DAD) and electrochemical (EC) detectors (Wulf and Nagel, 1976; Hayes and Smyth *et al.*, 1987; Jaworski and Chang, 1987; Kermasha *et al.*, 1995a,b; Goldberg *et al.*, 1996).

2.5.1. High Performance Liquid Chromatography (HPLC)

2.5.1.1. Principle of Reversed-Phase Chromatography (RPC)

Separation is similar to the extraction of different compounds from water into an organic solvent such as octanol, where more hydrophobic (non-polar) compounds preferentially extract into the non-polar octanol phase. The column (typically, a silica support modified with a C_8 or C_{18} bonded phase) is less polar than the water-organic mobile phase.

The elution sequence of the individual compounds can best be interpreted by assuming that the compounds are first adsorbed on the hydrophobic stationary phase by "hydrophobic interaction", and that they are subsequently eluted with the mobile phase according to the extent of hydrogen bond formation.

2.5.1.2. The Elution Profile for Phenolic Compounds and Flavonoids in RP-HPLC

A high-pressure liquid chromatography (HPLC) technique for the separation and quantitation of three classes of naturally occurring phenolic compounds has been developed by Wulf and Nagel (1976). The use of a reverse-phase column as well as dual wavelength ultraviolet detector enabled the elution order of the phenolic compounds and

flavonoids in the order typically seen in a reverse-phase system, that is, polar components eluting before non-polar components. They have shown that the structural difference between hydroxybenzoic acids (HBA) and hydroxycinnamic acids (HCA) is the presence of an extra ethylenic chain in the cinnamic acids which provides more hydrophobicity and therefore results in a higher selectivity compare to phenolic acids with a one-carbon chain (HBA).

2.5.1.3. Ultraviolet Detection

Many different detection methods are available in HPLC analysis. In general, ultraviolet (UV) detectors are most popular and have been extensively used in the detection of phenols. The limitations concerning the UV detector are that the compound must absorbs ultraviolet light and any other contamination that also absorbs UV radiation may interfere in the analysis. On the other hand, the analyte is not destroyed by this type of detection and can be recuperated after separation for further characterization (White, 1984).

Winter and Herrman, (1984) have developed a method for the separation of hydroxycinnamic acids derivatives from plant extracts. RP-18 column with a gradient elution system and UV detector set at 320 nm was used.

Diode-array detectors have gain tremendous popularity due to their ability to scan a spectrum region of interest and therefore provide the compound with a fingerprint. Each different compound has a specific scan with a maximum wavelength. Identification of phenolic compounds using HPLC and diode-array detector at specific wavelength was undertaken and showed reliable results (Jaworski and Chang, 1987; Oleszek *et al.*, 1988; Spanos *et al.*, 1990; Gao and Mazza, 1994; Pietrogrande and Kahie, 1994).

2.5.1.4. Comparison of Ultraviolet and Electrochemical Detectors

Electrochemical (EC) detectors are being used increasingly in HPLC analysis as they exhibit high sensitivity and selectivity. One of the limitations of EC detector is that the analyte must be electroactive, otherwise it will not be detected. Phenols are electro-oxidisable compounds and therefore are amenable to EC detection. Several analytical

methods based on HPLC with EC detection for phenolic compounds have been developed using isocratic conditions (Kenyherz and Kissinger, 1977; Roston and Kissinger, 1981).

A comparison between EC and UV detection has been applied to the determination of phenolic compounds in beer. Both detectors were placed in series and gradient rather than isocratic conditions were used. The best detection limits were achieved with the EC detector. It has been stated that using a fixed rather than a variable wavelength for UV detector increases the sensitivity because it produces less noise (Laurence, 1981; Hayes *et al.*, 1987).

Combined UV-diode array and electrochemical analyses of standard phenolic and furfural compounds were made at three different detection conditions for UV at 280 and 320 nm and EC at 600 mV. The results showed that 2-furfural, 5-methylfurfural and HMF were monitored only with UV detection, whereas phenolic compounds were detected by both UV and EC. In addition, EC analyses provided a dramatic increase in the limits of detection of all phenolic compounds, compared to those obtained by UV analyses (Kermasha *et al.*, 1995b).

Determination of phenolic compounds in maple products using UV diode-array and EC detection was performed (Kermasha *et al.*, 1995a). The EC analyses provided a dramatic increase in the limits of detection of all phenolic compounds compared with those obtained by UV analyses. In addition, the detection limits are of the order of previous work on UV/EC comparison (Hayes *et al.*, 1987; Galetti *et al.*, 1990).

2.5.2. Gas Chromatography (GC)

Gas chromatography is that form of chromatography in which a gas is the moving phase. The components of the sample separate from one another based on their relative vapor pressures and affinities for the stationary bed. Many advantages arise from the use of GC as an analytical technique, it is fast, efficient, sensitive and can easily detect ppm and often ppb. In addition, this method is nondestructive and allows on-line coupling such as the use of a mass spectrometer. One major limitation is that the sample analyzed

in GC must be volatile and therefore derivatization is often required (McNair and Miller, 1998).

2.5.2.1. Gas Chromatography and Flame Ionization Detection (FID)

Flame ionization detector is the most widely used GC detector and is part of the universal detectors. the analyte in the effluent enters the flame and passes through an hydrogen/air flame. Ions and electrons formed in the flame cause a current to flow in the gap between two electrodes in the detector by decreasing the gap resistance. By amplifying this current flow a signal is produced (Kitson *et al.*, 1996). The FID responds to all organic compounds that burn in the oxy-hydrogen flame. The signal is approximately proportional to the carbon content. giving rise to the so-called *equal per carbon* rule (McNair and Miller. 1998).

2.5.2.2. Derivatization Procedure

Derivatization causes a nonvolatile sample to become volatile, or it improves the detectability of the derivative. Furthermore. the derivatives may also be more thermally stable. Silylation reactions are very popular. A variety of reagents are commercially available. and most are designed to introduce the trimethylsilyl group into the analyte to make it volatile. A typical reaction is the one between bis-trimethylsilylacetamide (BSA) and an alcohol. A closely related reagent contains the trifluoroacetamide group and produces a more volatile reaction by-product (not a more volatile derivative); the reagent is bis(trimethylsilyl)-trifluoroacetamide (BSTFA). The latter has a greater reactivity than BSA but has a lower reactivity than trimethylsilylimidazole (TSIM). If a solvent is used it is usually a polar one: the bases DMF and pyridine are commonly used to absorb the acidic by-products (McNair and Miller. 1998).

Generally. derivatization of phenols and dihydroxybenzenes requires the addition of 250 μL of N-methyl-trimethylsilyltrifluoroacetamide (MSTFA) or TRI-SIL/BSA to approximately 1 mg of sample in a septum-stoppered vial followed by a heat treatment at 60° C for 15 min (Kitson *et al.*, 1996).

A comparison was made between different combinations of derivatizing agents: bis(trimethylsilyl)-trifluorocetamide (BSTFA) only, BSTFA with 1% trimethylchlorosilane (TMCS) (v/v), and BSTFA/pyridine (1:1) by volume in attempt to eliminate some interference and improve recoveries. Some matrix interference was eliminated, and a significant increase in recoveries was observed with BSTFA/pyridine (Soleas *et al.*, 1997a).

In 1997, Soleas *et al.* derivatized fifteen phenolic constituents using BSTFA but first to ensure complete removal of water, 0.5 mL of methylene chloride was added, and the resultant mixture was vortexed and evaporated to dryness (azeotropic removal of water). The extracts were then further dried in an oven at 70°C for 15 min and derivatized by incubating with 1mL of 1:1 BSTFA/pyridine (v/v) using vigorous vortexing and incubating at 70 °C for 30 min.

2.5.3. Gas Chromatography-Mass Spectrometry

Gas chromatography with mass spectrometry (GC/MS) is the synergistic combination of two powerful analytic techniques. The gas chromatograph separates the components of a mixture in time, and the mass spectrometer provides information that aids in the structural identification of each component (Kitson *et al.*, 1996).

A mass spectrometer is an instrument that measures the mass-to-charge ratio (*m/z*) of gas phase ions and provides a measure of the abundance of each ionic species. A mass spectrum is a graphic representation of the ions observed by the mass spectrometer over a specified range of *m/z* values. The mass spectrum will contain peaks that represent fragment ions as well as molecular ion. Interpretation of a mass spectrum identifies, confirms, or determines the quantity of a specific compound (Kitson *et al.*, 1996).

Free, esterified, and insoluble-bound phenolic acids in oilseeds were subjected to GC/MS analysis using a capillary column of fused silica. In this study the GC/MS analyses were used to confirm the presence or absence of phenolic compounds in nature (Krygier *et al.*, 1982).

Goldberg *et al.* (1994) have developed a novel assay for *trans*-resveratrol that may have a wide application. Direct injection of the underivatized extract into a gas chromatograph/mass spectrometer (GC/MS) with the detector in the selective ion monitoring mode (SIM). The molecular ion was detected and quantitated at a mass of 228, with qualifier ions at *m/z* 227 (M-H) and 229. Similarly a method for the measurement of *cis*-resveratrol in wine was developed. The limit of detection for *trans*-resveratrol was found to be 50 μg/L (Goldberg *et al.*, 1994), whereas for *cis*-resveratrol it was 10 μg/L (Goldberg *et al.*, 1995).

A multiresidue derivatization gas chromatographic assay for fifteen phenolic constituents with mass selective detection was developed by Soleas *et al.* (1997a). The TMS derivatives of each phenolic compound were analyzed on a GC/MSD coupled to a DB-5HT capillary column using one target and two qualifying ions for each compound. This GC/MS method was developed to simultaneously measure the concentration of fifteen biologically active phenolic components of wine and has been applied to solid vitaceous plant material.

3.MATERIALS AND METHODS

3.1. Materials

3.1.1. Reagents and Standards

All chemicals used throughout this study were of ACS reagent grade or higher. Phenolic standards of protocatechuic, vanillic, chlorogenic, *p*-coumaric and ferulic acids as well as vanillin, rutin, phloridzin, quercitrin, (+)-catechin, (-)-epicatechin, *trans*-resveratrol, hesperetin and kaempferol were purchased from Sigma Chemical Co. (St. Louis, MO). Coniferyl alcohol (coniferol), coniferyl aldehyde (coniferal), syringaldehyde, fisetin, caffeic, homovanillic, sinapic and syringic acids were obtained from Aldrich Chemical Co. (Milwaukee, WI). Hesperidin and quercetin were obtained from ICN Biochemicals (Cleveland, OH).

Trifluoroacetic acid (TFA) (ACP Chemicals Inc., St-Leonard, QC) and methanol of HPLC grade (Fisher Scientific Ltd., Negean, CA) were used. Bis(trimethylsilyl)-trifluoroacetamide (BSTFA), used for the derivatization of all standards and samples prior to GC analysis, was purchased from Sigma Chemical Co.

3.2. Methods

3.2.1. Development of Methodologies for the Analysis of Phenolic and Flavonoid Standards

3.2.1.1. Optimization of HPLC Analysis

An HPLC analytical method was developed for the separation and identification of phenolic and flavonoid standards. The standard mixture was analyzed with an HPLC system (Bekman Model 126, Beckman Instruments Inc., San Ramon, CA) equipped with a UV diode-array (UV-DAD) detector (Beckman, Model 168) and an electrochemical (EC) detector (Coulochem II, Esa Inc., Bedford, MA) assembled in series and computerized integration with data handling was used for the analysis. A Beckman analog interface Model 406 was used to transfer data from the EC detector to the HPLC system. The UV detection was performed at 280 and 320 nm. Scanning of the analytes was also performed from 190 to 400 nm and was monitored at 1 sec interval. The EC

detector was set at an output of 1 V, and the detection was performed at 200 and 600 mV at 10 μA. Automatic injection (Varian, Autosampler 9095. Varian Associates, Inc., Walnut Creek, CA) was carried out with a 50 μL loop onto a Zorbax SB-C18 reverse phase column (250 x 4.6 mm i.d., pore size 5 μm) protected with a guard column of the same material (Chromatographic Specialities Inc., Brockville, ON).

The analysis was performed with a flow rate of 0.75 mL/min, using 0.2% trifluoroacetic acid (TFA) as solvent A and methanol as solvent B, with a linear gradient from 5 to 80% methanol in 50 min. All solvent used were filtered on AcetatePlus (0.22 μm) (Fisher Scientific Ltd., Negean, CA) before analysis. The selected flavonoid standards required a greater concentration of methanol (80%) and a longer HPLC run for their proper elution than phenolic acids.

Each standard was first injected individually to determine the exact retention time and chromatographic characteristics (λ_{max}, absorbance ratio and EC response) followed by the analysis of the standard mixture.

3.2.1.1.1. The Limit of Detection for Phenolic and Flavonoid Standards

Calculation of the limit of detection (LOD) for each phenolic and flavonoid standard was based on the external standard method. Dilutions of methanolic solutions containing 25 μg/mL of all standards, injected in triplicate, were used to create a standard curve (peak area versus concentration in micrograms per mililiter), with a linear regression for each compound using the method previously described for HPLC analysis. The determination of the LOD of phenolic and flavonoid standards, responding to the UV detection at 280 and 320 nm as well as the EC detection at 600 mV, was hence performed. Calculations were based on the minimum detectable concentration of standard compounds calculated on the basis of a signal-to-noise ratio of 3. Furthermore, the range of linearity was determined by the lower and higher limits of quantification from the calibration graph, r-square value and precision were calculated for each compound.

3.2.1.2. Optimization of GC Analysis

3.2.1.2.1. The Derivatization Procedure

The derivatization of phenolic and flavonoid standards for GC analysis was performed according to a modification of the procedure described by Soleas *et al.* (1997). Azeotropic removal of water was achieved by adding 0.5 mL of dichloromethane and drying completely the sample under nitrogen. The trimethylsilylation (TMS) reaction was performed, using 100 μL of 1:1 (v/v) BSTFA/pyridine, with vigorous vortexing and capped under nitrogen for incubation at 40°C for 30 min. The derivatized samples were dried under a stream of nitrogen and were re-dissolved in dichloromethane to be subjected to GC analysis.

3.2.1.2.2. GC Analysis Procedure

The analyses of phenolic and flavonoid standards were performed using Hewlett-Packard (HP) (Model GC 6890) GC equipped with a FID and a mass spectrometer (MS) (HP 5973). Ultrahigh-purity helium was used as a carrier gas; compressed air and hydrogen were used for the FID. The carrier gas line pressure was set at 80 psi, compressed air at 60 psi and hydrogen at 40 psi. Hydrogen flow was 30.0 mL/min, air at 300 mL/min and a make up flow of helium was set at 30 mL/min.

For GC/FID analysis, an EPC cool on-column injection in track oven mode was the injector parameter with an injection volume of 1 μL using 10 μL syringe. For GC/MS analysis, an EPC split/splitless injection mode was used with the same injection conditions for the GC/FID; however, the injector temperature was set at 280°C. The GC programmed temperature for the column was the same for both type of detectors. Initial oven temperature was 80°C reaching 250°C at a rate of 20.0°C/min, followed by 300°C at 6°C/min and finally 320°C at 20°C/min for a total run time of 25.8 min.

GC analysis was performed with a fused silica capillary column HP 5MS (30m x 0.25mm i.d., with 0.25 μm of film thickness and cross-linked with 5% PHME (phenylmethyl) siloxane), obtained from Hewlett-Packard; however, for GC/MS analysis

no guard column was used. Each standard was first injected individually in order to determine the exact retention time, followed by the injection of the standard mixture.

3.2.1.2.3. The Limit of Detection for Phenolic and Flavonoid Standards

Calculation of the limit of detection (LOD) for each phenolic and flavonoid standard, except the glycosylated ones, was based on the external standard method. Dilutions of methanolic solutions containing 83.33 µg/mL of all standards injected in triplicate were used to create a standard curve (peak area versus concentration in µg/mL), with a linear regression for each compound using the method previously described for GC analysis. The LOD of phenolic and flavonoid standards was determined from the GC/FID analysis. Calculations were based on the minimum detectable concentration of the standard compounds, calculated on the basis of a signal-to-noise ratio of 3. Furthermore, the range of linearity was determined by the lower and higher limits of quantification from the calibration graph, r-square value and precision were calculated.

3.2.2. Characterization of Maple Sap and Maple Syrup

3.2.2.1. Maple Product Samples

Samples of maple sap, collected at different periods of the 1999 maple sap season, were qualified as 0, 25, 50, 75 and 100% and obtained from "Le Centre de recherche, de développement et de transfert technologique en acériculture, ACER", St-Hyacinthe, Qc. Maple sap at 2°Brix was stored frozen in 4-L containers. It was thawed vigorously by shaking continuously the sap container under tap water at 25°C in the shortest time possible to prevent microorganisms multiplication which could create an alteration of the product. The maple sap was filtered on qualitative circles 70 mm ϕ, Whatman filter paper No. 1 (Whatman International Ltd., Maidstone, U.K.) under vacuum and subsequently on AcetatePlus filters (0.22 µm) to remove the undesirable contaminants. After performing the last filtration, the sap was ready for extraction with ethyl acetate. The maple syrup was handle differently since it was stored in glass container at 4°C and had been previously filtered by the producer. The maple syrup (66°Brix) was adjusted to 2°Brix with deionized water to perform the extraction under the same conditions as for the maple

sap by diluting 1.5 mL of maple syrup in a total volume of 50 mL. Thereby, diluting 33 times the maple syrup.

Maple saps and maple syrups were sampled in quadruplet for the different periods of the maple sap season of 1999 (0, 25, 50, 75 and 100%). The pH and degree Brix (°Brix) values were determined for each sample. The degree Brix was defined as the refractometric dry substance at 20°C and was provided by the producer in accordance with Québec regulations (Gouvernement du Québec, 1983), which state that maple syrup needs 66% of refractometric dry substance at 20 °C.

3.2.2.2. Extraction of Phenolic Compounds and Flavonoids

Extraction of phenolic compounds and flavonoids was carried out according to modifications of the methods reported by Kermasha *et al.* (1995a) as well as Dawes and Keene (1999). Maple sap and maple syrup (50 mL) were adjusted to pH 7 with 2 N NaOH. However, only slight adjustment was required since the pH of maple sap and syrup was already close to a neutral value. Subsequently, the sample was extracted three times with 50, 25 and 25 mL of ethyl acetate using a separating funnel. The organic upper phase, containing the phenolic compounds and flavonoids, was recovered after each extraction. The residual aqueous traces present in the ethyl acetate extract was removed using anhydrous sodium sulfate (Na_2SO_4). Evaporation of the ethyl acetate to dryness was performed by the automatic environmental SpeedVac system (System Savant, Holbrook, N.Y.).

The analysis of phenolic compounds and flavonoids present at different periods of the maple sap season for both maple sap and maple syrup was performed.

3.2.2.3. HPLC Analysis of Phenolic Compounds and Flavonoids Obtained from Maple Sap and Maple Syrup

Each phenolic compounds and flavonoids residue recovered from ethyl acetate extraction was dissolved in 125 µL of methanol, thereby concentrated 400-fold and subjected to HPLC analysis.

HPLC analysis of phenolic compounds and flavonoids at 0, 25, 50, 75 and 100% of the maple sap season was performed using the method previously described for the analysis of phenolic and flavonoid standards.

3.2.2.4. GC Analysis of Phenolic Compounds and Flavonoids Obtained from Maple Sap and Maple Syrup

Each phenolic compounds and flavonoids residue recovered from ethyl acetate extraction was derivatized, dried under a stream of nitrogen and dissolved in 125 µL of dichloromethane, thereby concentrated 400-fold, for GC analysis.

GC/FID analysis of phenolic compounds and flavonoids at 0, 25, 50, 75 and 100% of the maple sap season was performed using the method previously described for the analysis of phenolic and flavonoid standards.

3.2.2.5. GC/MS Analysis of Phenolic Compounds and Flavonoids Obtained from Maple Sap and Maple Syrup

Each phenolic compounds and flavonoids residue recovered from ethyl acetate extraction was derivatized, dried under a stream of nitrogen and dissolved in 125 µL of dichloromethane, thereby concentrated 400-fold, for GC analysis.

GC/MS analysis of phenolic compounds and flavonoids at 0 and 100% of the maple sap season was performed using the method previously described for the analysis of phenolic and flavonoid standards. Total ion chromatogram (TIC) and mass spectra of maple samples were recorded.

3.2.3. Identification of Phenolic Compounds and Flavonoids

Preliminary identification of phenolic compounds and flavonoids was based on comparing retention time data obtained with UV and EC detectors for standard compounds and sample analytes. Comparison of spectral characteristics (scans from 200 to 400 nm) of standards and sample components provided confirmation on the presence of phenolic compounds and flavonoids in maple sap and maple syrup. Additional

information was provided by the comparison of EC characteristics of standards and sample components.

In addition, retention time obtained with GC/FID for standard compounds and sample analytes was used to enhance the identification work. Furthermore, GC/MS on a full-scan mode, from 50 to 750 amu, allowed the establishment of TMS derivatives mass spectra for phenolic and flavonoid standards. Using the mass spectrum characteristics of the standards, such as the molecular ion (M), the base peak ion (BP) and the fragment ion (FI) was used to support the identification of phenolic compounds and flavonoids from maple products.

4. RESULTS AND DISCUSSION

4.1. Development of Chromatographic Analysis of Phenolic and Flavonoid Standards

Comparative HPLC and GC analyses of selected phenolic and flavonoid standards were developed, using a wide range of detectors, including UV diode-array (UV-DAD) and electrochemical (EC) detectors for HPLC and flame ionization detector (FID) and mass spectrometry (MS) for GC.

4.1.1. Optimization of HPLC Analysis

Figure 2 demonstrates the different chromatograms of HPLC analysis of phenolic and flavonoid standards, using UV-DAD detector at 280 and 320 nm, as well as EC detector at 200 and 600 mV. All selected phenolic and flavonoid standards absorbed at 280 nm, whereas 8 phenolic and 8 flavonoid compounds also absorbed at 320 nm. In addition, most standards responded at 600 mV except for quercitrin, protocatechuic and chlorogenic acids; however, kaempferol and quercetin were not determined by either voltage. White (1984) indicated that EC detection system affords an excellent selectivity because organic functional groups will electrolyze only at specific value of applied potential. In addition, certain aromatic hydroxyls require a higher applied voltage than phenols for a significant EC response.

The literature (Escarpa and Gonzalez, 1998; Markham and Bloor, 1998) indicated that the optimal detection of phenolic and flavonoid standards was at 280 and 320 nm. Previous work, undertaken in our laboratory, for the optimization and selection of the most appropriate potential values for setting the electrode of EC detector, indicated that both sensitivity and stable baseline were obtained for the analyses of phenolic compounds at 200 and 600 mV (Kermasha *et al.*, 1995a,b).

UV diode-array detector provides a scan spectrum of maximum absorbance wavelengths (λ_{max}) when scanning a specified region, as well as an absorbance ratio

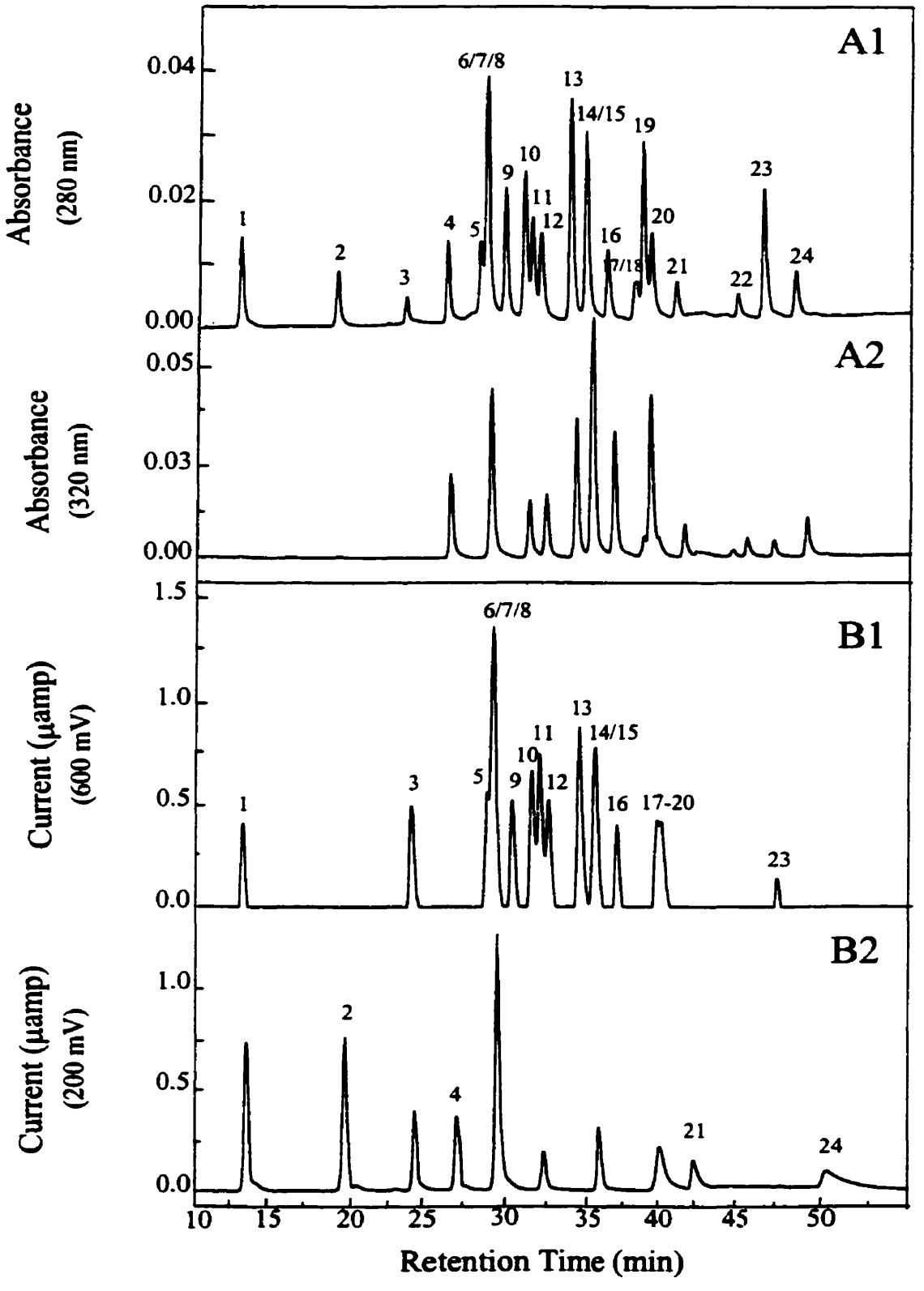


Figure 2. Chromatograms of HPLC analysis of phenolic and flavonoid standards using UV diode-array detector at 280 nm (A1) and 320 nm (A2) as well as electrochemical(EC) detector at 200 mV (B2) and 600 mV (B1) including, gallic acid (1), protocatechuic acid (2), (+)-catechin (3), chlorogenic acid (4), vanillic acid (5), caffeic acid (6), homovanillic acid (7), (-)-epicatechin (8) syringic acid (9), vanillin (10), coniferol (11), syringaldehyde (12), *p*-coumaric acid (13), sinapic acid (14), ferulic acid (15), coniferal (16), rutin (17), hesperidin (18), *trans*-resveratrol (19), phloridzin (20), quercitrin (21),quercetin (22), hesperetin (23) and kaempferol (24).

(280/320 nm). HPLC analysis of individual standards is depicted in Table 1. Comparison of retention time data, spectral and electrochemical characteristics of corresponding peaks from the standard mixture in HPLC to the standards characteristics provided by the different mode of detection enables accurate identification (Benavente-Garcia *et al.*, 2000).

4.1.1.1. Elution Profile of Phenolic and Flavonoid Standards

The results (Table 1) demonstrated that caffeic acid, homovanillic acid and (-)-epicatechin have similar retention time, 27.95, 27.98 and 28.13 min, respectively. This would explain their co-elution, represented by peaks 6, 7 and 8 (Fig. 2). The results also indicate that sinapic and ferulic acids also have similar retention time and therefore co-eluted just after *p*-coumaric acid. When injected alone, rutin and hesperidin eluted (Table 1) at 37.77 and 37.88 min, respectively, which resulted in a co-elution (Fig. 2) of peaks 17 and 18. Hydroxybenzoic and hydroxycinnamic acids as well as their derivatives were eluted before 35 min, whereas most of the flavonoids were eluted later, with the exception of (+)-catechin and (-)-epicatechin.

Preliminary trials, carried out for the optimization of HPLC analysis, indicated the retention time for standard phenolic acids were mostly pH dependent (Snyder *et al.*, 1997). Flavonoids are polyphenolic substances with three phenolic rings and interact more with the stationary phase, through hydrophobic interaction, as a function of their molecular structure and therefore they require a greater methanol concentration for their own elution. Hence, a gradient elution solvent system, consisting of 5 to 80% methanol and 95 to 20% of an aqueous solution containing 0.2% trifluoroacetic acid, was developed to provide a chromatogram of well separated and high resolution peaks (Fig. 2).

The results (Fig. 2) depict a general profile usually seen in reverse-phase (RP) system where most of the phenolic acids were eluted in the first 35 min of the run, whereas the flavonoids were eluted after 35 min mainly because of their higher hydrophobicity (Fig. 2). In RP system, the sample elution is retained for more hydrophobic compounds. Acid ionization increases its hydrophilic properties and as a

Table 1. HPLC analysis of phenolic and flavonoid standards, using UV diode-array and electrochemical (EC) detectors.

		Detector		
		UV diode-array		EC
Standard	Retention time (min)	λ_{max} (nm)[a]	Absorbance ratio (nm)[b]	Potential response (mV)
Gallic acid	12.56	214, 266	92.81	200, 600
Protocatechuic acid	18.74	218, 256, 294	10.41	200
(+)Catechin	23.30	224, 274	27.27	200, 600
Chlorogenic acid	25.52	222, 238, 288, 326	0.63	200
Vanillic acid	27.40	218, 256, 290	17.96	600
Caffeic acid	27.95	218, 244, 292, 326	0.60	200, 600
Homovanillic acid	27.98	228, 280	45.13	600
(-)Epicatechin	28.13	224, 274	23.97	200, 600
Syringic acid	29.15	218, 274	36.37	600
Vanillin	30.56	226, 274, 304	1.57	600
Coniferol	31.05	215, 262, 299	12.16	200, 600
Syringaldehyde	31.59	230, 305	0.23	600
p-Coumaric acid	33.23	228, 304	0.70	600
Sinapic acid	34.31	234, 324	0.30	200, 600
Ferulic acid	34.47	218, 236, 292, 322	0.54	200, 600
Coniferal	35.97	240, 294, 336	0.34	600
Rutin	37.77	256, 298, 356	0.61	600
Hesperidin	37.88	230, 284, 338	5.08	600
trans-Resveratrol	38.32	226, 300, 314	0.67	200, 600
Phloridzin	38.76	224, 280	3.05	600
Quercitrin	40.33	250, 346	0.70	200
Quercetin	44.23	248, 366	0.84	-[c]
Hesperetin	45.87	224, 282	4.64	600
Kaempferol	47.80	242, 360	0.75	-[c]

[a]Maximum absorbance wavelengths.

[b]Relative ratio of UV absorbance (280 / 320 nm).

[c]Not determined due to poor peak resolution.

result, its retention (k) in RP chromatography will be reduced 10 to 20-fold (Snyder *et al.*, 1997). The results (Fig. 2) suggest that as pH increases, the retention time for acid decreases. The gradient system, employed throughout this work, was initiated with a high concentration of solvent A (0.2% TFA) at pH 2, followed by a gradual increase in solvent B, methanol. The latter would suggest that more hydrophilic phenolic compounds and flavonoids will elute first, followed by those which are hydrophobic.

A similar order of the elution profile (Fig. 2) has also been demonstrated by Hayes *et al.* (1987). The two major differences in our study came from (-)-epicatechin, ferulic and sinapic acids. The results (Table 1) indicate a co-elution of (-)-epicatechin with caffeic and homovanillic acids. For ferulic and sinapic acids, the gradient system used, 5 to 80% of methanol in 40 min, did not allow the separation of the two compounds. The method used by Hayes *et al.* (1987) was slightly different, using a linear gradient of 0-50% (v/v) methanol in 30 min creating a baseline separation for ferulic and sinapic acids. In our study, ferulic and sinapic acid were not baseline separated nonetheless, a separation could be seen at the apex of the peak. Snyder *et al.* (1997) indicated that sample retention can be controlled by varying the solvent strength of the mobile phase. A strong solvent (80%) decreases the retention time whereas a weak solvent (40%) increases the retention time. A decrease in methanol concentration often results in a well-defined baseline for all peaks as well as improved resolution for most compounds under investigation, but results in longer run times.

The elution profile (Fig. 2) is similar to that reported by Kermasha *et al.* (1995b), where gallic acid, protocatechuic acid, catechin, chlorogenic acid, caffeic acid, epicatechin, *p*-coumaric acid, ferulic acid and phloridzin were eluted in the same order.

4.1.1.1.1. Hydroxybenzoic Acids and Their Derivatives

Table 1 shows that HBA were less retained by the stationary phase than HCA; Wulf and Nagel (1976) suggested that the structural difference between these two groups is the presence of an extra ethylenic chain in the HCA which provides more hydrophobicity and therefore results in a higher selectivity compared to that for HBA. It was suggested by Wulf and Nagel (1976) that gallic and protocatechuic acids, belonging

to the HBA family, were eluted before caffeic, chlorogenic, *p*-coumaric and ferulic acids, belonging to the HCA family. Furthermore, ferulic acid eluted after *p*-coumaric acid (Fig. 2); these findings may indicate that a methoxy-substituent is non-polar as it increases retention time whereas a decrease in retention time is produced by the addition of an hydroxyl group. Chlorogenic acid eluted before caffeic acid (Fig. 2) because chlorogenic acid is considered to be a more polar compound due to the presence of a quinic acid moiety in its structure (Macheix and Fleuriet, 1998).

4.1.1.1.2. Hydroxycinnamic Acids and Their Derivatives

Although, HBA have lower retention time than HCA, caffeic acid (HCA) was eluted before syringic acid (HBA) as well as coniferol (HCA) that also eluted before syringaldehyde (HBA) (Fig. 2). This could be explained by the fact that a linear increase in eluent strength during gradient elution can be achieved by increasing the concentration of methanol and consequently decreasing the separation between HBA and HCA. Hence, the retention time for cinnamic acids was lower than for benzoic acids, indicating that the separation factor between any cinnamic (HCA) and benzoic acids (HBA) decreases with increasingly methanolic solvents (Wulf and Nagel, 1976).

4.1.1.1.3. Flavonoid Aglycones

Table 1 indicates that flavonoids have higher retention time than that for HBA and HCA. A 4-keto group function substitution into the flavonoid nucleus would be the explanation, whereas (+)-catechin and (-)-epicatechin (lacking the 4-keto function) were eluted early with a low methanol conentration at 23.30 and 28.13 min, respectively; similar flavone or flavanone aglycone, having a 4-keto function in position 4, such as, hesperetin, quercetin and kaempferol, required at least a 30% methanol concentration for their elution. The 4-keto functional group increases the hydrophobicity of the flavonoid molecule by forming a planar non-polar six-member ring appearing less polar to the solvent. The non-polarity of the 4-keto compounds is indicated by their almost total insolubility in water (Wulf and Nagel, 1976).

Within the flavonol class, the elution order (Fig. 2) is the same as for the two classes of phenolic acids, HBA and HCA, being highly dependent upon hydroxylation and methylation. Quercetin (peak 22), with one more hydroxyl group was eluted before kaempferol (peak 24). Finally, the unsaturation between positions 2 and 3 of the pyran ring on the flavonoid molecule (Fig. 1), makes the compound much less polar due to a larger electron density on the oxygen atom of the 4-keto group resulting from resonance structures; explaining the higher retention time encountered for quercetin and kaempferol (Wulf and Nagel, 1976).

4.1.1.1.4. Flavonoid Glycosides

Figure 2 depicts a co-elution between rutin and hesperidin (peaks 17 and 18) just before resveratrol (peak 19), followed by phloridzin (peak 20) and quercitrin (peak 21). The presence of a disaccharide unit (rutin and hesperidin) creates a more hydrophilic compound, which were eluted faster than a flavonoid linked to a monosaccharide (phloridzin and quercitrin). These results are in agreement with those of Wulf and Nagel (1976) who reported that flavonoid glycosides are more polar than flavonoid aglycones and that glycosylation of quercetin with rhamnose creates quercitrin, which elutes faster than quercetin (quercitrin aglycone), due to an increase in polarity upon addition of rhamnose at position 3.

Rutin and hesperidin belong to two different classes of flavonoids, the first being a flavonol and the second a dihydroflavonol. It has been suggested by Wulf and Nagel (1976) that flavonols are more hydrophobic due to the presence of a double bond between position 2 and 3 when compared to dihydroflavonols. Hence, rutin would be expected to elute after hesperidin since they both have a disaccharide unit; the change in polarity due to the disaccharide, should be the same for both molecules. The experimental findings (Fig. 2) may suggest that it is the position to which the disaccharide unit is linked that plays a role in altering the polarity of the molecule and making rutin more hydrophillic. The attachment of a disaccharide at position 3 of the rutin molecule could alter and decrease the electron density on the oxygen atom of the 4-keto group and therefore, the lower electron density created will make the hydgrogen bond between the 5-hydroxyl

group and the 4-keto group weaker and make the functional group appear more polar to the solvent (Wulf and Nagel, 1976). This would explain the co-elution (Fig. 2) of rutin and hesperidin represented by peaks 17 and 18.

The overall elution sequence, depicted by Figure 2, of a standard mixture of phenolic compounds and flavonoids can best be interpreted by assuming that the compounds were first adsorbed on the hydrophobic stationary phase by "hydrophobic interaction", and that they were subsequently eluted with the mobile phase according to the extent of hydrogen bond formation. Hence, the hydrogen bond donating and/or accepting ability of a given substituent as well as its contribution to the hydrophobic interaction have to be considered. In a methoxyl group, for example, the oxygen is a hydrogen bond acceptor, whereas the methyl group contributes to the hydrophobic interaction. The strongest hydrogen bond acceptor in a flavone or isoflavone is the 4-keto group, which due to resonance, bears a partial negative charge. If an OH group is present at position 5, a strong internal hydrogen bond is formed between this group and the 4-keto group, and consequently the latter can no longer strongly interact with the solvent resulting in higher migration time. Hydrogen bonding between the 4-keto group and an OH group in position 3 is much weaker. This range applies to flavonoid aglycone because glycosides have the ability to form various hydrogen bonds due to the sugar moiety (Casteele *et al.*, 1982).

4.1.1.2. The Limit of Detection for Phenolic and Flavonoid Standards

4.1.1.2.1. Ultraviolet Diode-Array (UV-DAD) Detector

Table 2 shows the LOD of HPLC analysis of phenolic and flavonoid standards, using the UV-DAD. The limit of detection for all phenolic compounds and flavonoid standards was performed at 280 and 320 nm as well as 600 mV. The UV-DAD analysis at 320 nm provided an increase in the LOD for syringaldehyde, coniferal, rutin, hesperidin, *trans*-resveratrol, phloridzin, quercitrin and kaempferol when compared to the LOD performed at 280 nm; this statement is supported by the value obtained for the absorbance ratio provided in Table 1. When the absorbance ratio was less than 1, the compound showed higher absorbance higher at 320 nm than at 280 nm. Although

Table 2. Limit of detection of HPLC analysis of phenolic and flavonoid standards, using UV diode-array and electrochemical (EC) detectors.

Standard	Retention time (min)	Detection limit (ng/mL) [a]		
		UV diode-array		EC
		(280 nm)	(320 nm)	(600 mV)
Gallic acid	12.56	33.00	-[b]	2.20
Protocatechuic acid	18.74	50.00	-[b]	0.50
(+)Catechin	23.30	19.00	-[b]	0.40
Chlorogenic acid	25.52	46.00	140.00	0.90[c]
Vanillic acid	27.40	33.00	-[b]	0.10
Caffeic acid	27.95	42.00	67.00	0.10
Homovanillic acid	27.98	42.00	-[b]	0.10
(-)Epicatechin	28.13	15.00	-[b]	0.30
Syringic acid	29.15	24.00	-[b]	1.10
Vanillin	30.56	5.00	5.00	0.40
Coniferol	31.05	7.00	-[b]	1.00
Syringaldehyde	31.59	7.00	5.00	0.40
p-Coumaric acid	33.23	17.00	50.00	0.10
Sinapic acid	34.31	57.00	70.00	0.10
Ferulic acid	34.47	42.00	88.00	0.50
Coniferal	35.97	10.00	3.00	0.40
Rutin	37.77	22.00	17.00	5.70
Hesperidin	37.88	22.00	17.00	5.70
trans-Resveratrol	38.32	3.00	2.00	0.60
Phloridzin	38.76	7.00	2.00	0.70
Quercitrin	40.33	14.00	12.00	4.30[c]
Quercetin	44.23	11.00	12.00	-[b]
Hesperetin	45.87	4.00	15.00	0.90
Kaempferol	47.80	4.00	3.00	-[b]

[a]Detection limit is define as the minimum detectable concentration of a compound calculated on the basis of a signal-to-noise ratio of 3.

[b]Not determined due to poor peak resolution.

[c]Limit of detection calculated at 200 mV.

hesperidin and phloridzin were expected to have a higher sensitivity at 280 nm, the results (Table 2) indicate a higher sensitivity at 320 nm. The experimental findings for these two standards may be due to the co-elution of hesperidin with rutin. the latter absorbs highly at 320 nm with a ratio of 0.61. Phloridzin was not baseline separated from *trans*-resveratrol and once again *trans*-resveratrol demonstrated a greater absorption at 320 nm with a ration of 0.67.

All the other standards have a higher sensitivity at 280 nm. The range of values obtained for the LOD were between 3 to 57 ng/mL for the detection at 280 nm and 2 to 140 ng/mL for that at 320 nm. It was shown that any solute with UV absorption can be monitored, and for a highly absorbing species a detection limit of 1 ng is feasible (White, 1984). The advantage of the use of a continuously variable λ detector is that the selectivity can be enhanced by choosing the λ at which the analyte exhibits maximum absorption. It has been shown, however, that fixed λ detectors, even when operated at a λ that does not coincide with the absorption maximum of compound, will give greater sensitivity than a variable λ detector because they produce less background noise (Lawrence, 1981).

Figures 3 and 4 illustrate the different calibration curves of HPLC analysis of phenolic and flavonoid standards using UV-DAD at 280 and 320 nm. All standards depicted a good range of linearity (up to 25 μg/mL), with r-square values of 0.99 and precision less than 10 (data not shown). The minimum detectable concentrations of phenolic compounds and flavonoids were calculated on the basis of a 3:1 signal/noise ratio. The LOD was defined as the smallest concentration that can be detected reliably. It was related to both the signal and the noise of the system and defined as a peak whose signal-to-noise (S/N) ratio is at least 3:1 (Snyder *al.*, 1997).

When estimating the LOD it involves taking into account background noise, instrument sensitivity to the analyte and the signal to noise ratio (S/N). Many sources of errors can arise from determining the LOD. The value obtained is specific for the experimental conditions and different experimental conditions will result in a different estimate for the LOD. Short-term noise is of primary interest for S/N measurements. It

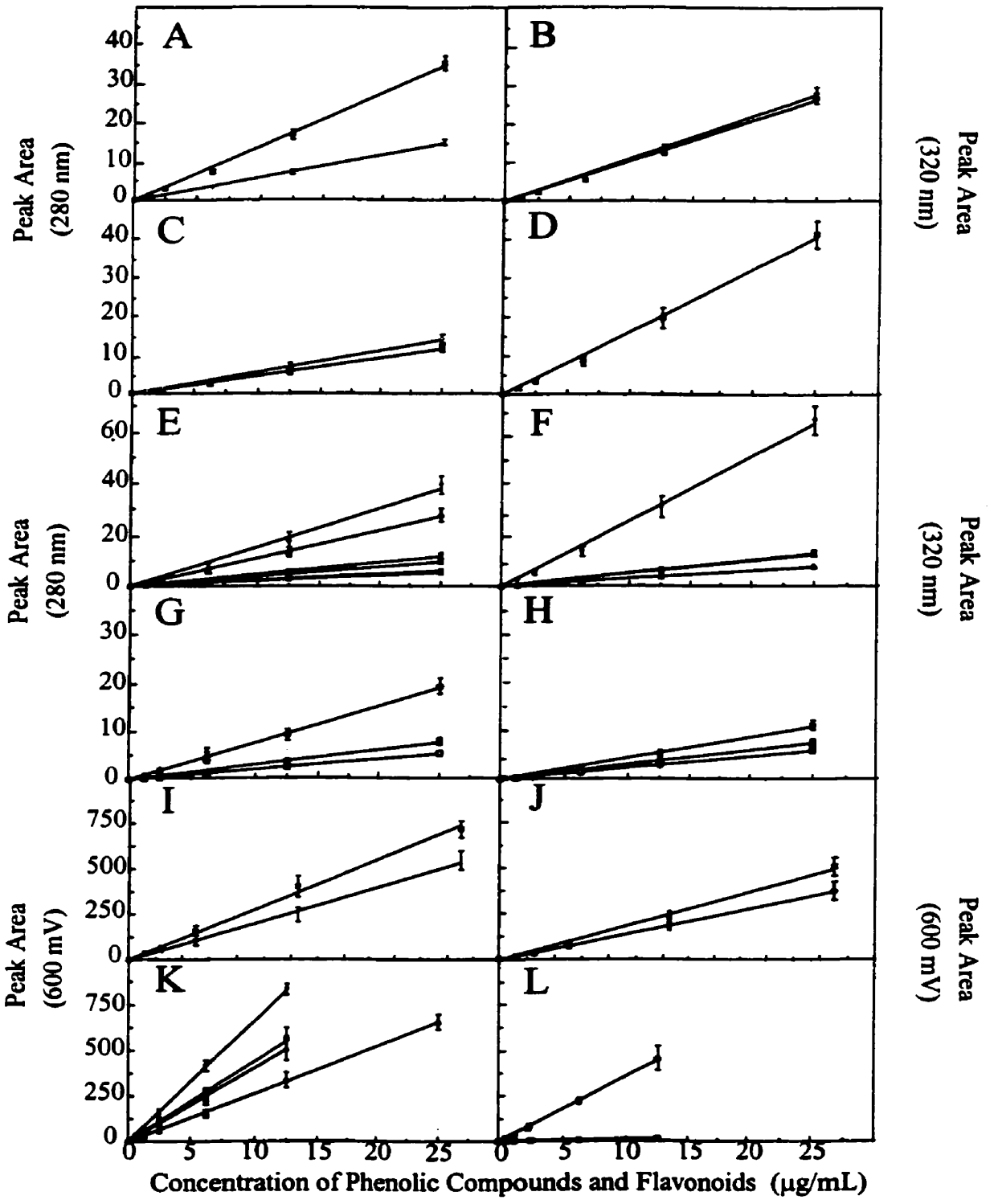


Figure 3. Calibration curves of HPLC analysis of phenolic and flavonoid standards,using UV diode-array detector at 280 nm (A) and 320 nm (B) for hydroxybenzoic acids: vanillin(X) and syringaldehyde (I); at 280 nm (C) and 320 nm (D) for hydroxycinnamic acids:coniferol (○), and coniferal (X); at 280 nm (E) and 320 nm (F) for flavonoid aglycones: (+)-catechin (■), (-)-epicatechin (▲), *trans*-resveratrol (▼), quercitin (●), hesperetin (△) and kaempferol (●); at 280 nm (G) and 320 nm (H) for flavonoid glycosides: rutin (□), phloridzin (○)quercitrin (✳) and hesperidin (□). Using electrochemical (EC) detector at 600 mV(I) for hydroxybenzoic acids; (J) for hydroxycinnamic acids; (K) for flavonoid aglycones and (L)for flavonoid glycosides.

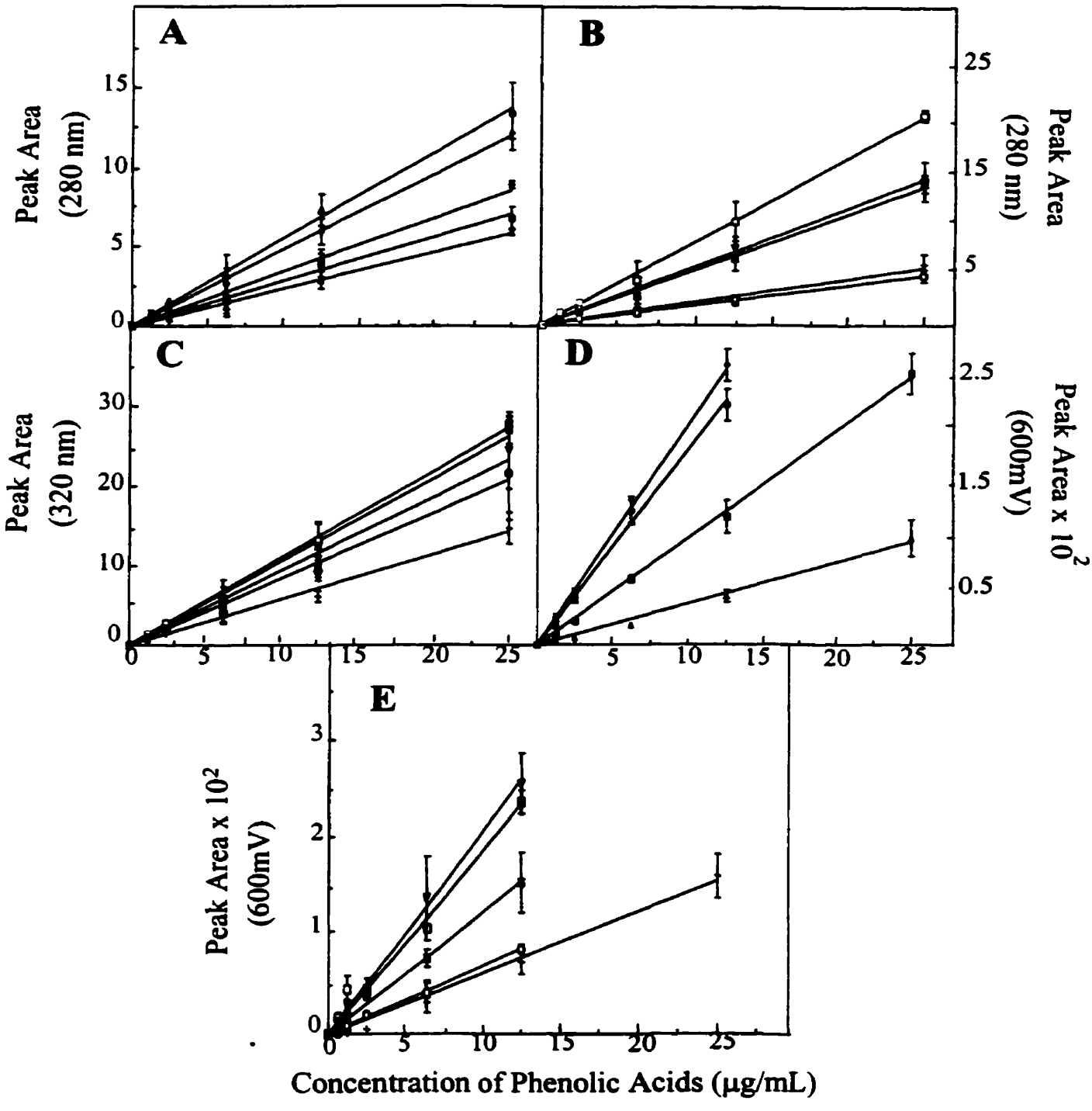


Figure 4. Calibration curves of HPLC analysis of phenolic acid standards using UV diode-array and electrochemical (EC) detectors . Detection at 280 nm for hydroxybenzoic acids (A); at 280 nm for hydroxycinnamic acids (B),at 320 nm for hydroxycinnamic acids (C) at 600 mV for hydroxybenzoic acids (D) and hydroxycinnamic acids (E) . Including, gallic acid (■), homovanillic acid (△), protocatechuic acid (▲), vanillic acid (●), syringic acid (◆), *p*-coumaric acid (□), caffeic acid (▽), ferulic acid (○), sinapic acid (✳) and chlorogenic acid (+).

can be due to a number of factors, including detector noise, pulsation of the pumping system and electronic noise in the integration system (White, 1984; Snyder *et al.*, 1997). The simplest way to measure the detector response is by determining the peak height of the signal. The method of peak measurement is the preferred approach and conditions for trace analysis of high sensitivity (Snyder *et al.*, 1997).

4.1.1.2.2. Electrochemical (EC) Detector

Table 2 depicts that the EC analysis provided an increase in the LOD as for all phenolic compounds and flavonoids, responding properly at 600 mV, compared to that obtained by UV analysis. The use of EC analysis provided a dramatic increase in the LOD for gallic, protocatechuic, chlorogenic, vanillic, caffeic/homovanillic, *p*-coumaric and sinapic acid by 15, 100, 50, 330, 420, 170 and 570 time, respectively. The results demonstrated that the EC detector was particularly sensitive for the detection of flavanols, HBA and HCA. The increase in LOD was similar that reported by Kermasha *et al.* (1995b), except for gallic acid.

EC is an extremely sensitive detector, with typical detection limits in the femptomole to subpicomole range, or about a 10 to 1000-fold improvement over UV/VIS detection sensitivity and at least 10-fold higher that of fluorescence detection (Hensley *et al.*, 1999). Snyder *et al.* (1997) have also reported that electrochemical detection is more sensitive (up to 100-fold) and compound-selective than UV detection.

Although most flavonoids showed an increase in the LOD using the EC detector, the increase was less pronounced than that obtained for HBA and HCA as well as for their derivatives. The detection of flavonoids with the EC detector was less successful than that with UV. The only two flavonoids that responded extremely well to the EC detection were (+)-catechin and (-)-epicatechin with a significant increase in the limit of detection of 50 times higher than the value obtained with UV analyses. The increase for (+)-catechin and (-)-epicatechin was less pronounced than the one observed by Kermasha *et al.* (1995b).

The poor response to electrochemical detection at both voltages for quercetin and kaempferol explains the absence of results for these two compounds. Furthermore, quercitrin responded only at 200 mV and therefore the limit of detection was calculated at that specific voltage instead of 600 mV (Table 2).

Figures 3 and 4 show the different calibration curves of HPLC analysis performed for phenolic and flavonoid standards using EC detector at 600 mV. All standards depicted a good range of linearity (up to 15 μg/mL) with r-square values of 0.99 and precision less than 10 (data not shown).

4.1.2. Optimization of GC Analysis

Individual phenolic compounds and flavonoids standards were subjected to GC analysis, using flame ionization detector. However, the glycosylated flavonoids were not subjected to GC analysis, due to their poor response. The flavonoid glycosides (rutin, hesperidin, phloridzin and quercitrin) have high molecular weights (436.40 to 610.55 g/mol) which after derivatization exceed 1000 Daltons. GC/FID instruments can analyze organic and inorganic material of molecular weights ranging from 2 to 1000 Daltons (McNair and Miller, 1998).

4.1.2.1. Elution Profile of Phenolic and Flavonoid Standards

Figure 5 shows a chromatogram of GC analysis of derivatized phenolic and flavonoid standards. The results show an excellent resolution between all compounds of interest. Vanillin (peak 1) displayed poor sensitivity, even at concentration as high as 25 μg/mL; vanillic and homovanillic acids were not completely baseline separated. The only co-elution observed was with coniferol and *p*-coumaric acid at 10.76 min. The elution profile of phenolic and flavonoid standards (Fig. 5) was somewhat different than the one previously seen in HPLC (Fig. 2); a better resolution and baseline separation of all peaks was seen with GC/FID when compared to analysis made by HPLC.

The elution order (Fig. 13) demonstrates shorter retention time for standards having lower molecular weight, such as vanillin (MW= 152.14 g/mol), and longer retention time for higher ones, such as chlorogenic acid (MW= 354.30 g/mol) and

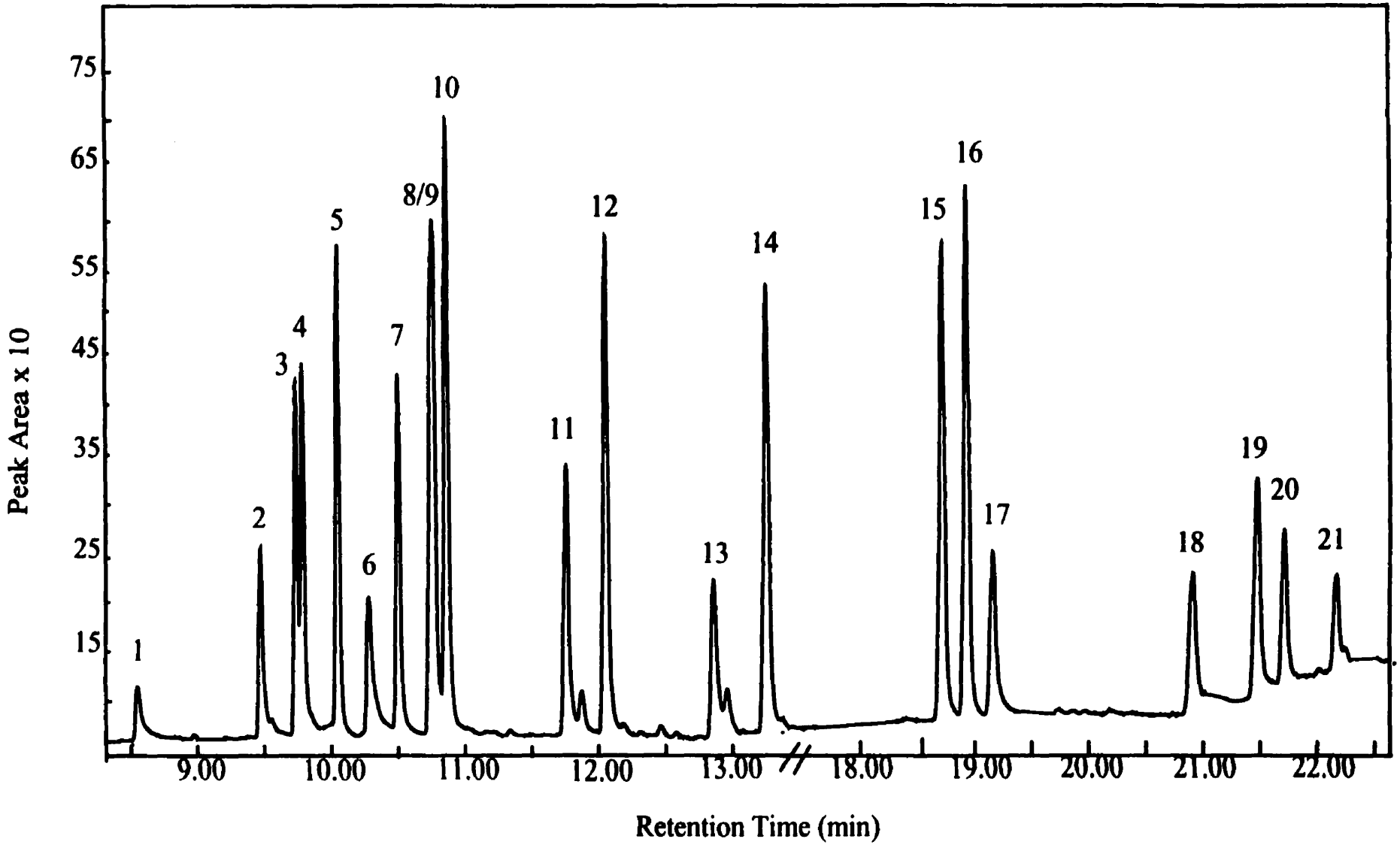


Figure 5. Chromatogram of GC analysis of phenolic and flavonoid standards including, vanillin (1), syringaldehyde (2), vanillic acid (3), homovanillic acid (4), protocatechuic acid (5), coniferal (6), syringic acid (7), coniferol (8), *p*-coumaric acid (9), gallic acid (10), ferulic acid (11), caffeic acid (12), sinapic acid (13), *trans*-resveratrol (14), (-)-epicatechin (15), (+)-catechin (16) hesperetin (17), kaempferol (18), fisetin (19), chlorogenic acid (20) and quercitin (21).

quercitin (MW= 302.23 g/mol). Although the molecular weight highly correlates with the elution order, the affinity towards the stationary phase is also important (McNair and Miller, 1998). This would explain why quercitin (peak 21) with a lower MW was eluted after chlorogenic acid (peak 20) (Fig. 5). The DB-5 column is relatively non-polar and retains quercitin more than chlorogenic acid, which is more polar.

Comparing the elution profile with the GC (Fig. 5) to that in HPLC (Fig. 2), the results indicate that chlorogenic acid behaved very differently; this behavior may be due to the fact that HPLC analysis depends mainly on the polarity of chlorogenic acid, which is high due to the quinic acid linked to the HCA molecule and makes hence the elution of this specific compound faster. In GC, the molecular weight of chlorogenic acid (even after derivatization) needed a higher temperature in order to be volatilized and therefore did not elute early as in HPLC, but on the contrary much later, towards the end of the chromatogram (Fig. 5).

As indicated above, the only co-elution observed in GC was with coniferol and *p*-coumaric acid. When injected separately, coniferol was eluted at 10.73 min whereas *p*-coumaric acid was at 10.76 min. The co-elution just before gallic acid was hence likely to have happen (Fig. 5). In HPLC, co-elution of selected standards was more widespread than in GC. The latter, is probably due to the fact that the polarity of a molecule is less specific than their individual vapor pressure.

4.1.2.2. The Limit of Detection for Phenolic Compounds and Flavonoid Standards

4.1.2.2.1. Flame Ionization Detection (FID)

The limit of detection of all selected phenolic and flavonoid standards are presented in Table 3. For all standards except vanillin and syringaldehyde, the LOD ranged from 0.02 to 0.71 µg/mL with gallic acid, (-)-epicatechin and (+)-catechin having the highest sensitivity and coniferal the lowest. Vanillin and syringaldehyde responded poorly to the FID with a LOD of 2.98 and 1.02 µg/mL, respectively. Previous work reported by Goldberg *et al.* (1994 and 1995) on resveratrol, found a LOD of 0.05 µg/mL

Table 3. Chromatographic parameters for GC analysis and limit of detection of phenolic compounds and flavonoid standards, using flame ionization detector (FID).

Standard	Retention time (min)	Relative (%)[a]	Detection limit (μg/mL)[b]	Range of linearity (μg/mL)[c]	R^{2d}	Precision[e]
Vanillin	8.55	1.18	2.98	3.00 - 83.33	0.96	10.60
Syringaldehyde	9.47	2.59	1.02	1.00 - 83.33	0.99	4.52
Vanillic acid	9.73	2.97	0.09	0.10 - 83.33	1.00	4.30
Homovanillic acid	9.78	3.21	0.10	0.10 - 83.33	1.00	3.71
Protocatechuic acid	10.04	6.15	0.05	0.05 - 83.33	1.00	3.77
Coniferal	10.28	3.07	0.71	0.70 - 83.33	0.99	3.82
Syringic acid	10.50	5.14	0.12	0.10 - 83.33	1.00	3.25
Coniferol + *p*-coumaric acid	10.76	10.46	0.20	0.20 - 83.33	0.99	3.48
Gallic acid	10.86	8.66	0.02	0.02 - 83.33	1.00	3.01
Ferulic acid	11.76	4.56	0.20	0.20 - 83.33	1.00	3.15
Caffeic acid	12.05	8.41	0.14	0.14 - 83.33	1.00	3.54
Sinapic acid	12.86	2.76	0.15	0.14 - 83.33	0.92	3.73
trans-Resveratrol	17.18	7.73	0.04	0.05 - 83.33	1.00	3.06
(-)Epicatechin	18.71	8.50	0.02	0.02 - 83.33	1.00	3.34
(+)Catechin	18.92	9.19	0.02	0.02 - 83.33	0.99	3.41
Hesperetin	19.16	3.54	0.05	0.05 - 83.33	1.00	3.63
Kaempferol	20.91	3.06	0.10	0.10 - 83.33	1.00	5.17
Chlorogenic acid	21.71	2.77	0.10	0.10 - 83.33	0.99	3.26
Quercetin	22.17	1.48	0.14	0.14 - 83.33	1.00	5.16

[a]Relative percentage peak area of each compound compared to the total peak area.

[b]Detection limit is defined as the minimum detectable concentration of a compound calculated on the basis of a signal-to-noise ratio of 3.

[c]Range of linearity is determined by the lower and higher limits of quantification from the calibration curve.

[d]Determination coefficient (R^2) of the calibration curve, calculated on the basis of triplicate injections of each product.

[e]Precision is the percentage deviation of the mean of the peak areas as obtained by three analyses of each standard; the standard contained 12.5 μg/mL of each compound after derivatization with bis(trimethylsilyl)trifluoroacetamide.

for *trans*-resveratrol and 0.01 μg/mL for *cis*-resveratrol; these findings are in agreement with our present results. *trans*-resveratrol has a LOD of 0.04 μg/mL (Table 3).

A study conducted by Soleas *et al.* (1997a) used MS instead of FID for the calculation of the LOD of phenolic compounds and flavonoids, using similar conditions to those used throughout the present study. An increase in the LOD for ferulic and caffeic acids was reported by Soleas *et al.* (1997a) when compared to that in our results (Table 3); however, similar LOD were reported for vanillic and *p*-coumaric acids. An increase in the LOD was seen with FID, when compared to that obtained by Soleas *et al.* (1997a) for gallic acid, *trans*-resveratrol, (-)-epicatechin, (+)-catechin and quercetin.

Relative retention volumes are more reproducible than individual retention volumes, so qualitative data should be reported on a relative basis (Blomberg, 1987). The relative percentage peak area of each standard compound is shown in Table 3. The highest relative percent is seen by the co-elution of coniferol and *p*-coumaric acid (10.46%), peaks 8/9 (Fig. 5) and the lowest by vanillin (1.18%) and quercitin (1.48%). The highest relative percentage observed with the co-elution of coniferol and *p*-coumaric acid is due to the presence of two compounds in one peak. Aside from the co-eluting of peak at 10.76 min, it is (+)-catechin and (-)-epicatechin that showed the greatest relative percentage peak area with 9.19 and 8.50%, respectively.

Figure 6 illustrates the calibration curves of phenolic and flavonoid standards of GC analysis using FID. The linearity of a method is a measure of how well a calibration curve response (peak area versus, concentration) approximates a straight line (Snyder *et al.*, 1997). From Table 3, a linear response was found for all compounds (up to 83.33 μg/mL) with high correlation coefficients, sometimes above 0.99. Precision less than 10 was observed for all standards, except for vanillin which had a precision of 10.60% that correlates with its poor resolution as indicated by the LOD.

4.1.2.3. Mass Spectrometry (MS) Analysis of Phenolic and Flavonoid Standards

The chromatographic parameters used for a qualitative GC/FID analysis are the retention volume or some closely related parameters. However, since the retention

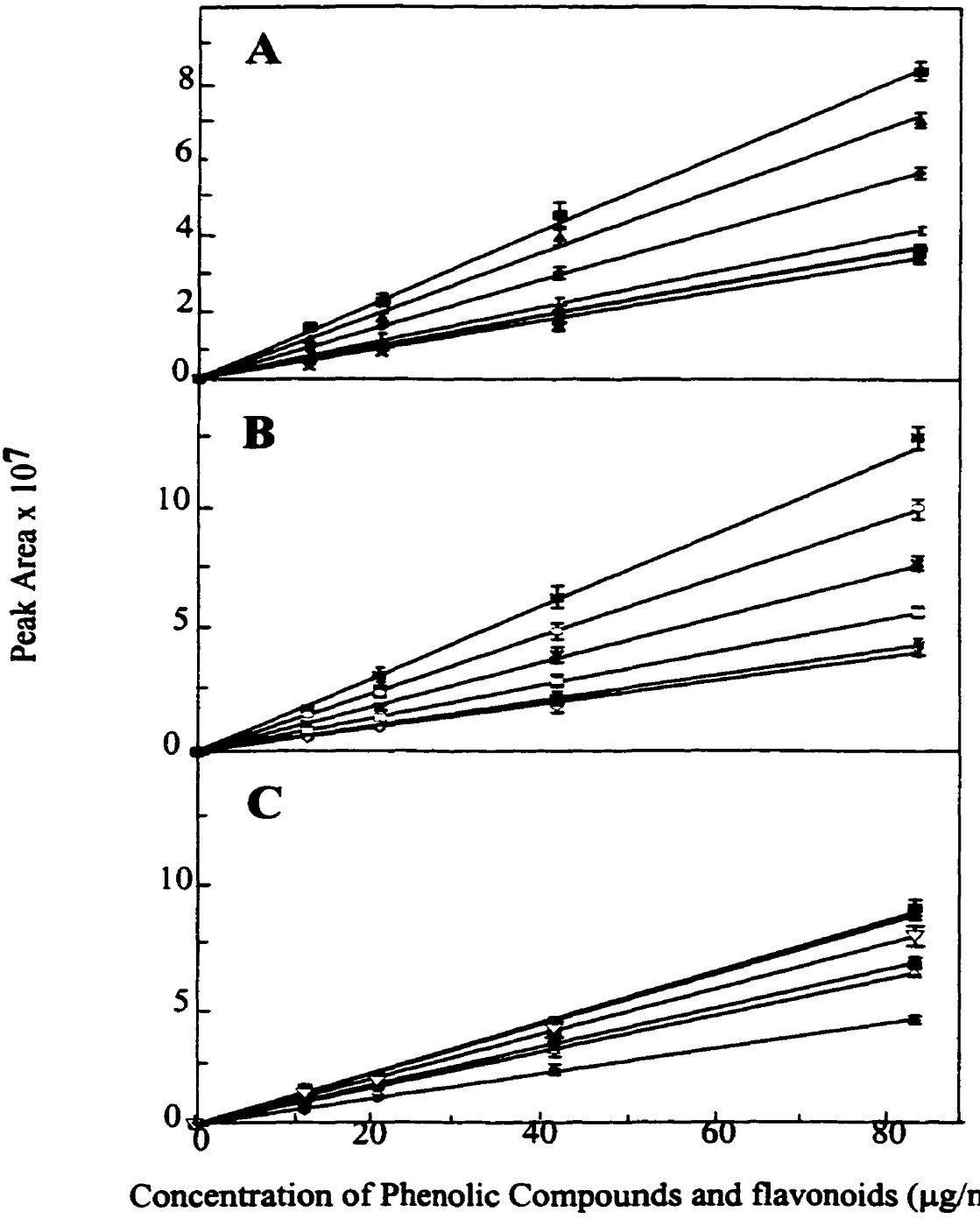


Figure 6. Calibration curves of GC analysis of phenolic and flavonois stanards including (A) Hydroxybenzoic acid and derivatives: gallic acid (■), protocatechuic acid (▲), vanillic acid (●), syringic acid (◆), syringaldehyde (⁞), homovanillic acid (△) and vanillin (x); (B) hydroxycinnamic acid and derivatives: *p*-coumaric acid (✱), ferulic acid (□), sinapic acid (▽), chlorogenic acid (x), caffeic acid (○), coniferol (✱) and coniferal (◇) and (C) flavonoids aglycone: *trans*-resveratrol (▽), kaempferol (●), hesperetin (△), quercitin (◆), (+)catechin (■) and (-)epicatechin (▲).

parameters cannot confirm the peak identity, it is common to couple a mass spectrometer (MS) to the GC (GC/MS) for qualitative analysis (McNair and Miller, 1998).

4.1.2.3.1. Conventional Electron Impact Mass Spectrometry (EI-MS)

Numerous ionization techniques are available for mass spectrometer analysis, however, for GC/MS almost all analyses are performed using either electron impact (EI) ionization or chemical ionization (CI) (Kitson *et al.*, 1996).

Table 4 shows the chromatographic parameters for GC/MS analysis of phenolic and flavonoid standards, using the mass selective detector (MSD). Electron impact ionization (EI) was used to identify phenolic compounds and flavonoid standards already analyzed by FID. Interpretation of mass spectra by choosing the molecular ion (M), base peak ion (BP) with relative abundance of 100% and a fragment ion (FI), the latter based upon abundance and the specificity for the compound, was undertaken for each standard. Some molecular ions (M) chosen were identical to the base peak ions, but it was not always the case.

Figure 7 depicts the GC/MS analysis of phenolic and flavonoid standards. The same elution profile was observed as that obtained with FID. As in GC/FID, the only co-elution encountered is for coniferol (peak 8) and *p*-coumaric acid (peak 9). The presence of additional peaks appearing in the TIC may be related to the impurities or background noise (Kitson *et al.*, 1996). The only major difference between GC/MS and GC/FID is the absence of quercitin (peak 21, Fig. 5) in GC/MS analysis. Quercitin eluted at the end of the run in GC/FID and needed a temperature of 300°C in order to be volatilized. In GC/FID the injection mode is on-column and the samples are directly introduce at the head of the column and will be volatilized when the oven reach their specific boiling point. In GC/MS, the injection mode is split/splitless and it was heated at 280°C. Therefore, quercitin could not be volatilized and was swept away when the valve opened after the injection was completed.

Once again the analysis of flavonoid glycosides was not undertaken due poor analysis. Wolfender *et al.* (1992) have shown that glycosides are thermally labile, polar

Table 4. Chromatographic parameters and mass spectrum characteristics for GC analysis of phenolic compounds and flavonoid standards, using GC/MS.

	FID	MS	Mass spectrum characteristics		
Standard	Retention time (min)	Retention time (min)	Molecular ion (M) $(m/z)^a$	Base peak ion (BP) $(m/z)^b$	Fragment ion (FI) $(m/z)^c$
Vanillin	8.55	7.74	224.00	194.00	209.00
Syringaldehyde	9.47	8.82	254.00	224.00	239.10
Vanillic acid	9.73	9.14	312.20	297.20	267.10
Homovanillic acid	9.78	9.20	326.20	326.20	311.00
Protocatechuic acid	10.04	9.47	370.20	193.10	355.20
Coniferal	10.28	9.66	250.00	220.10	235.00
Syringic acid	10.50	9.93	342.20	327.20	312.20
Coniferol	10.73	10.17	324.40	293.20	308.20
p-coumaric acid	10.76	10.19	308.20	293.00	293.10
Gallic acid	10.86	10.28	458.00	281.00	443.20
Ferulic acid	11.76	11.19	338.00	338.00	323.00
Caffeic acid	12.05	11.49	396.00	396.00	381.00
Sinapic acid	12.86	12.31	368.00	368.00	353.00
trans-Resveratrol	17.18	16.69	444.30	444.30	429.30
(-)Epicatechin	18.71	18.30	650.40	368.30	635.00
(+)Catechin	18.92	18.56	650.40	368.30	635.00
Hesperetin	19.16	18.82	446.30	209.20	431.00
Kaempferol	20.91	20.68	574.00	559.00	559.00
Fisetin	21.48	21.12	574.00	559.00	559.00
Chlorogenic acid	21.71	22.30	714.00	297.00	699.00
Quercetin	22.17	-[d]	-[d]	-[d]	-[d]

[a]Molecular ion, generated after an electron strikes the parent molecule and ejecting one electron, most representative of the derivatized molecular weight (MW).

[b]Base peak ion, representing 100% abundance.

[c]Fragment ion, chosen on the basis of their abundance and specificity for the compound.

[d]Not determined due to poor peak resolution.

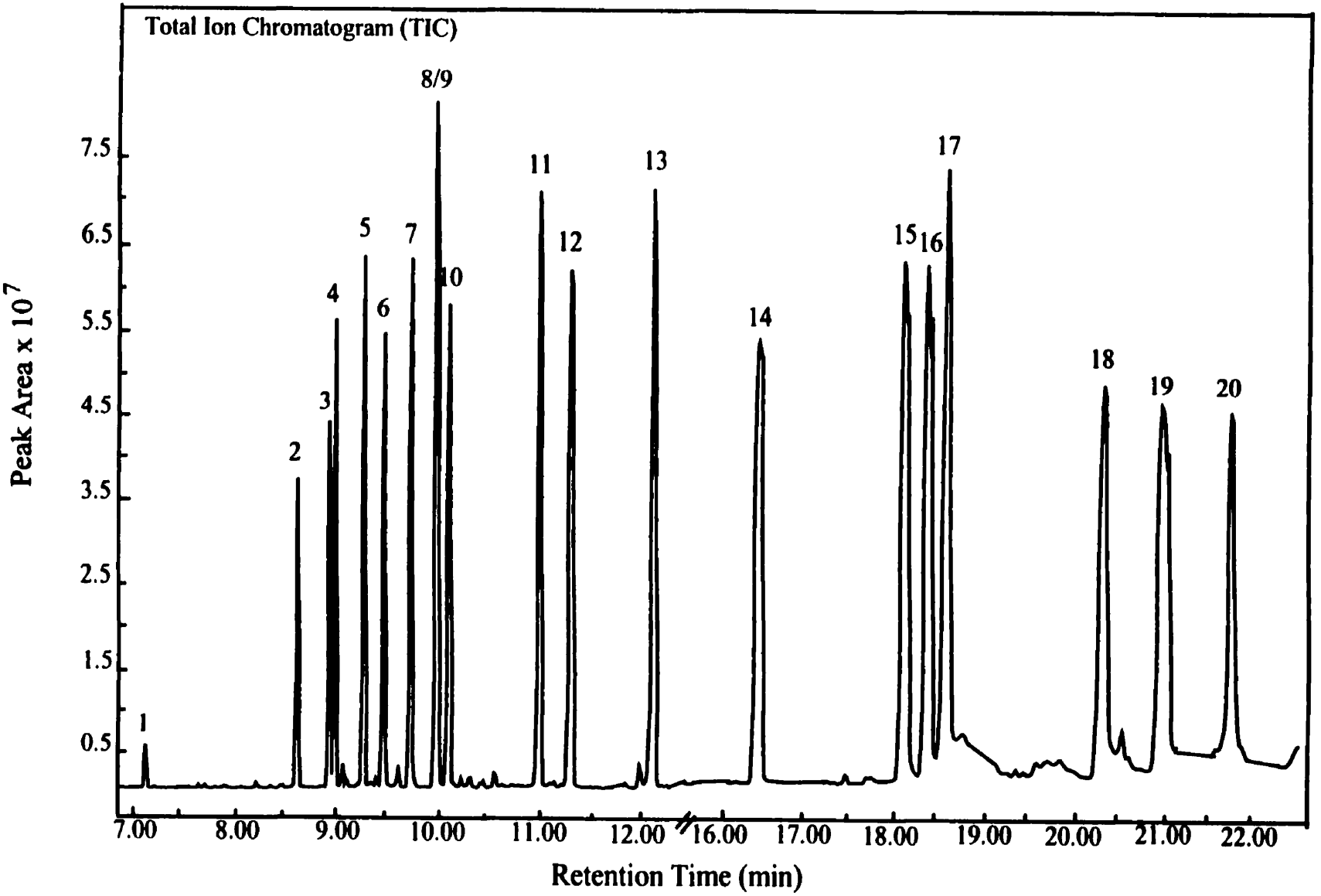


Figure 7. GC/MS analysis of phenolic compounds and flavonoid standards including, vanillin (1), syringaldehyde (2), vanillic acid (3), homovanillic acid (4), protocatechuic acid (5), coniferal (6), syringic acid (7), coniferol (8), *p*-coumaric acid (9), gallic acid (10), ferulic acid (11), caffeic acid (12), sinapic acid (13), *trans*-resveratrol (14), (-)-epicatechin (15), (+)-catechin (16), hesperetin (17), kaempferol (18), fisetin (19), chlorogenic acid (20).

and non-volatile compounds and require derivatization for their analysis by conventional electron impact mass spectrometry (EI-MS). Even after derivatization, the molecular ions of very large molecules were not present in the EI spectra. In addition, the derivatization methods often produce mixtures of partially derivatized compounds, which require subsequent purification. Finally, the development of "soft" ionization techniques, mainly in the early 1980s, has allowed the analysis of glycosides without derivatization (Wolfender *et al.*, 1992).

The results (Table 4) indicate a different retention time for phenolic and flavonoid standards, when detected by MS and compared to FID. The slight difference is due to the absence of a pre-column when GC/MS analysis was performed, resulting in shorter retention time. Molecular ion, base peak ion and fragment ion, are reported for each standard compounds; however, quercetin was not detected and therefore no data are present for this specific flavonol.

In order to facilitate the identification of the standard compounds, a calculation of the theoretical molecular weight after TMS derivatization was performed. It is possible to do so by adding 72 (the mass of C_3H_9Si minus the mass of an hydrogen ion) mass units to the original molecular weight, for each active hydrogen present (Kitson *et al.*, 1996). Using the retention time and matching the molecular ion (M) with the theoretical value, calculated for the derivatized MW, an accurate identification can be attempted.

An attempt to match retention time and theoretical molecular weight with the molecular ion (M) for all standards except quercetin, was performed.. The high temperature at the end of the run creates more noise in the baseline and hence it becomes harder to identify accurately the different peaks (Kitson *et al.*, 1996).

Watson and Pitt (1997) developed a method for the analysis and characterization of quercetin and kaempferol in urine. A similar HP instrument was used but they worked in the negative ion chemical ionization mode (NICI) with methane as the reagent gas introduced.

Soleas *et al.* (1997) developed a conventional GC/MS method, in which the phenolic compounds from wine was analyzed by derivatization with bis(trimethylsilyl)trifluoroacetamide (BSTFA); their preliminary trials, using *trans*-resveratrol standard .showed a molecular ion (M) of $m/z = 444$ with a relative abundance of 100% which is in agreement with our results (Table 4). Moreover, ferulic and caffeic acids standards also showed similar results than those of our study, $m/z = 338$ and 396, respectively.

4.2. Characterization of Phenolic Compounds and Flavonoids in Maple Sap and Maple Syrup

The analysis of phenolic compounds and flavonoids from maple sap and maple syrup at different periods of the season was performed with HPLC and GC systems.

4.2.1. Analyses of Phenolic Compounds and Flavonoids from Maple Sap

4.2.1.1. HPLC Analysis

Figures 8 and 9 illustrate the chromatograms of HPLC analysis of phenolic compounds and flavonoids from maple sap at 0 and 100% of the season, respectively. The HPLC analysis, using UV-DAD detector at 280 and 320 nm as well as EC detector at 200 and 600 mV, indicate the presence of 16 major peaks all at different periods of the maple sap season. At the start of the season (0%), peak 12 was absent; whereas peak 7 was not detected using UV-DAD at 280 nm (Table 5), but could be integrated with the EC detector at 600 mV (Table 6).

4.2.1.1.1. Tentative Identification of Phenolic Compounds and Flavonoids

Table 7 shows the tentative identification of phenolic compounds and flavonoids from maple sap, using HPLC with UV-DAD. A comparison of maximum absorbance λ of standard compounds with the identified peaks in maple sap products is depicted in Table 7. Spectral characteristics of peaks 1 and 2 suggest the presence of hydroxycinnamic acid (HCA) derivatives due to their absorbance in the 300-320 nm region. Peaks 1 and 2 could be esterified to quinic acid or simply present as glucosides, which would explain their lower retention time. Hence, peaks 1 and 2 were tentatively assigned as HCA derivatives.

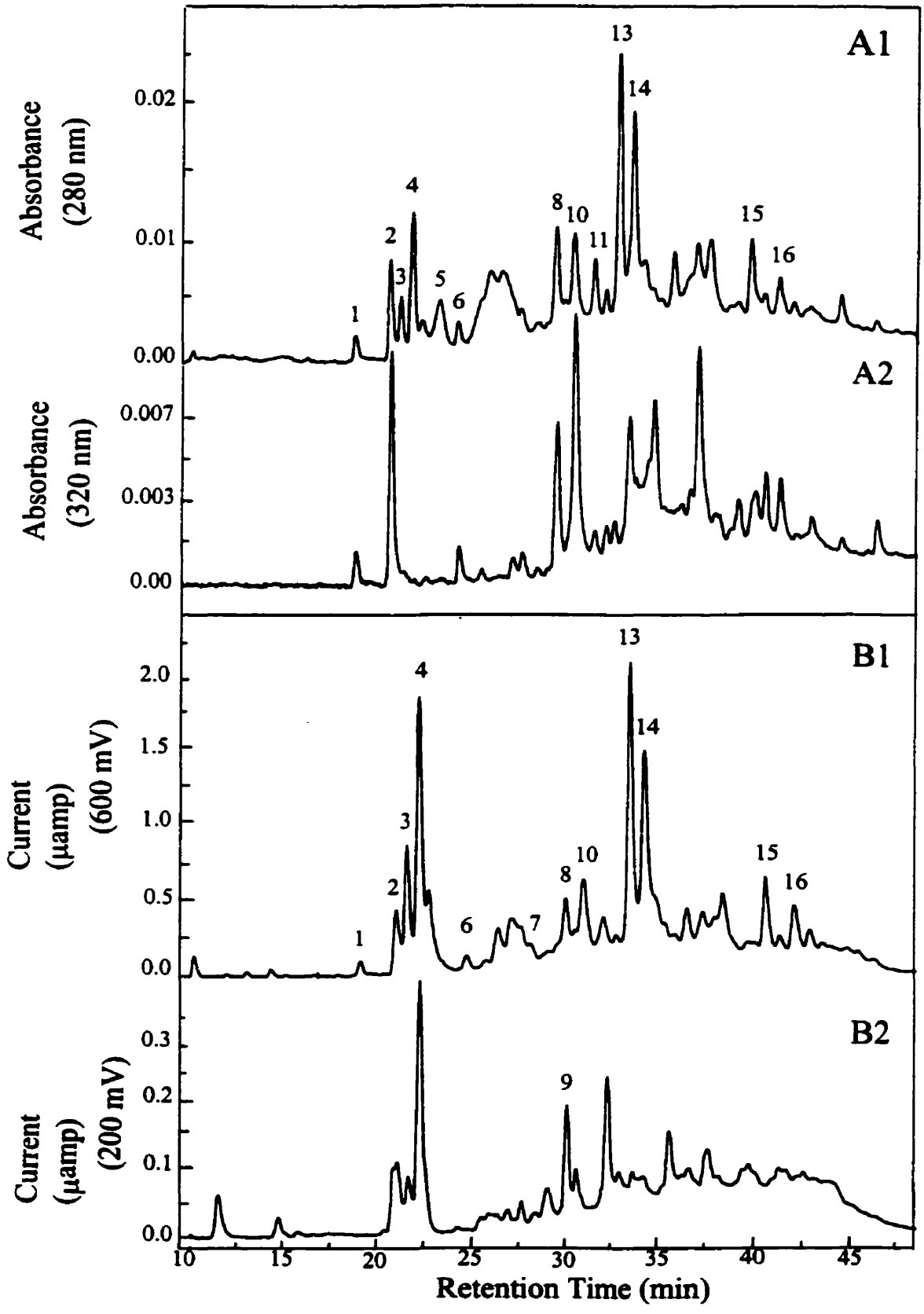


Figure 8. Chromatograms of HPLC analysis of phenolic compounds and flavonoids obtained from maple sap at 0% of the season, using UV diode-array detector at 280 nm (A1) and 320 nm (A2) as well as electrochemical (EC) detector at 200 mV (B2) and 600 mV (B1).

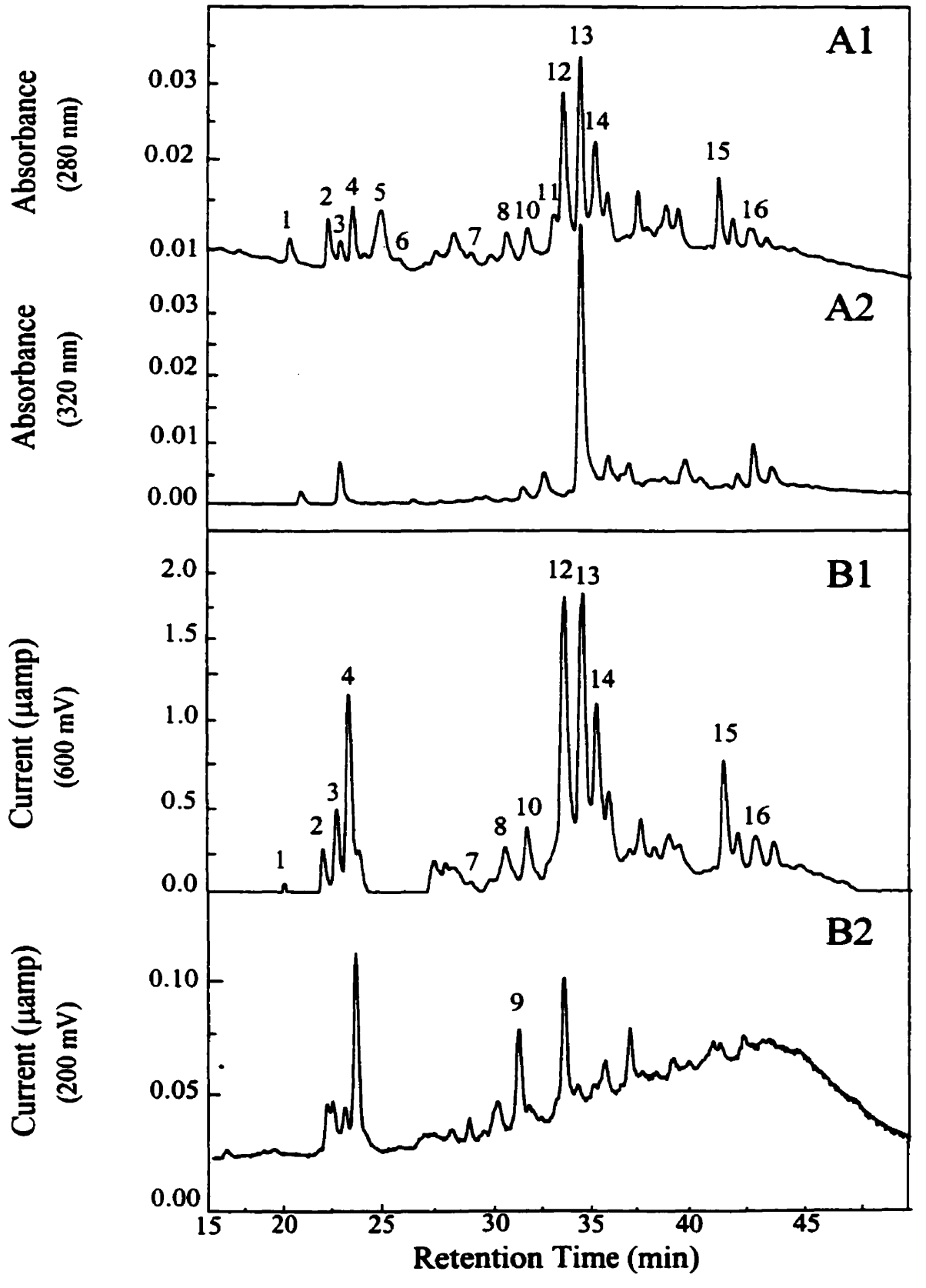


Figure 9. Chromatograms of HPLC analysis of phenolic compounds and flavonoids obtained from maple sap at 100% of the season, using UV diode-array detector at 280 nm (A1) and 320 nm (A2) as well as electrochemical (EC) detector at 200 mV (B2) and 600 mV (B1).

Table 5. HPLC analysis of phenolic compounds and flavonoids obtained from maple sap at different periods of the season, using UV diode-array detector.

Peak No[a]	Retention time (min)[b]	Standard deviation[c]	Precision[d]	λmax (nm)[e]	Absorbance ratio (nm)[f]	Relative percent of analytes at different periods of the maple sap season (%)[g] Season (%) 0	25	50	75	100
1	18.77	0.39	2.07	230, 274, 312	2.84	1.36	1.39	1.49	1.26	1.71
2	20.69	0.37	1.81	214, 300	1.07	4.86	4.55	5.38	2.41	3.10
3	21.37	0.32	1.49	226, 274	23.43	2.60	2.24	2.87	3.24	1.75
4	21.97	0.33	1.49	224, 276	88.74	7.15	5.67	8.03	7.27	4.09
5	23.37	0.36	1.53	228, 284	48.94	5.56	1.70	5.35	3.94	9.74
6	24.20	0.43	1.77	226, 286	2.42	1.28	0.80	1.26	0.32	0.18
7	28.02	0.70	2.49	226, 274	3.07	-[h]	1.26	0.52	0.72	0.99
8	29.43	0.44	1.50	226, 272, 308	2.20	4.96	5.32	4.76	3.34	2.59
10	30.51	0.38	1.24	222, 276, 304	1.06	5.60	5.94	5.09	4.54	2.49
11	31.89	0.40	1.25	230, 270	3.91	4.39	5.16	-[h]	4.25	2.22
12	32.32	0.64	1.97	228, 302	1.05	-[h]	-[h]	4.00	6.63	13.53
13	33.18	0.31	0.92	222, 274	27.86	15.41	14.30	14.75	15.12	12.46
14	33.95	0.32	0.94	222, 274	5.06	12.01	11.43	13.45	14.88	9.23
15	40.27	0.27	0.67	224, 278	5.02	3.70	3.36	4.33	4.58	4.83
16	41.81	0.25	0.60	222, 274, 336	1.92	2.38	1.79	2.38	2.63	2.20

[a] Peak number are referring to figures 8, 9, 14 and 15.

[b] Average retention time of the different periods of the maple sap season for each peak identified.

[c] Standard deviation from the average retention time.

[d] Precision is the percentage deviation of the mean of retention time as obtained by the different periods of the maple sap season.

[e] Maximum absorbance wavelengths.

[f] Relative ratio of UV absorbance (280/ 320 nm).

[g] Area percent of each peak compared to the total peak area of each different periods of the maple sap season.

[h] Not determined due to poor peak resolution

Table 6. HPLC analysis of phenolic compounds and flavonoids obtained from maple sap at different periods of the season, using electrochemical (EC) detector at 600 mV.

Peak No[a]	Retention time (min)[b]	Standard deviation[c]	Precision[d]	Relative percent of analytes at different periods of the maple sap season (%)[e] Season (%) 0	25	50	75	100
1	19.57	0.22	1.15	0.65	0.68	[f]	0.28	0.15
2	21.52	0.22	1.00	3.03	2.84	2.48	1.26	1.53
3	22.12	0.19	0.84	5.90	5.03	5.25	6.15	3.34
4	22.69	0.20	0.90	13.13	11.91	23.46	22.38	9.52
6	25.14	0.31	1.23	0.55	0.32	0.51	0.12	[f]
7	28.59	0.19	0.66	0.20	1.12	[f]	0.25	0.12
8	30.39	0.29	0.96	3.02	3.83	3.05	3.19	3.22
9[g]	30.69	0.45	1.45	6.40	6.22	12.02	5.16	10.07
10	31.39	0.26	0.84	5.44	5.19	4.75	4.39	3.63
12	33.07	0.30	0.91	[f]	[f]	4.52	6.22	18.73
13	33.99	0.28	0.83	14.72	13.16	16.28	12.33	16.04
14	34.74	0.26	0.75	13.67	12.20	14.91	12.11	10.23
15	41.04	0.24	0.59	3.36	4.26	4.46	4.27	5.02
16	42.54	0.29	0.67	2.19	2.33	2.54	2.48	2.00

[a] Peak number are referring to figures 8, 9, 14 and 15.

[b] Average retention time of the different periods of maple sap season for each peak identified.

[c] Standard deviation from the average retention time.

[d] Precision is the percentage deviation of the mean of retention time as obtained by the different periods of the maple sap season.

[e] Area percent of each peak compared to the total peak area of each different periods of the maple sap season.

[f] Not determined due to poor peak resolution.

[g] Detection at 200 mV.

Table 7. Tentative identification of phenolic compounds and flavonoids obtained from maple sap and maple syrup, using HPLC with UV diode-array as well as electrochemical (EC) detectors.

		UV detector	Standard	Maple products	
Peak No[a]	Compound	Retention time (min)[b]	λ_{max} (nm)[c]	λ_{max} (nm)[c]	Absorbance ratio (nm)[d]
Ia	Protocatechuic acid[e]	18.60	218, 256, 294	214, 256, 290	11.25
1	HCA derivative	19.10	300-320	230, 274, 312	2.84
2	HCA derivative	21.02	300-320	214, 300	1.07
3	Flavanol	21.68	280	226, 274	23.43
4	(+)-Catechin	22.27	280	224, 276	88.74
5	Flavanol[f]	23.37	280	228, 284	48.94
6	Flavanol	24.64	280	226, 286	2.42
7	(-)-Epicatechin	28.28	280	226, 274	3.07
8	Vanillin	29.80	226, 274, 304	226, 272, 308	2.20
9	Coniferol[g]	30.67	215, 262, 299	218, 260, 300	8.30
10	Syringaldehyde	30.85	230, 305	222, 276, 304	1.06
11	Vanillic acid derivative	32.12	218, 256, 290	230, 270	3.67
12	*p*-Coumaric acid	32.68	228, 304	228, 302	1.05
13	Flavanol	33.47	280	222, 274	27.86
14	Flavanol	34.25	280	222, 274	5.06
15	Flavanol	40.53	280	224, 278	5.02
16	Dihydroflavonol	42.07	320-355	222, 274, 336	1.92
17	Dihydroflavonol	45.83[g]	320-355	228, 288, 326	0.62

[a] Peak number referring to figures 8, 9, 14 and 15.

[b] Average retention time of maple sap and maple syrup of different periods of the season.

[c] Maximum absorbance wavelengths.

[d] Relative ratio of UV absorbance (280/ 320 nm).

[e] Only present in maple syrup starting at 50%.

[f] Average retention time in maple sap.

[g] Average retention time in maple syrup.

Wulf and Nagel (1976) reported the presence of HCA derivatives with sugars and hydroxy acids in numerous fruits, such as apples, tomatoes and cherries. In most cases, glycosylated derivatives were distinctively less abundant than quinic esters (Macheix and Fleuriet, 1998). The esterification of HCA with either quinic acid or a sugar moiety would explain their lower retention time compared to other HCA which is due to a change in the polarity of the molecule upon esterification with a more hydrophilic moiety (Wulf and Nagel, 1976).

Peak 4, eluted at 22.27 min with maxima absorbance at 224 and 276 nm (Table 7), was tentatively identified as (+)-catechin; peak 7, eluted at 28.28 min, depicted similar characteristics to (-)-epicatechin. Individual standard properties (Table 1) have shown that (+)-catechin and (-)-epicatechin absorbed mainly at 280 nm and responded at both electrochemical voltages; the experimental findings (Fig. 8 and Fig. 9) indicate that peaks 4 and 7 have also these characteristics. Figure 10 shows a scan spectrum comparison between (+)-catechin standard and peaks 4 and 7 which are almost identical. These results suggest the presence of (+)-catechin and (-)-epicatechin, since their respective standards were eluted at 23.30 and 28.13 min, respectively, which are in close proximity to the retention times of peaks 4 and 7.

Peaks 3, 5 and 6, eluted between 20 and 25 min, were designated as flavanols because of their maxima absorbance in the region of 224 - 280 nm (Table 7). Dawes and Keene (1999) suggested that the major flavanols and procyanidins (maximum absorbance at 280 nm) was eluted between 17 and 37 min, analyzed under similar conditions as those described in our study. Furthermore, these authors identified two eluting peaks between (+)-catechin and (-)-epicatechin as procyanidins. Other studies conducted by Jaworski and Lee (1987) and Oszmianski *et al.* (1988) using an increasing gradient of acetonitrile in water on RP-HPLC demonstrated the presence of procyanidins in wine sample were the eluting order was procyanidin B1 < B3 < catechin < B4 < B2 and epicatechin. The experimental results (Table 7) suggest the presence of procyanidins in maple sap; peak 3 could be either procyanidin B1 or B3, whereas peaks 5 and 6, are procyanidin B4 and B2, respectively. However, further investigation would be necessary to confirm their

identification by selecting a range of procyanidin standards and injecting them in the HPLC system under the same conditions. In summary, the presence of flavanol related compounds in maple sap could not be disregarded.

The major flavanols present in fruits are epicatechin, catechin, gallocatechin, and epigallocatechin. Flavanols participate in the structure of proanthocyanidins (condensed tannins) as their monomers. In grapes and wine procyanidins are the major proanthocyanidins encountered (Lea *et al.*, 1979; Macheix *et al.*, 199; Escribano-Bailon *et al.*, 1992).

Peak 8, eluted at a similar retention time (29.80 min) to that of vanillin standard, absorbed at both wavelengths (280 and 320 nm) and appeared at the higher voltage (600 mV). Preliminary work done with standard compounds (Table 1) showed that vanillin eluted at 30.56 min, absorbed at 280 and 320 nm and responded at 600 mV. In addition, scan spectrum comparison of the vanillin standard with peak 8 (Fig. 10) resulted in a positive identification.

Peak 9, eluted at 30.67 min was tentatively identified as coniferol; however, its appearance at 280 nm was scarce (Fig. 8 and Fig. 9) and was only detected at 200 mV. Peak 10 was eluted at 30.85 min with absorbance maxima at 222, 276 and 304 nm, and an EC response at 600 mV; it was identified as syringaldehyde. To support the identification, Figure 11 shows a scan spectrum of peak 10 that correspond to syringaldehyde standard.

Although the retention time was quite different, peak 11 exhibited a scan spectrum almost identical to that of vanillic acid standard and was assigned as a vanillic acid derivative. Schuster and Herrman (1985) reported that any molecule linked to the aromatic ring could greatly affect the retention time. Benzoic acids are frequently present in bound form and constitute either complex structures like hydrolyzable tannins or simple molecules by combining with sugars or organic acids (Schuster and Herrman, 1985). Moreover, the UV absorbance spectrum of an aglycone (free molecule) esterified with a carboxylic acid or sugar moiety remains substantially unchanged; however, the

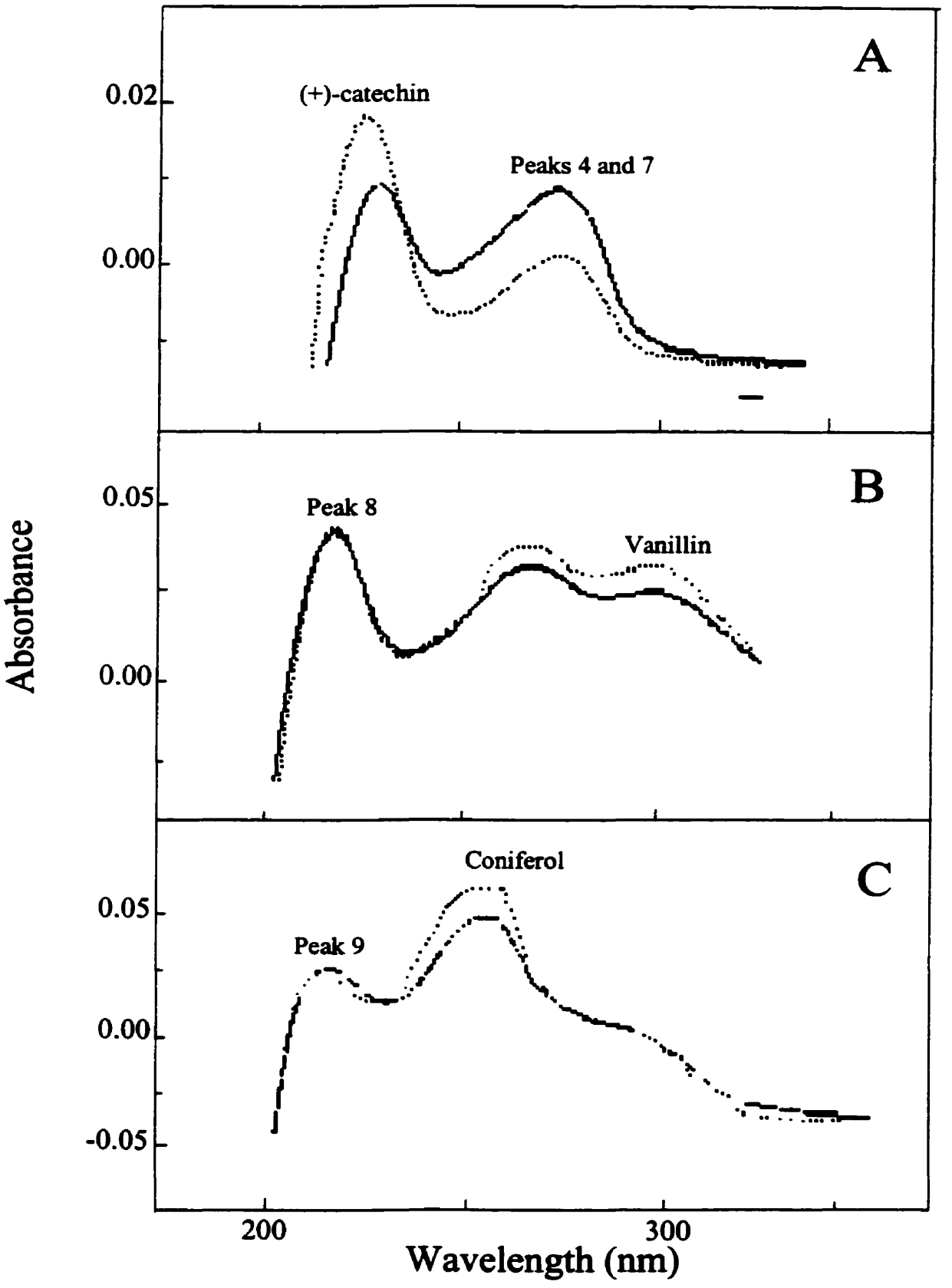


Figure 10. Scan spectrum comparison of phenolic and flavonoid standards and identified peaks from Figures 8, 9, 14 and 15 obtained from maple sap and maple syrup at different periods of the season. (A) (+)-catechin and peaks 4 and 7, (B) vanillin and peak (8) and (C) coniferol and peak (9).

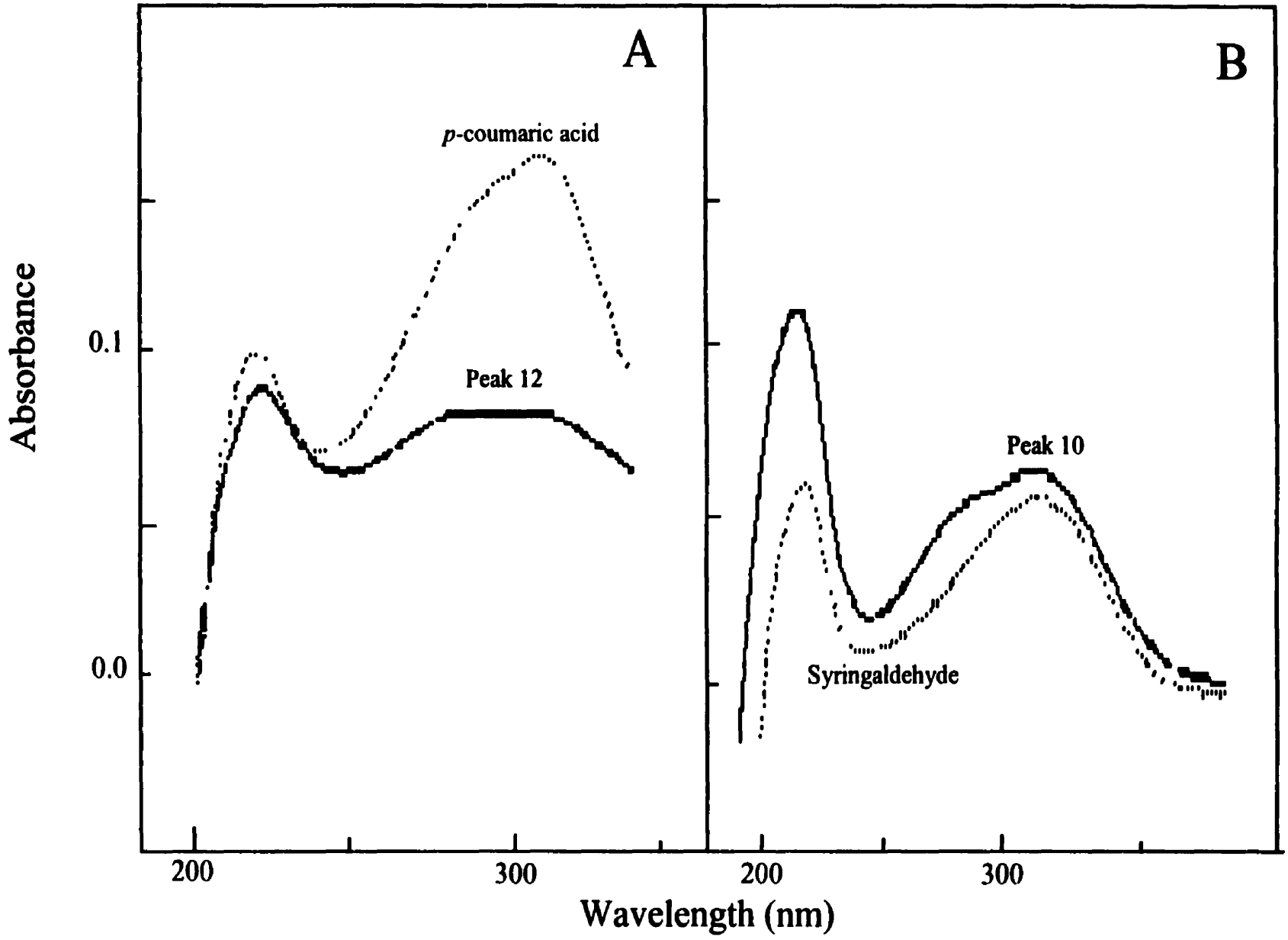


Figure 11. Scan spectrum comparison of standard phenolic compounds and identified peaks from figure 8, 9, 14 and 15 obtained from maple sap and maple syrup at different periods of the season. (A) *p*-coumaric acid and peak 12, (B) syringaldehyde and peak 10.

alteration of the retention times may be due to changes in the polarity of the molecule (Nagels *et al.*, 1979; Moller and Herrmann, 1982; Spanos *et al.*, 1990). On the basis of these assumptions, it was more crucial to rely on the retention time and further ensure the identification of analyses with reference to the absorbance spectrum since many different compounds issued from the same family have similar scan spectra.

From the absorbance spectrum (Fig. 11), peak 12 was identified as *p*-coumaric acid with absorbance maxima at 228 and 302 nm, to support the identification similar electrochemical response and retention time as for *p*-coumaric acid standard were found.

Hydroxybenzoic acids (HBA) in fruits commonly occur as free as well as derivatives glycosides and esters derivatives (Macheix *et al.*, 1990; Fernandez de Simon *et al.*, 1992). HBA can occur in the free form after hydrolysis (acid/base/enzymatic); however, they are frequently present as derivatives such as glycosidic derivatives. Hydroxycinnamic acids (HCA) occur as free acids only after exceptional conditions such as brutal extraction, contamination by microorganisms and technological processing. In addition, HCA are present very often as glucose esters, glucosides and can be linked to flavonoids or lignins (Macheix *et al.*, 1990).

Peaks 13, 14 and 15 showed an absorption maximum at 280 nm and displayed spectra identical to (+)-catechin standard (Fig. 12); these peaks were assigned as flavanol related compounds. Peak 16, eluted at 42.07 min displayed a longer λ at 336 nm with a lower intensity shoulder and was characterized as dihydroflavonol; however, other chromatographic characteristics, such as retention time and EC parameters, did not match those of the selected standards. Although, peak 16 cannot be accurately identified, the scan spectrum, can related it to the class of flavonoids, having the same profile. Markham and Bloor (1998) showed that the most informative λ band for absorption spectroscopy of flavonoids is the UV-VIS (210-600 nm). Within this range most flavonoids exhibit absorption peaks in two regions, the short λ region at 210-290 nm (band II) and the longer λ at 320-380 nm or 490-540 nm for anthocyanins (band I). The exact λ of band I can give good indication of the class of flavonoids under study. The λ band I for

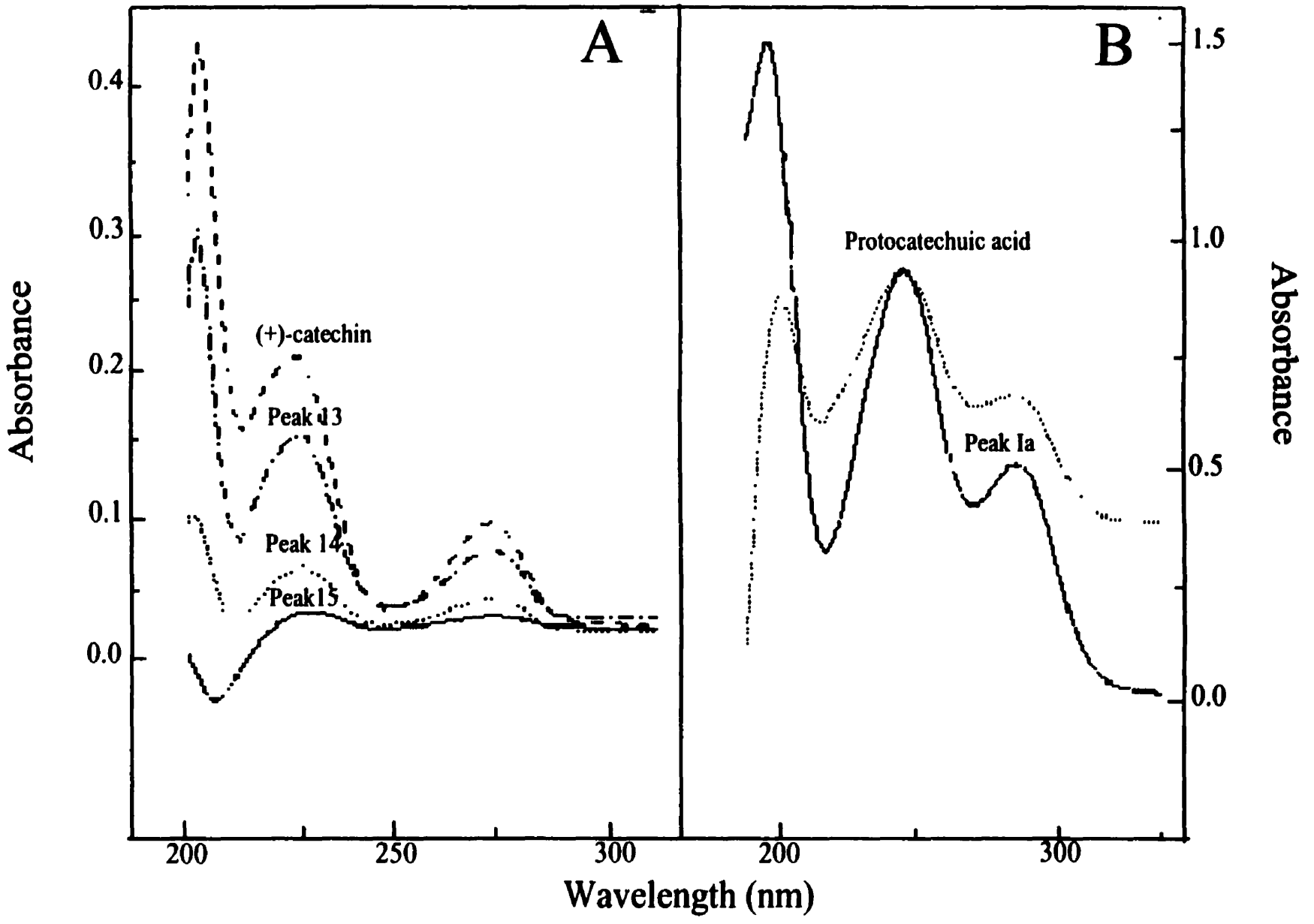


Figure 12. Scan spectrum comparison of phenolic and flavonoid standards and identified peaks from figure 8, 9, 14 and 15 from maple sap and maple syrup at different periods of the season. (A) (+)-catechin and flavanol related compounds, peak 13,1 4 and 15. (B) protocatechuic acid and peak Ia identified in maple syrup only starting at 50% of the season.

dihydroflavonols was shown to be in the range 310-330nm with a low intensity shoulder (Markham and Bloor, 1998); these characteristics correspond to that of peak 16.

4.2.1.2. GC Analysis

Figure 13 shows typical chromatograms of GC/FID analysis of phenolic compounds and flavonoids, obtained from maple sap at 0 and 100% of the season. The experimental findings for all periods of the season (0, 25, 50, 75 and 100%) are presented in Table 8. The presence of 22 selected peaks, chosen for their similar retention time to that of standards compounds (Table 3) showed a consistency throughout the maple sap season. At the beginning of the season (0%), peaks 5, 14 and 19 were not integrated due to poor peak resolution whereas peak 17 showed the greatest relative percent (Table 8).

4.2.1.2.1. Tentative Identification of Phenolic Compounds and Flavonoids

When comparing retention time of the different peaks obtained from maple sap (Table 8) to that of standards (Table 3), a tentative identification of phenolic compounds and flavonoids could be made. Similar retention times were found for vanillin (peak 2, 8.53 min), syringaldehyde (peak 4, 9.47 min), vanillic acid (peak 5, 9.81 min), homovanillic acid (peak 6, 9.84 min), protocatechuic acid (peak 7, 10.07 min), coniferal (peak 8, 10.21 min), syringic acid (peak 10, 10.54 min), coniferol/*p*-coumaric acid (peak 11, 10.74 min), ferulic acid (peak 13, 11.87 min), caffeic acid (peak 14, 12.07 min), sinapic acid (peak 16, 12.93 min), (-)-epicatechin (peak 18, 18.71 min) and (+)-catechin (peak 19, 19.02 min).

The literature, Soleas *et al.* (1997a), indicates that one major problem underlying the separation of phenolic compounds is their similarity in chemical characteristics. Many phenolic compounds show similar UV spectra with maxima absorbance in a narrow range of 280 and 320 nm. Gas chromatography, with or without mass spectrometric detection has been employed for the analysis of phenolic compounds in wines using the retention time data as a mean for their identification (Soleas *et al.*, 1997a).

Comparison of the GC/FID analysis to that of HPLC showed that the GC instrument is more precis in term of retention time repeatability. The retention time

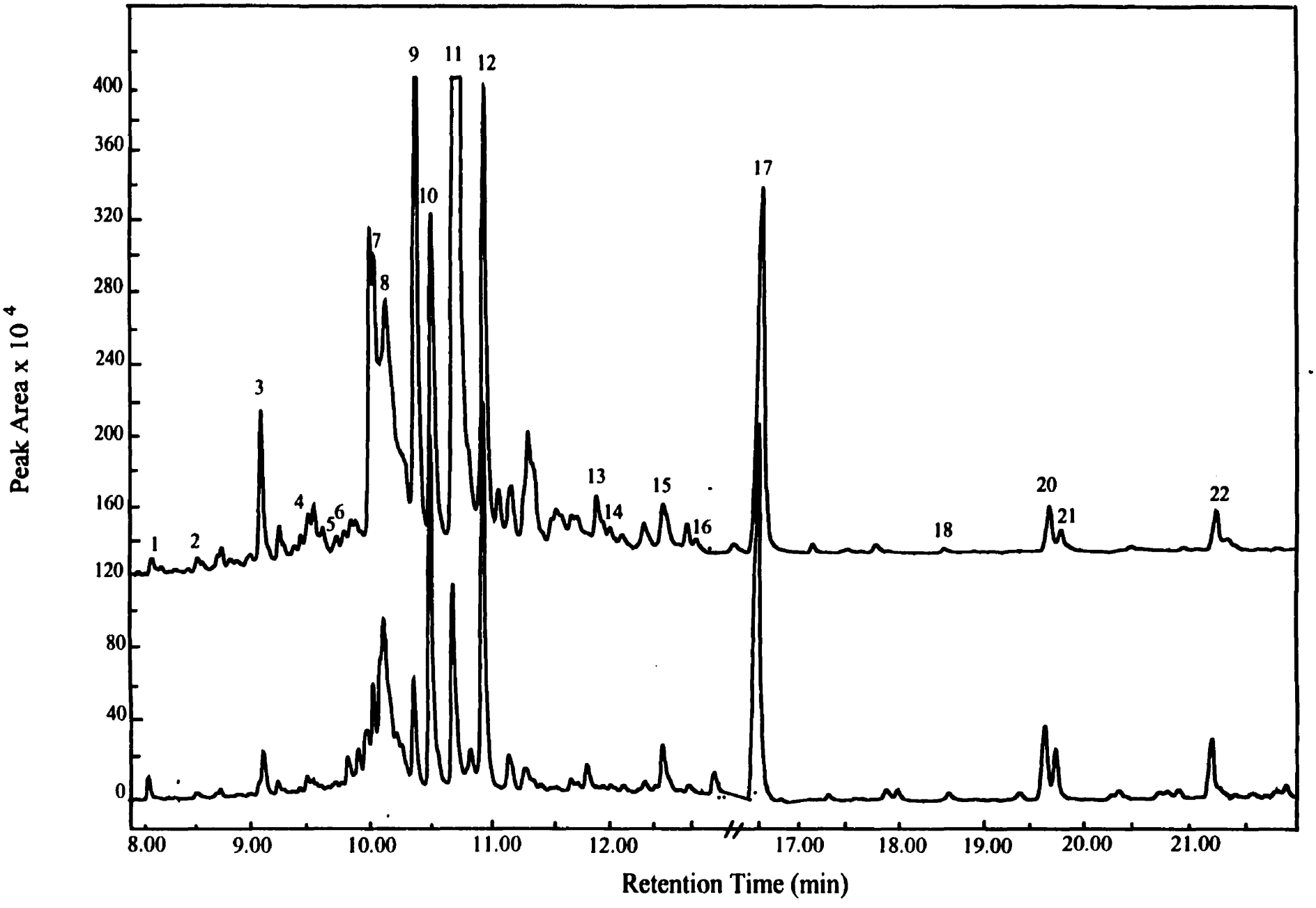


Figure 13. Chromatograms of GC analysis of phenolic compounds and flavonoids obtained from maple sap at 0% (lower line) and 100% (upper line) of the maple season.

Table 8. GC analysis of phenolic compounds and flavonoids obtained from maple sap at different periods of the season.

Peak No[a]	Retention time (min)[b]	Standard deviation[c]	Precision[d]	Relative percent of analytes at different periods of the maple sap season (%)[e] Season (%) 0	25	50	75	100
1	8.124	0.009	0.110	0.276	0.889	0.970	1.132	0.196
2	8.530	0.000	0.000	0.086	0.717	0.583	0.404	0.246
3	9.094	0.013	0.148	0.766	2.085	1.593	1.601	1.264
4	9.472	0.004	0.047	0.287	0.645	0.529	0.415	0.441
5	9:813	0.081	0.824	-[f]	-[f]	0.240	0.274	0.348
6	9.838	0.038	0.390	0.639	0.664	0.250	0.330	0.310
7	10.072	0.025	0.247	1.398	0.615	1.088	1.186	1.988
8	10.212	0.066	0.644	6.858	0.912	1.255	0.980	6.372
9	10.392	0.004	0.043	1.735	1.427	2.420	3.457	4.474
10	10.542	0.022	0.206	5.113	0.285	0.380	0.759	3.214
11	10.736	0.019	0.182	3.272	2.593	5.485	8.949	19.952
12	10.980	0.012	0.112	5.707	1.55	1.307	1.442	4.075
13	11.886	0.042	0.350	0.641	0.827	0.849	0.856	0.855
14	12.068	0.005	0.041	-[f]	0.755	0.727	0.299	0.288
15	12.524	0.005	0.044	1.075	2.291	2.041	1.215	0.947
16	12.926	0.065	0.506	0.381	0.787	0.510	0.423	0.217
17	16.834	0.036	0.213	29.070	7.012	12.279	15.505	25.128
18	18.714	0.005	0.029	0.742	1.344	1.103	1.145	0.269
19	19.017	0.006	0.030	-[f]	0.281	0.334	0.222	-[f]
20	19.792	0.008	0.042	5.724	10.254	8.643	7.803	2.672
21	19.912	0.008	0.042	3.835	7.995	6.387	5.880	1.701
22	21.502	0.016	0.076	5.532	12.456	13.001	11.258	2.551

[a] Peak number are referring to figure 13.

[b] Average retention time of the different periods of maple sap season for each peak identified.

[c] Standard deviation from the average retention time.

[d] Precision is the percentage deviation of the mean of retention time as obtained by the different periods of the maple sap season.

[e] Area percent of each peak compared to the total peak area of each different periods of the maple sap season.

[f] Not determined due to poor peak resolution.

precision calculated for the GC analysis of phenolic compounds and flavonoids from maple sap (Table 8) ranged from 0.00 to 0.82%; whereas that for HPLC ranged from 0.60 to 2.49% for UV detection (Table 5) and 0.59 to 1.45% for EC detection (Table 6).

4.2.2. Analyses of Phenolic Compounds and Flavonoids from Maple Syrup

4.2.2.1. HPLC Analysis

Figures 14 and 15 illustrate typical chromatograms of HPLC analysis of phenolic compounds and flavonoids obtained from maple syrup at 0 and 100% of the season, using UV-DAD at 280 and 320 nm. as well as EC detector at 200 and 600 mV. The separation of 17 major peaks was observed for maple syrup, obtained from different periods of the maple sap season. A consistent presence with slight variation of these major peaks was found in the maple syrup obtained from different periods.

Table 9 shows the HPLC analysis of phenolic compounds and flavonoids of maple syrup, obtained at different periods of the season, using the UV-DAD detector at 280 nm. The results (Fig. 14 and Fig. 15) demonstrate that all peaks characterized in maple sap are present in maple syrup, except that for peak 5, which was absent as well as peak 17 which newly appeared in maple syrup. At the beginning of the maple sap season (0%), all peaks are present with peaks 13 and 14 showing the greatest relative percent. Table 10 shows the HPLC analysis of phenolic compounds and flavonoids of maple syrup, obtained at different periods of the season, using the EC detector at 600 mV; the results are consistent with those obtained with UV-DAD.

4.2.2.1.1. Comparison of Identified Peaks from Maple Sap and Maple Syrup

Table 7 shows the tentative identification of phenolic compounds and flavonoids, obtained from maple sap and maple syrup, using UV-DAD detector. The results indicate that all peaks identified in maple sap were also present in maple syrup with the confirmation that peak 9, eluted at 30.67 min, was coniferol. A scan spectrum comparison of peak 9 from maple syrup with coniferol standard is presented in Figure 10. In addition, peak 17 was only characterized in maple syrup; from its maximum absorbance wavelength (326 nm, band I) it was identified as dihydroflavonol.

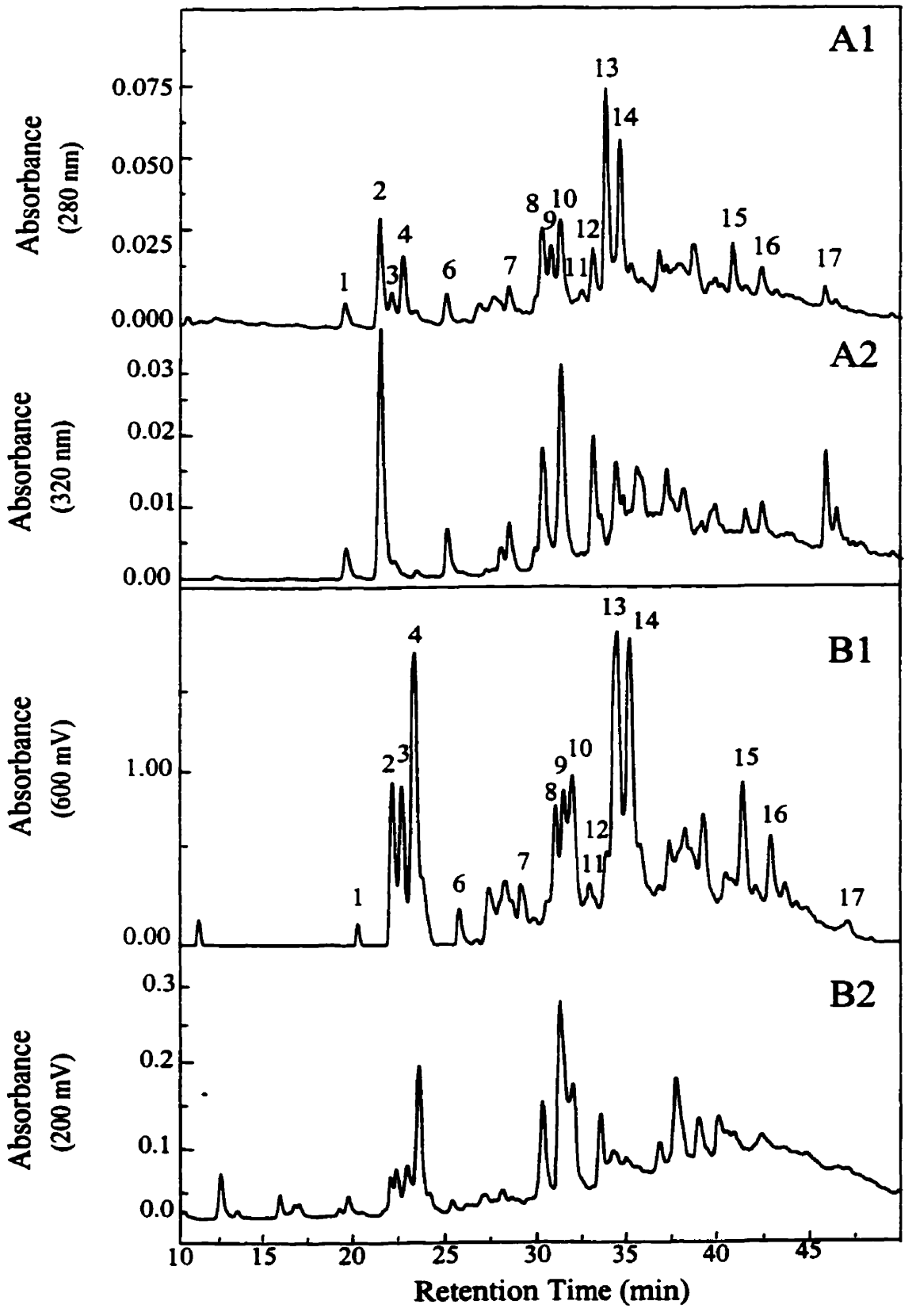


Figure 14. Chromatograms of HPLC analysis of phenolic compounds and flavonoids obtained from maple syrup at 0% of the season using UV diode-array detector at 280nm (A1) and 320 nm (A2) as well as electrochemical (EC) detector at 200 mV (B2) and 600 mV (B1).

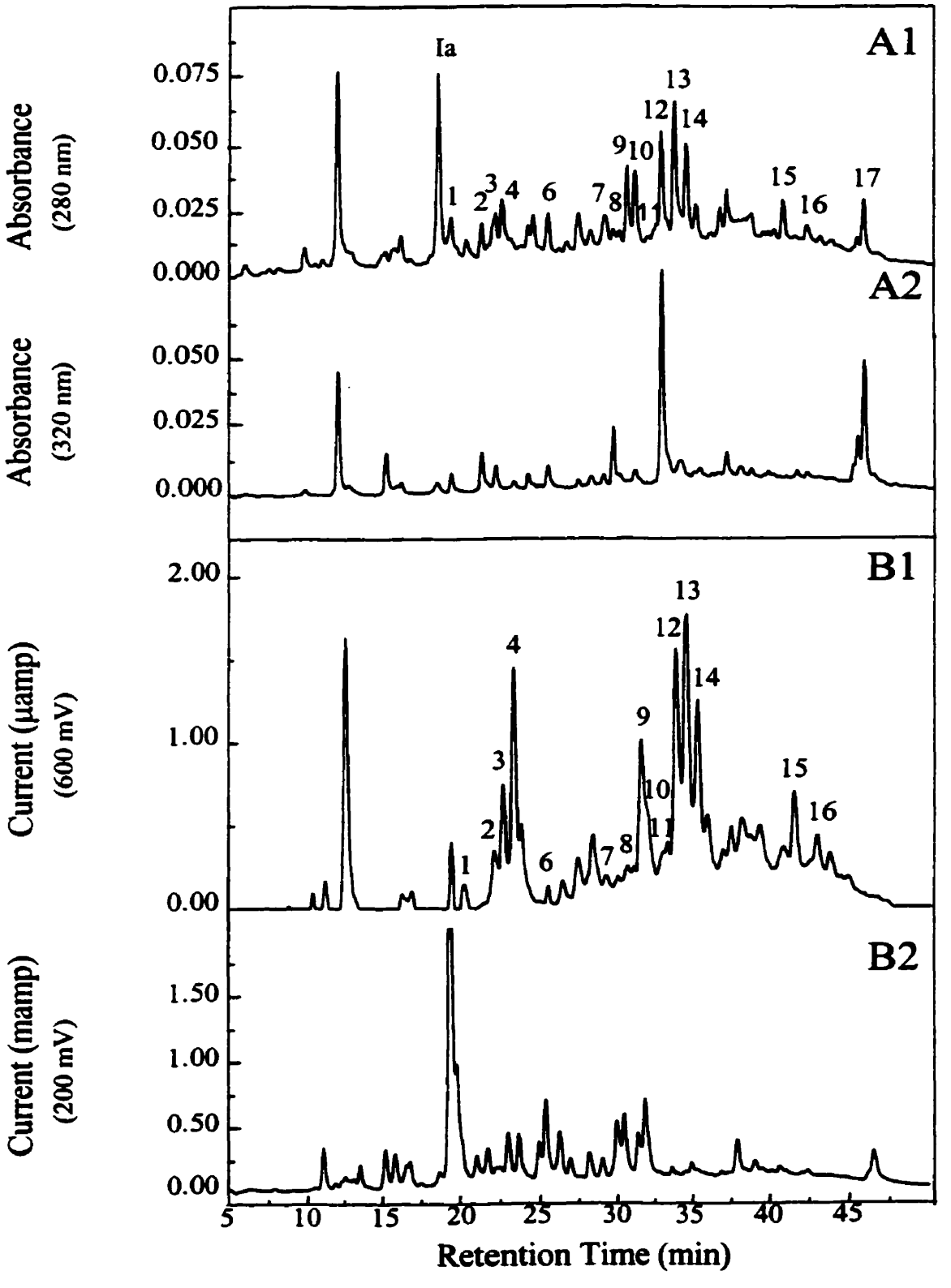


Figure 15. Chromatograms of HPLC analysis of phenolic compounds and flavonoids obtained from maple syrup at 100% of the season using UV diode-array detector at 280 nm (A1) and 320 nm (A2) as well as electrochemical (EC) detector at 200mV (B2) and 600 mV (B1).

Table 9. HPLC analysis of phenolic compounds and flavonoids obtained from maple syrup at different periods of the season using, UV diode-array detector.

Peak No[a]	Retention time (min)[b]	Standard deviation[c]	Precision[d]	λ_{max} (nm)[e]	Absorbance ratio (nm)[f]	Relative percent of analytes at different periods of the maple sap season (%)[g] Season (%) 0	25	50	75	100
Ia	18.60	0.30	1.61	214, 256, 290	11.25	-[h]	-[h]	2.32	8.98	10.51
1	19.42	0.19	0.99	230, 274, 312	2.84	1.52	1.23	1.74	2.10	3.62
2	21.34	0.19	0.87	214, 300	1.07	5.89	5.67	4.31	2.37	1.37
3	21.99	0.15	0.69	226, 274	23.43	2.03	1.70	1.93	1.30	-[h]
4	22.56	0.16	0.72	224, 276	88.74	3.92	3.25	4.16	2.76	3.75
6	25.07	0.22	0.86	226, 286	2.42	1.60	1.36	0.90	0.69	1.97
7	28.54	0.28	0.98	226, 274	3.07	1.81	1.00	0.97	0.94	2.47
8	30.16	0.28	0.92	226, 272, 308	2.20	5.57	7.15	4.49	4.45	0.75
9	30.67	0.19	0.63	218, 260, 300	8.30	3.79	3.82	4.55	4.12	3.66
10	31.19	0.18	0.58	222, 276, 304	1.06	6.03	7.87	8.67	8.05	3.76
11	32.34	0.21	0.64	230, 270	3.91s	1.93	2.27	2.13	2.20	0.38
12	33.04	0.24	0.72	228, 302	1.05	3.87	3.30	4.40	5.33	7.44
13	33.76	0.13	0.38	222, 274	27.86	11.52	9.95	11.17	9.62	7.64
14	34.55	0.14	0.40	222, 274	5.06	11.23	11.63	11.48	9.75	5.07
15	40.78	0.12	0.29	224, 278	5.02	3.27	2.78	2.54	2.22	1.59
16	42.32	0.10	0.24	222, 274, 336	1.92	2.24	1.56	2.38	1.68	0.95
17	45.83	0.13	0.29	228, 288, 326	0.62	0.99	3.99	4.92	4.72	3.16

[a] Peak number are referring to figures 8, 9, 14 and 15.

[b] Average retention time of the different periods of the maple sap season for each peak identified.

[c] Standard deviation from the average retention time.

[d] Precision is the percentage deviation of the mean of retention time as obtained by the different periods of the maple sap season.

[e] Maximum absorbance wavelengths.

[f] Relative ratio of UV absorbance (280/ 320 nm).

[g] Area percent of each peak compared to the total peak area of each different periods of the maple sap season.

[h] Not determined due to poor peak resolution.

Table 10. HPLC analysis of phenolic compounds and flavonoids obtained from maple syrup at different periods of the season, using EC detector at 600 mV.

Peak No[a]	Retention time (min)[b]	Standard deviation[c]	Precision[d]	Relative percent of analytes at different periods of the maple sap season (%)[e] Season (%) 0	25	50	75	100
Ia	19.44	0.11	0.58	[f]	[f]	0.47	0.93	1.18
1	20.11	0.10	0.50	0.28	0.26	0.23	0.11	0.64
2	21.99	0.08	0.37	3.48	3.60	2.40	1.69	1.92
3	22.52	0.06	0.27	3.49	3.53	3.60	3.16	3.38
4	23.17	0.05	0.20	8.71	7.84	14.40	8.78	6.87
6	25.59	0.13	0.50	0.71	0.65	0.62	0.10	2.83
7	29.11	0.08	0.28	1.82	1.96	1.11	0.61	1.02
8	30.89	0.23	0.76	3.43	4.66	2.78	1.27	1.27
9[g]	31.41	0.08	0.26	3.45	3.59	4.25	5.45	7.08
10	31.90	0.08	0.27	5.74	6.57	6.01	4.82	1.97
11	32.97	0.17	0.52	1.93	2.27	2.13	2.20	0.38
12	33.83	0.10	0.29	1.71	1.81	4.08	4.45	8.48
13	34.50	0.07	0.19	11.23	9.31	10.32	13.14	11.10
14	35.22	0.08	0.22	11.09	10.56	9.55	10.15	7.11
15	41.39	0.05	0.12	4.47	4.16	5.38	5.83	3.47
16	42.90	0.05	0.13	3.34	3.12	4.21	4.24	2.78
17	46.75	0.26	0.56	0.40	1.13	0.37	0.80	[f]

[a] Peak number are referring to figures 8, 9, 14 and 15.

[b] Average retention time of the different periods of maple sap season for each peak identified.

[c] Standard deviation from the average retention time.

[d] Precision is the percentage deviation of the mean of retention time as obtained by the different periods of the maple sap season.

[e] Area percent of each peak compared to the total peak area of each different periods of the maple sap season.

[f] Not determined due to poor peak resolution.

[g] Detection at 200 mV.

Peak Ia was only identified in maple syrup, appearing at 50, 75 and 100% of the season, with a relative percent ranging from 2.32 to 10.51% when analyzed at 280 nm; this new peak eluted at 18.60 min, absorbed mainly at 280 nm and responded strongly at 200 mV, which are the exact characteristics depicted by protocatechuic acid standard (Table 1). The scan spectrum comparison of peak Ia revealed a positive identification for protocatechuic acid (Fig. 12). Macheix *et al.* (1990) reported that the hydroxybenzoic acids content of fruits is generally low, except in certain fruits of the *Rosaceae* family and in particular blackberry, in which protocatechuic acid and gallic acid contents may be very high. Protocatechuic acid is found in soft fruits in the form of glucosides (Macheix *et al.*, 1990).

4.2.2.2. GC Analysis

Figure 16 illustrates the typical chromatograms of GC/FID analysis of phenolic compounds and flavonoids from maple syrup obtained at 0 and 100% of the season. Table 11 shows that all identified peaks were present at the beginning of the season (0%) except for peaks 13 and 16. Peak 17 depicted the greatest relative percent at the early stage of the maple sap season (%).

4.2.2.2.1. Comparison of Identified Peaks from Maple Sap to Maple Syrup

When comparing the retention time of different peaks present in maple syrup (Table 11) to that of standards (Table 3), a tentative identification of phenolic compounds and flavonoids could be made. Similar retention times were found for vanillin (peak 2, 8.55 min), syringaldehyde (peak 4, 9.46 min), vanillic acid (peak 5, 9.82 min), homovanillic acid (peak 6, 9.86 min), protocatechuic acid (peak 7, 10.05 min), coniferal (peak 8, 10.28 min), syringic acid (peak 10, 10.53 min), coniferol/*p*-coumaric acid (peak 11, 10.74 min), caffeic acid (peak 14, 12.03 min), (-)-epicatechin (peak 18, 18.72 min) and (+)-catechin (peak 19, 18.85 min).

The major difference between GC/FID analysis of phenolic compounds and flavonoids, obtained from maple sap and maple syrup was, the absence of integration for peaks 13 and 16 (Table 11) which correspond to ferulic and sinapic acids retention time,

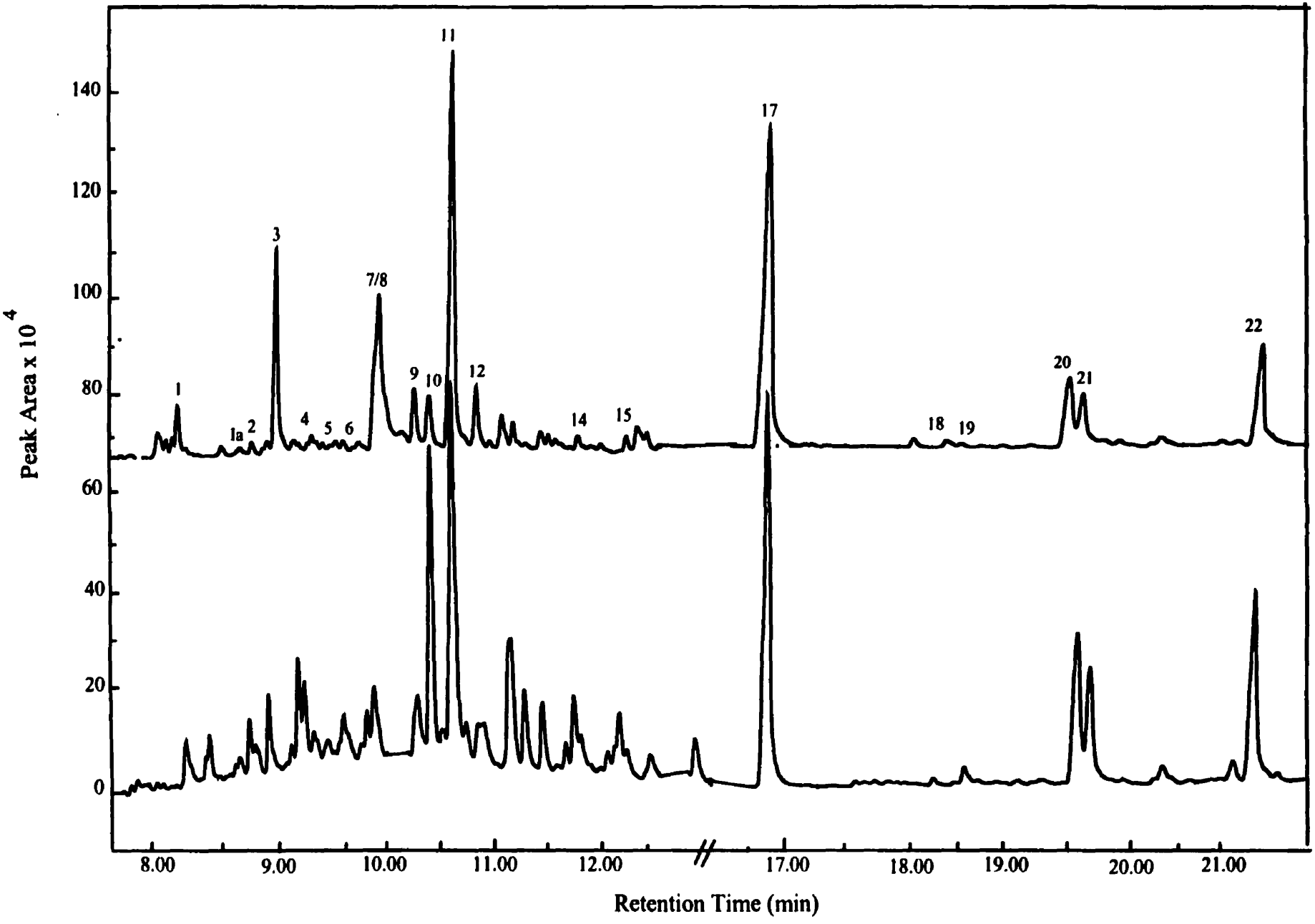


Figure 16. Chromatograms of GC analysis of phenolic compounds and flavonoids obtained from maple syrup at 0% (lower line) and 100% (upper line) of the season using flame ionization detector (FID).

Table 11. GC analysis of phenolic compounds and flavonoids obtained from maple syrup at different periods of the season.

Peak No[a]	Retention time (min)[b]	Standard deviation[c]	Precision[d]	Relative percent of analytes at different periods of the maple sap season (%)[e] Season (%) 0	25	50	75	100
1	8.132	0.008	0.103	0.025	1.238	1.337	0.085	1.718
Ia	8.45	0.015	0.127	0.301	0.457	0.196	0.159	0.652
2	8.546	0.011	0.133	0.381	0.499	0.188	0.155	0.506
3	9.074	0.009	0.099	0.712	0.413	0.445	0.986	5.601
4	9.460	0.022	0.236	0.689	0.469	0.215	0.346	1.252
5	9.824	0.013	0.137	0.198	0.057	0.046	0.108	0.294
6	9.856	0.013	0.136	0.386	0.244	0.208	0.421	0.516
7	10.046	0.009	0.089	0.670	0.290	1.260	5.357	10.057
8	10.282	0.011	0.107	0.978	0.590	1.260	5.357	10.057
9	10.396	0.005	0.053	0.988	0.538	0.310	0.637	1.488
10	10.526	0.015	0.144	2.240	1.696	1.862	3.860	2.410
11	10.744	0.015	0.141	3.386	1.666	2.598	6.958	13.663
12	10.990	0.010	0.091	1.136	0.756	0.593	1.125	2.531
13	-[f]	-[f]	-[f]	-[f]	-[f]	-[f]	-[f]	-[f]
14	12.030	0.028	0.235	0.202	0.187	0.107	0.162	0.520
15	12.530	0.007	0.056	2.890	1.659	0.973	1.350	1.306
16	-[f]	-[f]	-[f]	-[f]	-[f]	-[f]	-[f]	-[f]
17	16.916	0.063	0.371	22.898	55.687	66.605	39.059	15.735
18	18.722	0.004	0.024	1.167	0.558	0.351	0.655	0.297
19	18.852	0.004	0.024	0.348	0.151	0.130	0.360	0.166
20	19.816	0.011	0.058	10.555	5.213	3.596	5.935	3.293
21	19.934	0.011	0.057	8.024	4.485	3.028	4.470	2.520
22	21.526	0.011	0.053	12.675	6.815	5.059	8.348	4.776

[a] Peak number are referring to figure 16.

[b] Average retention time of the different periods of maple sap season for each peak identified.

[c] Standard deviation from the average retention time.

[d] Precision is the percentage deviation of the mean of retention time as obtained at different periods of the maple sap season.

[e] Area percent of each peak compared to the total peak area of each different periods of the maple sap season.

[f] Not determined due to poor peak resolution.

respectively. However, no major difference in phenolic compounds profile was observed when comparing maple sap and maple syrup, except those additional peaks appearing in the maple syrup at 100% of the season. The overall results showed certain consistency was seen between maple sap and maple syrup for the 22 selected peaks.

The experimental findings suggest that the GC analysis was more precis, in term of retention time repeatability, than that of the HPLC. The retention time precision, calculated for the analysis of phenolic compounds and flavonoids present in maple syrup, was from 0.02 to 0.37% for GC/FID (Table 11) and 0.24 to 1.61% for HPLC/UV (Table 9) and 0.12 to 0.76% for HPLC/EC (Table 10).

4.2.3. GC/MS Analysis of Phenolic Compounds and Flavonoids from Maple Sap and Maple Syrup

The analysis of phenolic compounds and flavonoids present in maple sap and maple syrup was also carried out with GC/MS for selected periods of the maple sap season (0 and 100%).

GC/MS analysis, using electron impact (EI) ionization, of phenolic compounds and flavonoids for maple sap and maple syrup at 0 and 100% of the maple sap season was done. It was possible to relate the 22 peaks identified in GC/FID to those in GC/MS, using retention time data. Hence, the tentative identification made by GC/FID was supported by mass spectrum characteristics obtained from the GC/MS analysis.

4.2.3.1. Identification of Phenolic Compounds and Flavonoids

Figure 17 shows a GC/MS analysis of phenolic compounds and flavonoids obtained from maple sap and maple syrup at 100% of the season. Table 12 shows the mass spectrum characteristics of the identified peaks. Peak 2 (7.74 min) which corresponds to the retention time of vanillin standard when analyzed in GC/MS (Table 4) was not identified as vanillin but instead has an homology of 99% with 1,2,3-trioxybenzene using the commercial library (WILEY138.L). Vanillin peak (Ia) eluted at 7.72 min. was tentatively identified by comparing its mass spectrum (Fig.18) to that of standard compound (Table 4). Figure 18 depicts a mass spectrum of vanillin (peak Ia:

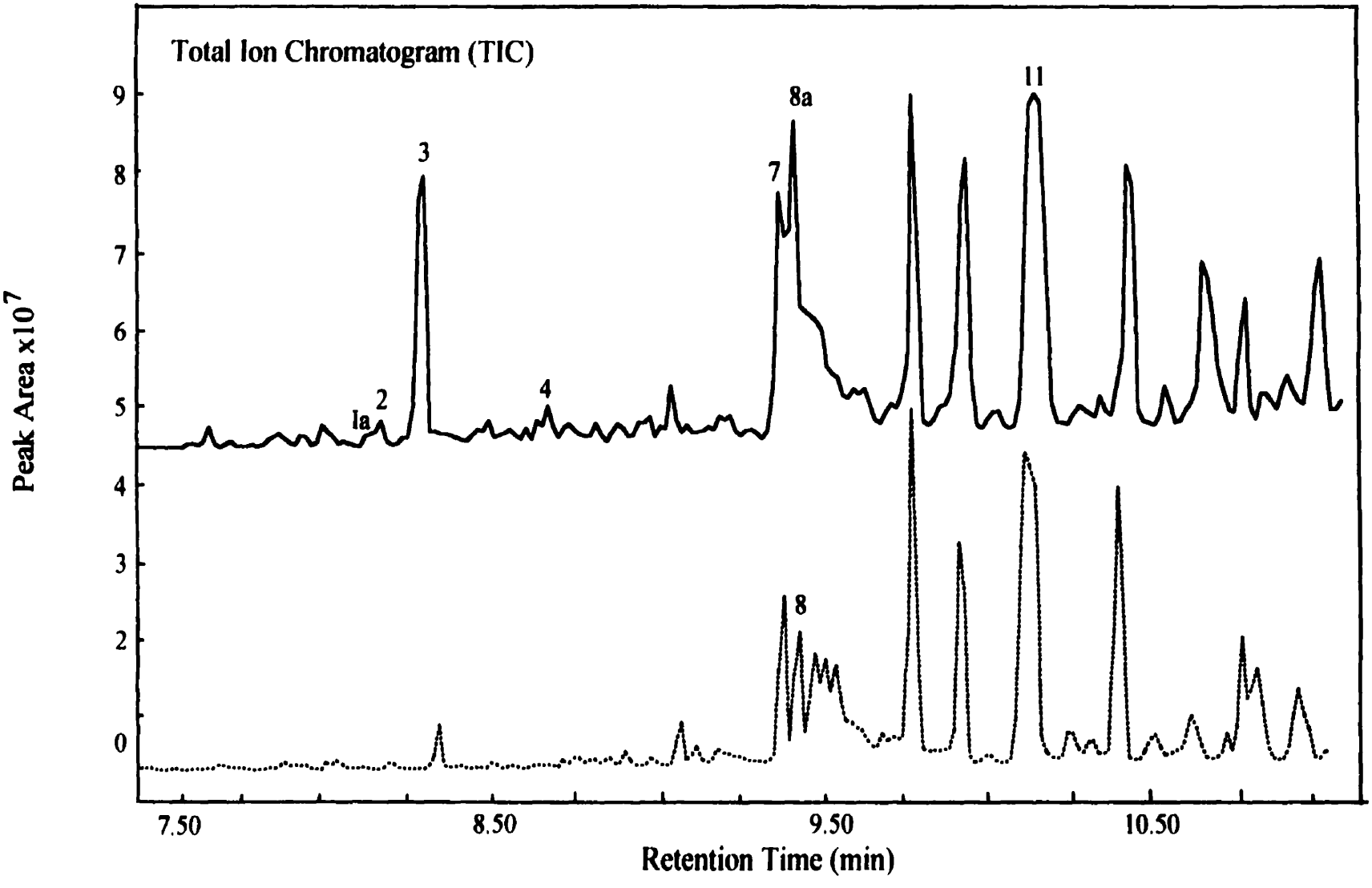


Figure 17. GC/MS analysis of phenolic compounds and flavonoids obtained from maple sap (·····) and maple syrup (—) at 100 % of the season. Including, vanillin (Ia), 1, 2, 3-trioxybenzene (2), 3-hydroxy-capric acid (3), syringaldehyde (4), protocatechuic acid (8a) and coniferol / *p*-coumaric acids (11) from Table 12.

Table 12. GC/MS analysis of phenolic compounds and flavonoids obtained from maple sap and maple syrup at 100 % of the season, using mass spectrum characteristics from TIC.

Peak No[a]	Derivitized compound	Elution time (min)	Mass spectrum characteristics: Molecular ion (M) $(m/z)^b$	Base peak ion (BP) $(m/z)^c$	Fragment ion (FI) $(m/z)^d$
Ia	Vanillin[e]	7.72	224	194	209
2	1, 2, 3-Trioxybenzene[e]	7.74	342	239	327
3	3-Hydroxycapric acid[e]	8.39	317	147	302
4	Syringladehyde[e,f]	8.80	254	224	239
8a	Protocatechuic acid[e]	9.46	370	193	355
11	Coniferol[e,f]	10.18	324	324	309
11	*p*-Coumaric acid[e,f]	10.19	308	299	293
22	Fisetin[e,f]	21.12	559	559	471

[a]Peak number are referring to figure 17.

[b]Molecular ion, generated after an electron strikes the parent molecule and ejecting one electron, most representing the derivitized molecular weight (MW).

[c]Base peak ion, representing 100 % abundance.

[d]Fragment ion, chosen on the basis of their abundance and specificity for the compound.

[e]Presence in maple syrup.

[f]Presence in maple sap.

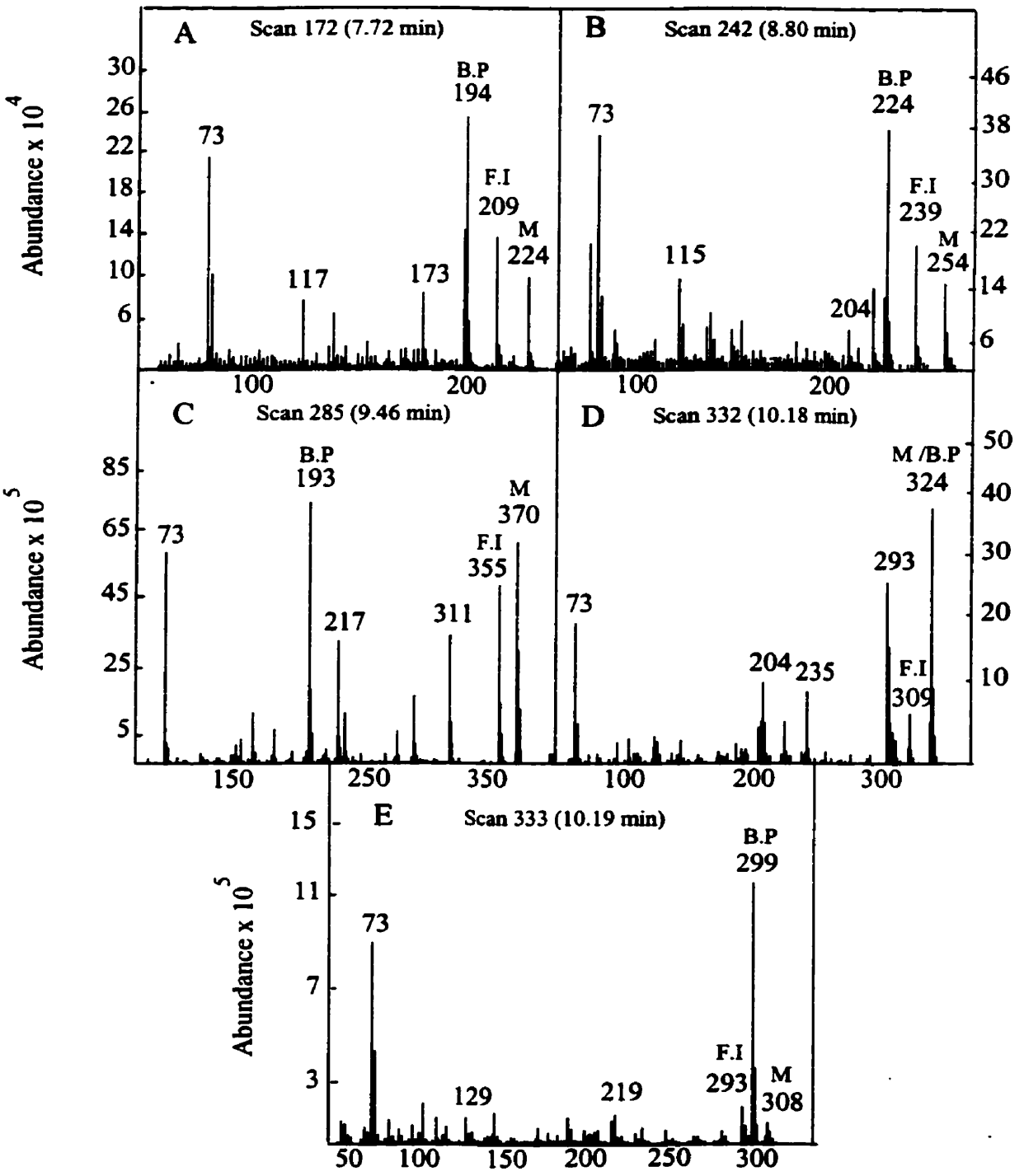


Figure 18. Mass spectrum of identified peaks from TIC obtained from maple sap and maple syrup at 100 % of the season. Including, vanillin (A), syringaldehyde (B), protocatechuic acid (C), coniferol (D) and *p*-coumaric acid (E). Molecular ion (M), base peak ion (BI) taken as 100% abundance and fragment ion (FI).

7.72 min) with a molecular ion of 224, a base peak ion of 194 and a fragment ion of 209. These characteristics are identical to those found for GC/MS analysis of vanillin standard (Table 4).

Peak 3, eluted at 8.39 min was only present in maple syrup and was identified as 3-hydroxycapric acid, with reference to the commercial library. In addition, peak 4, eluted at 8.80 min, was present in both maple sap and maple syrup and was tentatively identified as syringaldehyde by matching its mass spectrum (Fig. 18) with that of standards (Table 4). Figure 18 indicates a molecular ion of 254, a base peak ion of 224 and a fragment ion of 239 for scan 242 at 8.80 min, which are the matching characteristics for GC/MS analysis of syringaldehyde standard.

Figure 17 indicates an interesting difference occurring within peak 8. It has a completely different mass spectrum in maple sap at 100% of the season (peak 8) when compared to that in maple syrup of the same season (peak 8a). In addition, the mass spectrum (Fig. 18) of peak 8a from maple syrup at 100% of the season is identical to protocatechuic acid standard (Fig. 18) with a molecular ion of 370, a base peak ion of 193 and a fragment ion of 355, which correlates with protocatechuic acid standard (Table 4). These results may explain the sudden appearance of protocatechuic acid in maple syrup at 50 to 100% of the season previously described for HPLC analysis (Table 7). The results (Fig. 18) indicate the presence of protocatechuic acid (peak 8a) having a very different mass spectrum than peak 8 in maple sap of the same season.

Peak 11 contained two different compounds, which were identified as coniferol and *p*-coumaric acid by the comparison of their mass spectra (Fig. 18) with those of standards (Table 4). Coniferol scan was recorded at 10.18 min, whereas *p*-coumaric acid was at 10.19 min within the same peak. The GC/FID and GC/MS analyses of standards showed that coniferol eluted slightly prior to *p*-coumaric acid.

Figure 18 demonstrates the scan 332 at 10.18 min, with an identical molecular ion and base peak ion of 324 and a fragment ion of 309; Table 4 depicts these characteristics for coniferol standard when analyzed by GC/MS. Figure 18 also indicates the scan 333 at

10.19 min with a molecular ion of 308, a base peak ion of 299 and a fragment ion of 293; these characteristics are in agreement with the GC/MS analysis of *p*-coumaric acid standard (Table 4), except that the base peak ion and fragment ion are identical (293).

After preparing the TMS derivatives (derivatized analytes) and obtaining a spectrum of the sample, it was possible to find which GC peak (s) contain the TMS derivatives. The GC analysis shows (Fig. 18) an ion mass of *m/z* 73, which is characteristic of the molecular weight of TMS, used as reagent for derivatization. The molecular weight of the TMS derivative was determined by subtracting 15 units from the molecular ion (M-15) peak, which would be a prominent high-mass ion in the spectrum. When two high-mass peaks separated by 15 mass units were observed, the highest-mass peak was considered as the molecular weight of the TMS derivatives (Kitson *et al.*, 1996). The results indicate (Fig. 18) that by subtracting 15 from the molecular ion (M) of each different scan, the selected fragment ions (FI) was obtained, including 209, 239 and 355, 309 and 293 for vanillin, syringaldehyde, protocatechuic acid, coniferol and *p*-coumaric acid, respectively.

Goldberg *et al.* (1994 and 1995) reported a direct GC/MS method to measure *cis* and *trans*-resveratrol in wine, by determining the selective ion monitoring (SIM) of the molecular ion at mass 228. Subsequently, Soleas *et al.* (1997a) developed a conventional GC/MS method for both isomers using BSTFA as a derivatization procedure; the experimental results obtained throughout our studies are in agreement with those reported by these authors.

In addition, new TMS derivatized compounds that were not selected as standards in our preliminary trails (Table 4), revealed a match quality higher than 90% when compared to that of the commercial library (Table 12). 1,2,3-Trioxybenzene (peak 2), 3-hyrdroxy-capric acid (peak 3), α-D-mannopyranose (peak 10), glucose-5TMS (peak 12), sucrose-octaTMS (peak 17), vanillylmandelic acid (peak 18 and 19) and 2,6-dibromo-4-nitrophenol (peak 20 and 21) were hence identified. Although the aim of the research was not directed towards these compounds, 1,2,3-trioxybenzene (peak 2) has a benzene ring and a very similar structure to that of gallic acid and was suggested to be a phenol related

compound. GC/MS enabled the identification of selected standard compounds and facilitate thereby the tentative characterization of other compounds present in maple sap and maple syrup extract.

Although the GC/MS method did not reveal the presence of selected flavonoids, standards their tentative identification by HPLC cannot be ignored. The EI ionization mode used throughout this study was probably not the most appropriate method for the analysis of flavonoid glycosides, which are probably present in maple sap and maple syrup.

Mass spectrometry measures the mass and abundance of ions: it is a powerful analytic instrument mainly because it is highly sensitive. A complete mass spectrum can be obtained with a few nanograms of analyte whereas selected ions can be observed with a few picograms. The ability to obtain the molecular weight and characteristic fragment ions is relatively sufficient to identify analytes without the need for other analytical methods (Kitson *et al.*, 1996).

4.3. Changes in Phenolic Compounds and Flavonoids Profile for the Maple Sap Season

Phenolic compounds and flavonoids in fruit are important contributors to the color, flavor and aging characteristics of fruit products (Dawes and Keene, 1999) as well as changes occurring with maturation (Brenes *et al.*, 1999). In addition, Kermasha *et al.* (1995a) demonstrated that there was a significant effect of harvest time on the concentration of total phenolic compounds present in maple saps, concentrates, and syrups: these authors indicated a seasonal increase in the amount the phenolic compounds.

4.3.1. Changes in Phenolic Compounds and Flavonoids Profile in Maple Sap

4.3.1.1. HPLC Analysis

Table 5 shows the HPLC analysis of phenolic compounds and flavonoids of maple sap, obtained at different periods of the season, using the UV detector at 280 nm. Peaks 7 and 12 were not detected at 0% of the maple sap season; however, they appeared

at 25 and 50% of the season, respectively. An interesting observation, depicted by peak 11, was its complete disappearance at 50% of the season. Peak 9 was present but only responding at 200 mV, with a scarce absorption at 280 nm (Fig. 8). At 100% of the maple sap season, all major peaks were present with a significant increase in the relative percent for peak 12. Peaks 13 and 14: these peaks were highly present at all different periods of the maple sap season, with values ranging from 15.41 to 12.46% for peak 13 and from 12.01 to 9.23% for peak 14 at 0 and 100% of the season, respectively.

Table 6 shows the HPLC analysis of phenolic compounds and flavonoids of maple sap, obtained at different periods of the season, using the EC detector at 600 mV. The higher sensitivity of the EC detector enabled the detection of peak 7 at 0% of the season, which was absent from UV detection at 280 nm (Fig. 8). Peak 12 was absent at 0 and 25% of the season for EC and UV analyses. Peaks 5 and 11 were not detected with EC detector and consequently are absent in Figures 8 and 9. In addition, peaks 1 and 7 disappeared at 50% of the season, whereas peak 6 was not detected at 100% of the season. Peaks 13 and 14 presented the highest relative percent at all different periods of the maple sap season, with values ranging from 14.72 to 16.04% for peak 13 and 13.67 to 10.23% for peak 14 at 0 and 100% of the season, respectively.

The average retention time for each peak at the different periods of the maple sap season was calculated and statistical analysis was performed. Precision which represents the relative standard deviation (RSD), was never greater than 2.49 at 280 nm (Table 5) and 1.45 at 600 mV (Table 6), presenting an acceptable repeatability of the HPLC analysis using UV-DAD and EC detectors. Repeatability is the precision of a method under the same operating conditions over a short period of time and represents only one aspect of instrumental precision: it is measured by the sequential, repetitive injection of the same homogenous sample, followed by the averaging of the peak area or peak height values and determination of the relative standard deviation of all injections (Snyder *et al.*, 1997).

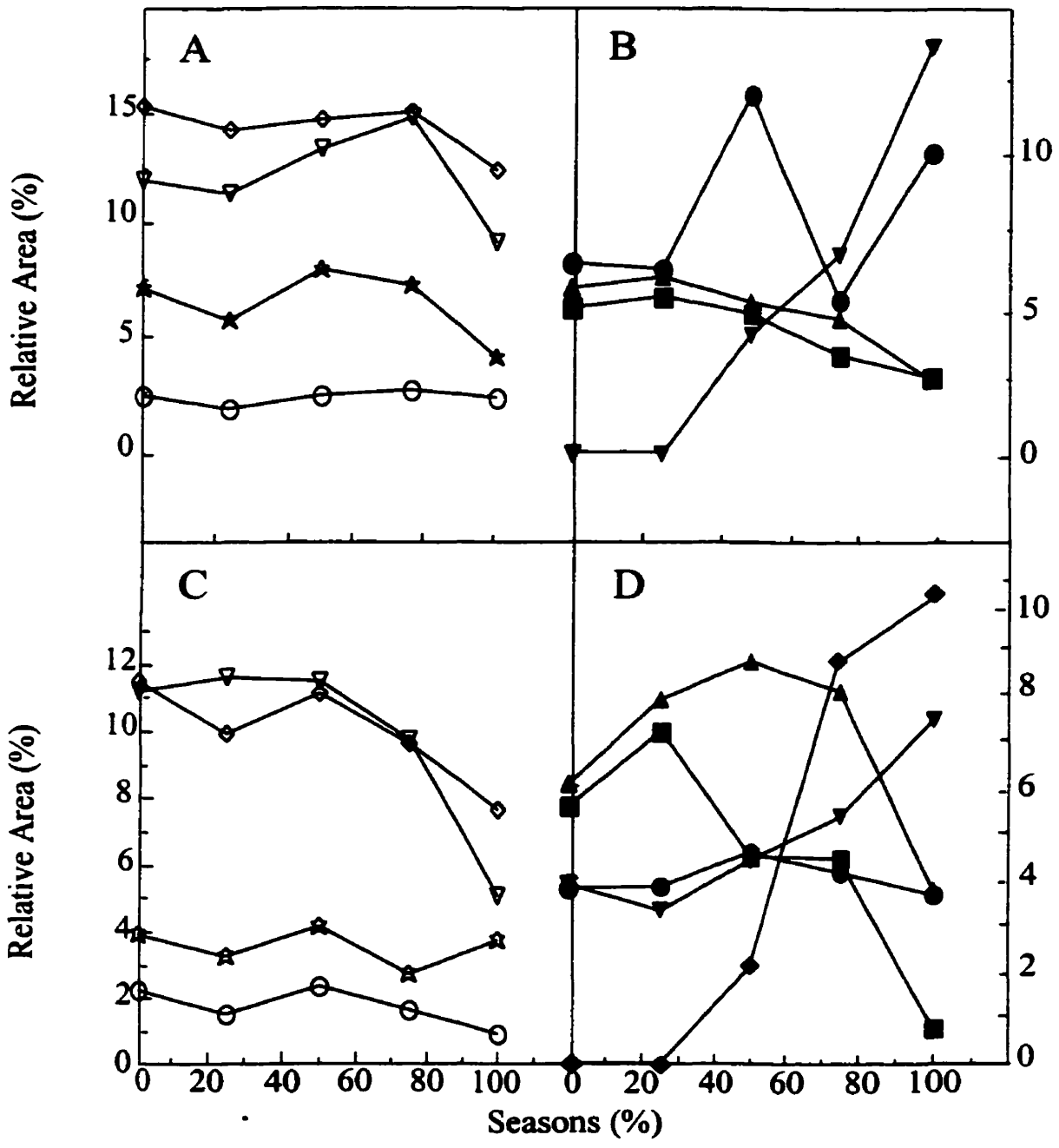


Figure 19. Seasonal profile of selected phenolic compounds and flavonoids from maple sap (A,B) and maple syrup (C,D) analyzed by HPLC at 280 nm. Flavonoid related compounds (A,C): (+)-catechin (☆), flavanol (peak 13) (◇), flavanol (peak 14) (▽), and dihydroflavonol (peak 16) (○). Phenolic compounds (B,D): vanillin (■),coniferol(●), syringaldehyde (▲), protocatechuic acid (◆) and *p*-coumaric acid (▼).

4.3.1.1.1. HBA and HCA Derivatives in Maple Sap

Figure 19 shows a seasonal profile of HBA and HCA derivatives present throughout the season. Vanillin and syringaldehyde showed concomitant decreases at the end of the season whereas coniferol showed a drastic increase at 50% of the season, followed by a drop to 75% before another major increase at 100% of the season. Finally, *p*-coumaric acid was absent at the beginning of the season (0 and 25%), appeared at 50% of the season and then increased in a linear fashion until the end of the season (100%) to reach the highest relative percent of all identified HBA and HCA derivatives. The evaluation (data not shown) with the EC detector at 600 mV (Table 6) indicated similar results to those obtained at 280 nm (Table 5).

4.3.1.1.2. Flavonoids in Maple Sap

Figure 19 shows the changes in phenolic and flavonoids profile in maple sap obtained by HPLC analysis, using the UV detector at 280 nm. The results indicate that flavonoids, such as (+)-catechin, flavanols and dihydroflavonols, show little variation over the different periods (0, 25, 50, 75 and 100%) of the maple sap season. However, a slight drop in (+)-catechin and flavanols represented by peaks 13 and 14 (Figs. 8 and 9) was observed from 75 to 100% of the season. More important was their corresponding profile throughout the season. Macheix *et al.* (1990) demonstrated that (+)-catechin and (-)-epicatechin were shown to vary in a similar manner. The variation for the selected flavonoids, using the EC detector at 600 mV (Table 6), presented a similar profile but was not presented graphically.

4.3.1.2. GC Analysis

Table 8 shows the GC analysis of phenolic compounds and flavonoids, obtained from maple sap at different periods of the season, using FID. The highest relative percent obtained throughout the different periods of the maple sap season was shown by peak 17; other major peaks were 9, 10, 11 and 12. At the beginning of the season (0%), peaks 5, 14 and 19 were not integrated due to poor peak resolution and at the end of the season (100%) peak 19 was absent. Fisetin (15 µg/mL) was used as an internal standard and

spiked in all extracts (peak 22). The response of fisetin was not used to correct results or to provide quantitative data but rather to monitor the unusual instrument fluctuation.

The average retention time for each peak, at the different periods of the maple sap season, was calculated and statistical analysis was performed. Precision was never greater than 0.82 (Table 8), presenting hence an excellent repeatability of the GC/FID system, as compared to that obtained with HPLC/UV-DAD at 280 nm.

Figure 20 shows the changes in phenolic compounds and flavonoids profile, obtained from maple sap, analyzed by GC/FID. The seasonal profile indicates a similar behavior between vanillin and syringaldehyde, as they simultaneously increased from 0 to 25% of the maple sap season where they reached their highest relative percent and than decreased gradually towards the end of the season; this trend was also encountered in HPLC analysis (Fig. 19). The experimental findings for GC analysis of coniferol and *p*-coumaric acid can not be compared to those of HPLC, this is due to the co-elution of both compounds in GC. Moreover, an increase in coniferol and *p*-coumaric acid was observed at the end of the season, using both analytical methods.

4.3.1.3. General Evolution Profile

Most flavonoids identified appeared consistently stable throughout the maple sap season with a slight decrease approaching 100% of the season. The HBA and HCA depicted a slight seasonal increase as well represented by coniferol and *p*-coumaric acid. The general profile of phenolic compounds and flavonoids is in agreement with the statement reported by Kermasha *et al.* (1995a); indicating a concommitant seasonal increase in phenolic compounds with maple sap season. This increase may be due to factors, including genetics, climatic and soil conditions, combined to provide variations in qualitative and quantitative profile of phenolic compounds (Belford and Lindsay 1992).

Although it has been shown that the production of free aglycones could increase during maturation (Amiot *et al.*, 1986), the production of glucosides of flavonoids could decrease during the same period and hence lower concentrations of flavonoid glycosides

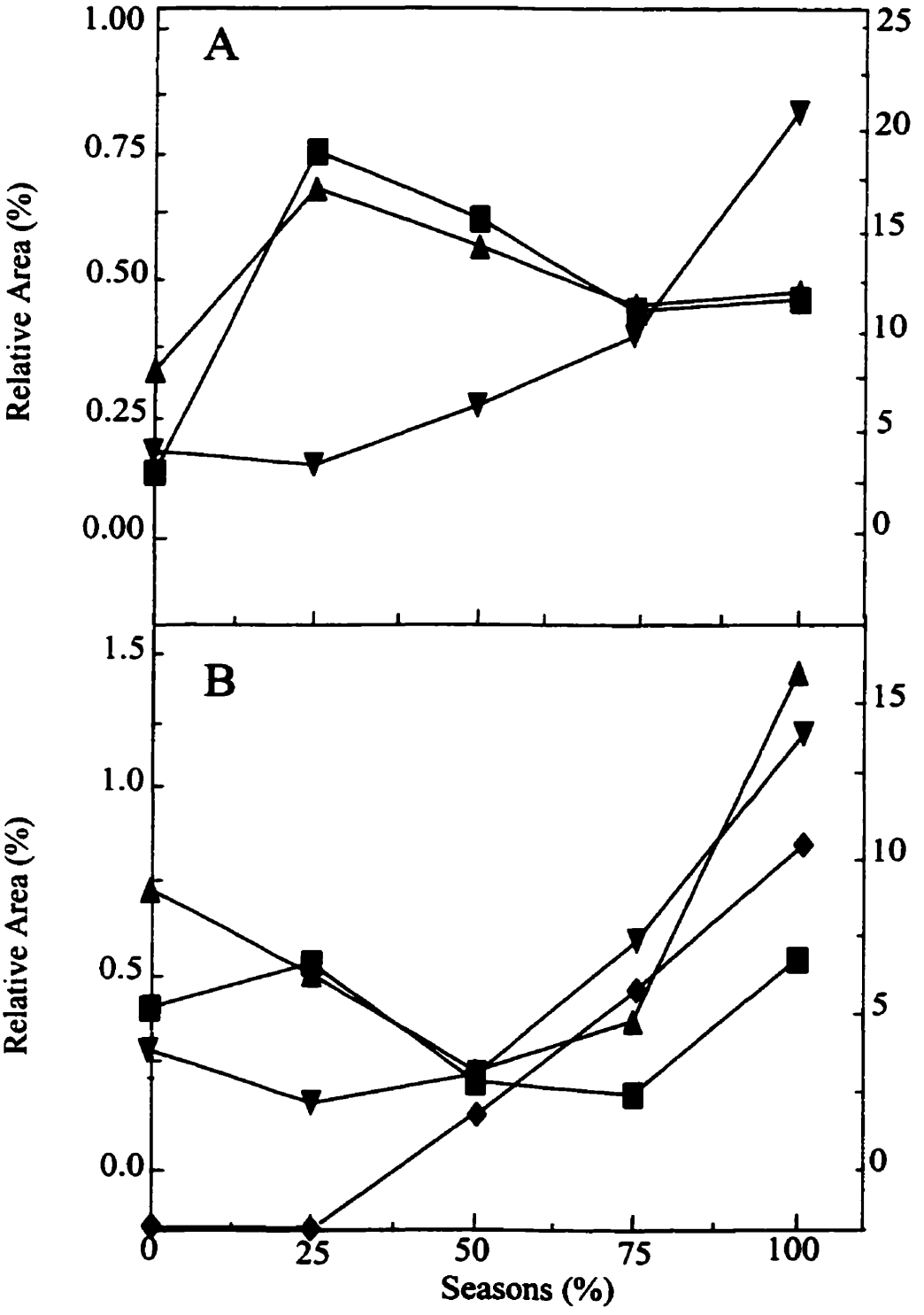


Figure 20. Seasonal profile of selected phenolic compounds from maple sap (A) and maple syrup (B) using GC-FID. Including vanillin (■), syringaldehyde (▲), protocatechuic acid (◆) and coniferol/ *p*-coumaric acid (▼).

may be reported. Belford and Lindsay (1992) have confirmed the presence of vanillin glucoside in acid and enzymatic hydrolysates of maple sap and maple syrup. The presence of glycosilated flavonoids in maple products cannot be ignored. However, this transformation needs an enzymatic activity to break down the glycosidic linkages (Brenes and de Castro, 1998). The literature (Amiot *et al.*, 1986) indicated the presence of such enzymatic activity during milling or malaxation of olives. In maple sap, the enzymatic activity may be triggered by the wound created from tapping the maple tree which disrupts the plant cell wall and releases intra-cellular enzymes. Hence, the variation in phenolic compounds and flavonoids present in maple sap could be due to the relative ratio of their existence as glycosides or free aglycones. In addition, phenolic compounds have been known to play a role in resistance of plants to different stresses such as wounding, various chemical treatments and microbiological infections (Bell, 1981). A possible explanation for the increase in phenolic compounds may be related to a physiological response of the maple tree to wounding during the tapping process. No matter what type of stress imposed, one of the most common responses is the increase in total phenolic content (Macheix *et al.*, 1990).

4.3.2. Changes in Phenolic Compounds and Flavonoids Profile in Maple Syrup

4.3.2.1. HPLC Analysis

Table 9 shows the HPLC analysis of phenolic compounds and flavonoids of maple syrup, obtained at different periods of the maple sap season using the UV-DAD detector at 280 nm. The results (Fig. 14 and Fig. 15) show that all peaks characterized in maple sap were also present in maple syrup, except for peak 5. Peak 17, newly appeared in maple syrup, and peak Ia (Fig. 15) started their appearance at 50% of the maple sap season. All the other peaks (1 to 16) were present throughout the season with peaks 13 and 14 having the highest relative percent ranging from 11.52 to 7.64 % for peak 13 and 11.23 to 5.07% for peak 14 at 0 and 100% of the season, respectively.

Table 10 shows the HPLC analysis of phenolic compounds and flavonoids of maple syrup, obtained at different periods of the maple sap season using the EC detector at 600 mV. The results are consistent with those obtained by UV-DAD detector since

peak 5 was still absent and peaks 17 and Ia was newly appeared. Furthermore, peak 11, which was absent in maple sap, with EC detection at 600 mV, could be identified in maple syrup at each different periods of the season using the same voltage. Finally, peaks 13 and 14 presented the highest relative percent with values ranging from 11.23 to 11.10% for peak 13 and 11.09 to 7.11% for peak 14 at 0 and 100% of the season, respectively.

The average retention time for each peak of the different periods of the maple sap season was calculated and statistical analysis was performed. Precision was never greater than 1.61, using UV at 280 nm (Table 9) and 0.76, using EC at 600 mV (Table 10), conferring hence a good repeatability of the HPLC/UV-DAD and EC detectors.

4.3.2.1.1. HBA and HCA Derivatives in Maple Syrup

Figure 19 indicates a pronounced variation for HBA and HCA in maple syrup throughout the maple sap season. The novel appearance of protocatechuic acid in maple syrup at 50% of the season was well depicted by a steady increase until 100% of the season. Vanillin and syringaldehyde varied simultaneously, starting with a slight increase to continue with a steady drop until the end of the season whereas coniferol presented a more consistent profile across all seasons. Finally, *p*-coumaric acid followed the same changes in profile that the one observed in maple sap, with a steady increase until the end of the season (100%), but at lower level of relative percent than protocatechuic acid. The evaluation with the EC detector at 600 mV (Table 10) showed similar results to those one obtained with the UV-DAD at 280 nm (data not shown).

4.3.2.1.2. Flavonoids in Maple Syrup

Figure 19 shows the changes in phenolic compounds and flavonoids profiles from maple syrup, using HPLC/UV detector at 280 nm. The results indicate that flavonoid compounds, such as (+)-catechin, flavanols and dihydroflavonols, vary in a similar manner with that encountered in maple sap. A more pronounced drop at 100% of the maple sap season was seen for flavanols, represented by peaks 13 and 14; the seasonal

variation of these selected flavonoids using the EC detector at 600 mV (Table 10) was similar to that with UV-DAD at 280 nm (data not shown).

4.3.2.2. GC Analysis

Table 11 shows the GC analysis of phenolic compounds and flavonoids, obtained from maple syrup at different periods of the season, using FID. Peak 17 showed the greatest relative percent; other major peaks 3, 7/8 and 11 were also present. Peaks 13 and 16 were not detected due to poor peak resolution and hence no data are recorded for these two peaks. Fisetin (15 µg/mL), was used as an internal standard, and was spiked in all extracts (peak 22). The response of fisetin was not used to correct results or provide quantitative data, but rather to monitor the unusual instrument fluctuation.

The average retention time for each peak, at the different periods of the maple sap season, was calculated and statistical analysis was performed. Precision was never greater than 0.37 using GC/FID indicating an excellent repeatability of the system. The precision encountered for the maple syrup samples were better than that for the maple sap.

Figure 20 shows the changes in phenolic compounds and flavonoids profiles in maple syrup, using GC/FID. The changes in vanillin and syringaldehyde were similar throughout the season and showed a consistent increase. The novel appearance of protocatechuic acid at 50 until 100% of the season was clearly demonstrated. Coniferol and *p*-coumaric acids were also increased, particularly towards the end of the season (100%).

4.3.2.3. General Evolution Profile

Similar profiles were observed for flavonoids in maple sap and maple syrup throughout the season, except that in maple syrup, the drop toward the end of the season (100%) was slightly more pronounced. The presence of HBA and HCA in maple syrup demonstrated (Fig. 20) a trend towards a slight seasonal increase, as the one observed in maple sap, especially for *p*-coumaric and protocatechuic acid which are both consistent in their increment. The levels of phenolic compounds and flavonoids found in maple syrup are somewhat lower than the ones observed in maple sap; this could be a result of the

oxidation resulted from the heat treatment used for the concentration of the maple sap into a maple syrup.

It was demonstrated that clarification and concentration of the kiwifruit juice resulted in some reduction in the concentration of the flavanols and procyanidins due to the use of elevated temperatures (Spanos *et al.*, 1990). It was clearly demonstrated by the chromatogram (Fig. 15) of maple syrup at 100% of the season for the peaks that were tentatively identified as flavonoids (peaks 3, 6, 13, 14 and 15) were smaller than the corresponding peaks observed in the chromatogram (Fig. 9) for maple sap.. In addition, the appearance of two new major peaks at 100% of the season could affect the value for the relative percent since the calculation is based on the total peak area.

Since the production of concentrated maple syrup involves heating, evaporation and storage, any changes in the compositional profile of phenolic compounds could be potentially used as a marker for monitoring any adulteration and assessing the quality of maple syrup; the same way it was used for distinguishing between fresh and concentrated apple juices (Kermasha *et al.*, 1995b).

It is interesting to observe the sudden appearance of protocatechuic acid in maple syrup at 50% of the maple sap season and consequently the simultaneous decrease in vanillin and syringaldehyde (Fig. 19). These findings indicate a correlation between *p*-coumaric acid (HCA family) from maple sap and protocatechuic acid (HBA family) from maple syrup; they follow a similar seasonal trend, both were absent at 0 and 25% of the season and subsequently appeared at 50% of the season to continuously increase up to 100% of the season. The results may suggest that *p*-coumaric acid, present in maple sap, could be a possible precursor for protocatechuic acid in maple syrup. To support such hypothesis, Macheix *et al.* (1990) have demonstrated that HBA (protocatechuic acid) can be produced by the degradation of HCA (*p*-coumaric acid), in a similar manner to β-oxidation of fatty acids. On the other hand, *p*-coumaric acid was still present in maple syrup, supporting the statement that maple syrup contained a flavor reserve, which theoretically could be activated by further oxidation (Belford and Lindsay, 1992). Another pathway for the biosynthesis of benzoic acids, such as protocatechuic acid, is

through the shikimate pathway and especially from dehydroshikimic acid which is derived from sugars (Macheix *et al.*, 1990).

CONCLUSION

Comparative HPLC and GC analyses of selected phenolic and flavonoids standards using a wide range of detectors was found to be successful in providing the information needed for the identification of phenolic compounds and flavonoids in maple sap and maple syrup.

A series of selected phenolic compounds, including vanillin, coniferol, syringaldehyde and *p*-coumaric acid were identified in maple sap and maple syrup extracts of various seasons studied using HPLC with UV-DAD and EC detectors. Additionally, we have found in maple sap and maple syrup the possible presence of protocatechuic acid, (+)-catechin, (-)-epicatechin, flavanols and dihydroflavonols related compounds which is the first time that it has been described in maple product.

Moreover, the use of GC/MS provided a confirmation on the identification of vanillin, syringaldehyde, protocatechuic acid, coniferol and *p*-coumaric acids. Unfortunately, the use of GC/MS for the analysis of flavonoids related compounds did not provide additional identity confirmation for this specific class of compounds. A more extensive study of the different classes of flavonoid aglycones and glycosides as well as a different method for their proper analysis should be undertaken using the GC/MS. Although it is hard too accurately name a specific flavonoid present in maple products, their presence, as secondary metabolites cannot be disregarded.

A slight seasonal increase for most phenolic compounds was seen using both analytical techniques whereas the flavonoids related compound appeared more stable throughout the whole season with a slight decrease at 100% of the maple sap season suggesting the presence of flavonoid glycosides.

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