SEPARATION AND CHARACTERIZATION OF GLYCOSYLATED PHENOLIC COMPOUNDS AND FLAVONOIDS FROM MAPLE PRODUCTS

by

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SHORT TITLE

Glycosylated Phenolic Compounds and Flavonoids From Maple Products

ABSTRACT

M.Sc. Jacinthe Côté

Using a model system of glycosylated and aglycon standards consisting of rutin and quercetin respectively, and a series of pre-packed solid phase extraction cartridges, including C18 Extra-Clean, DSC-18, DPA-6S, Oasis HLB and Amberlite XAD-2, Amberlite XAD-2 was found to be the most appropriate column for the recovery of these compounds. The experimental findings also showed that use of a commercial hesperinidase preparation, resulted in adequate hydrolysis of the glycosylated standard rutin. Based on these findings, the phenolic compounds and flavonoids from maple sap and syrup were separated using the Amberlite XAD-2 column, where the glycosylated fractions eluted with 60% aqueous methanol solution and the aglycon fractions eluted with a methanol:acetonitrile mixture (1:1, v/v). The recovered glycosylated fractions were subjected to enzymatic hydrolysis using the hesperinidase preparation and the liberated phenolic compounds and flavonoids, as well as the sugar components were analyzed by high-performance liquid chromatography (HPLC). HPLC analysis indicated that the phenolic compounds and flavonoids from the glycosylated fractions of maple products eluted between 16.78 and 43.15 min, whereas those from the aglycon fractions eluted between 31.26 and 47.51 min. The results also indicated the presence of rhamnose, fructose, glucose and sucrose in the glycosylated fractions of the maple products; however, as the maple season evolved, the relative predominance of sucrose decreased while that of the other sugars increased. HPLC analysis also indicated that in maple sap, the majority of phenolic compounds and flavonoids, including hydroxycinnamic acid, hydroxybenzoic acid, flavanol and flavone derivatives, were present in the form of aglycons, although some compounds were found glycosylated. A similar trend was found in maple syrup, but fewer glycosylated phenolic compounds and flavonoids appeared to be present. HPLC analysis of the sugar components of the maple products indicated that rhamnose bound to the phenolic compounds and flavonoids was liberated by enzymatic hydrolysis. The overall results indicated that over the 0, 25, 50, 75 and 100% of the maple sap season, maple sap harvested at the later stages contained higher levels of glycosylated phenolic compounds and flavonoids compared to that found at the earlier stages, whereas the opposite trend was observed for maple syrup.

RÉSUMÉ

M.Sc. Jacinthe Côté

En utilisant des produits modèles d'un composé glycosylé, la rutine, et d'un aglycone, la quercetine, ainsi qu'une série de cartouches d'extraction en phase solide, incluant C18 Extract-Clean, DSC-18, DPA-6S, Oasis HLB et Amberlite XAD-2, les résultats ont démontré que l'Amberlite XAD-2 a été la colonne la plus appropriée pour la séparation de ces composés. Les résultats ont également démontré qu'une hydrolyse enzymatique adéquate du composé glycosylé, la rutine, pouvait être obtenue avec une préparation enzymatique commerciale d'hespérinidase. À partir de ces résultats, les composés phénoliques et flavonoïdes extraits de la sève et du sirop d'érable, furent séparés en utilisant une colonne d'Amberlite XAD-2. Les fractions glycosylées étant éluées par une solution aqueuse de 60% de méthanol tandis que les fractions aglycones ont été éluées avec un mélange de méthanol:acétonitrile (1 :1, v/v). Les fractions glycosylées ainsi obtenues ont été soumises à une hydrolyse enzymatique par l'hespérinidase, et les composés phénoliques, flavonoïdes ainsi que les sucres libérés furent analysés par chromatographie liquide à haute performance (HPLC). Les résultats d'analyse HPLC ont démontré que les composés phénoliques et flavonoïdes, présents dans les fractions glycosylées, ont été éluées entre 16,78 et 43,15 min, tandis que ceux des fractions aglycones ont été éluées entre 31,26 et 47,51 min. De plus, les résultats ont démontré que les fractions glycosylées des produits de l'érable contiennent du rhamnose, du fructose, du glucose et du sucrose. Cependant, au cours de l'évolution de la saison d'écoulement de la sève d'érable, les résultats ont montré que la prédominance du sucrose tend à diminuer au profit des autres sucres. L'analyse HPLC des fractions glycosylées hydrolysées des produits de l'érable a permis de déterminer que la majorité des composés phénoliques et flavonoïdes, incluant des dérivés d'acide cinnamique, d'acide benzoïque, de flavanol et de flavone, sont présents sous la forme d'aglycone. De même, de l'hydrolyse enzymatique des composés glycosylés, peu présents dans les produits d'érable, a résulté en la libération du rhamnose. Enfin, une évaluation de la variation des composés phénoliques et flavonoïdes glycosylés, présents dans les produits de l'érable, fut réalisée au cours de la saison d'écoulement de la sève d'érable. Bien qu'il y avait une légère augmentation pour la plupart des composés phénoliques et des flavonoides dans la sève d'érable, il y avait diminution de ces composés dans le sirop d'érable.

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LIST of ABREVIATIONS

RI: Refractive Index

ACER: Centre de Recherche, de Développement et de Transfert Technologique en Acériculture

DAD: Diode-Array Detection

EC: Electrochemical

HBA: Hydroxybenzoic Acid

HCA: Hydroxycinnamic Acid

HPLC: High Performance Liquid Chromatography

LLSD: Laser Evaporative Light Scattering Detection

LOD: Limit of Detection

NP: Normal-Phase

RI: Refractive Index

RP: Reverse-Phase

SPE: Solid-Phase Extraction

TFA: Trifluoroacetic Acid

UV: Ultraviolet

Vis: Visible

1. INTRODUCTION

Phenolic acids and flavonoids are phytochemicals ubiquitous in food of plant origin (Lugasi and Hovari, 2000). Recently, these compounds have attracted a great deal of attention mainly because of their antioxidant role in preventing many diseases, including cardiovascular malfunction, cataracts, various cancers, rheumatism and auto-immune diseases (Kaur and Kapoor, 2001; Rice-Evans, 2001).

Qualitative and quantitative determination of phenolic compounds and flavonoids is problematical due to their immense diverse properties and variations in analytical methodologies. Many studies have investigated the phenolic acid and flavonoid content of fruits and vegetables, but studies on specific products such as maple products are scarce (Philipic and Underwood, 1964; Ali *et al.*, 1992; Potter and Fagerson, 1992; Kermasha *et al.*, 1995; Koelling and Heiligmann, 1996c; Deslauriers, 2000). Different techniques have been used for the determination and quantification of phenolic compounds and flavonoids in fruit and vegetable samples. Although modern separation systems, such as capillary zone and micellar electrokinetic chromatography, have shown good results for the measurement of these compounds in food, the most widely used technique has been high-performance liquid chromatography (HPLC) (Merken and Beecher, 2000a; Rodriguez-Delgado *et al.*, 2000).

For HPLC analysis, the detection systems used is of tremendous importance in the characterization of phenolic compounds and flavonoids (Escarpa and Gonzalez, 2001). The most common means of detection of phenolic acids and flavonoids are undoubtedly ultraviolet-visible (UV/Vis) and electrochemical (EC) detections. Kermasha *et al.* (1995) recovered and characterized certain phenolic compounds in maple products by HPLC with diode array (DAD) and EC detectors and found that maple sap had lower concentrations of phenolic compounds in comparison to maple syrup and concentrate. In maple sap, the major phenolic compound was sinapic acid, while maple concentrate contained sinapic acid, homovanillic acid and coniferal and maple syrup had ferulic acid, syringaldehyde and vanillin.

The majority of studies on phenolic compounds and flavonoids in maple products has been focused on characterizing the total content and are based on the assumption that these compounds are found in their free form as aglycons. However, in nature these compounds also occur in bound form where they are linked to either sugar molecules (mono and disaccharides) or to other phenolic acids or flavonoids. The attachment of sugars to these compounds is an important part of the economy of the plant as the glycosylated forms have greater sap solubility and more mobility than the parent compounds (Harborne, 1964; Shahidi and Naczk, 1995).

A recent study on aromatic compounds in maple products (Deslauriers, 2000) suggested that maple products may contain glycosylated phenolic compounds and flavonoids; these glycosylated conjugates are considered to be potential aroma precursors from which volatiles are released by enzymatic or chemical hydrolysis during the industrial pre-treatment or processing stage of maple sap. The literature also suggests that glycosylated compounds may have greater organoleptic and nutritional potential compared to the free molecules (Gunata *et al.*, 1988; Boulanger *et al.*, 1999; Mateo and Jimenez, 2000). It is therefore of great interest to develop methodologies for the separation and characterization of glycosylated phenolic compounds and flavonoids.

The overall objectives of this research were to recover glycosylated phenolic compounds and flavonoids from maple products for subsequent hydrolysis and characterization.

The specific objectives of this work were:

(1) To develop and optimize methodology for the extraction and recovery of glycosylated phenolic compounds and flavonoids from maple sap and syrup extracts.
(2) To optimize hydrolysis of the glycosylated phenolic compounds and flavonoids from maple sap and maple syrup extracts, using an enzymatic preparation.
(3) To evaluate changes in the profile of glycosylated phenolic compounds and flavonoids in maple sap and syrup during the harvest season.

2. LITERATURE REVIEW

2.1. Phenolic compounds and Flavonoids

2.1.1. Structure and Distribution

2.1.1.1. Phenolic Compounds

2.1.1.1.1. Hydroxybenzoic Acid Derivatives

This class of phenolic compounds includes hydroxybenzoic, protocatechuic, syringic, vanillic, ellagic, gallic and homovanillic acids, vanillin and syringaldehyde. They are the simplest structure within the congregation of polyphenolic compounds, with their 6 carbons structure. Vanillic and gallic acids are the most representative and widely distributed phenolic structures (Macheix and Fleurinet, 1998; Escarpa and Gonzalez, 2001).

The content of hydroxybenzoic acid (HBA) derivatives of food of plant origin is generally low, except for the blackberry, raspberry, black currant, red currant, strawberry, onion and horseradish, whose protocatechuic, ellagic and gallic acids content is very high (Shahidi and Naczk, 1995). Protocatechuic and ellagic acids found in soft fruits are generally glycosylated, being a product of lignin monomer oxidation or alkaline hydrolysis. These phenolic acids contribute to the characteristic flavor of blackberry, raspberry, black currant, red currant, and strawberry (Shahidi and Naczk, 1995; Tomas-Barberan and Clifford, 2000).

Vanillin and syringaldehyde have been detected in wood where they usually occur as methylated derivatives (Shahidi and Naczk, 1995). Some herbs and spices also contain certain HBA. Protocatechuic acid dominates in cinnamon bark, whereas gallic acid dominates in clove buds. Cereals also contain a wide range of HBA: wheat flours contain vanillic and syringic acid, and oats contains vanillic acid, 4-hydroxybenzoic acid and salicylic acid (Tomas-Barberan and Clifford, 2000).

2.1.1.1.2. Hydroxycinnamic Acid Derivatives

Hydroxycinnamic acids (HCA) are the most widely distributed group of phenolic compounds, otherwise known as phenylpropanoids (Shahidi and Naczk, 1995; Escarpa

and Gonzalez, 2001). Among this family of phenolic compounds, four basic structures exist: coumaric, caffeic, ferulic, and sinapic acids (Harborne and Simmonds, 1964; Harborne, 1998; Escarpa and Gonzalez, 2001).

HCA naturally occur with an aromatic ring onto which a three-carbon side-chain is attached (Harborne, 1998). They are rarely found in free form, but instead, associated to other types of compounds (Escarpa and Gonzalez, 2001); they can occur as simple acid, but only after brutal extraction processes, where contamination by micro-organism or technological processing, such as freezing, sterilization and fermentation in wine making, can occur (Macheix *et al,* 1990; Shahidi and Naczk, 1995).

2.1.1.2. Flavonoids

Flavonoids are defined as naturally occurring organic species that posses two aromatic centers, the A and the B rings, and a central oxygenated heterocyclic moiety, the C ring, with one or more hydroxyl groups attached to it (Gee and Johnson, 2001). They are generally classified according to the level of oxidation of their central C ring, but despite the plethora of structures presented in the scientific literature, the number of common, basic structural units remains limited. Flavanol and flavonol-related compounds are by fare the most common structural type of flavonoid (Bloor, 2001), but the occurrence of flavanones and flavone is also frequent (Nijveldt *et al.*, 2001; Rice-Evans, 2001).

The flavonoids polarity is greatly influenced by the presence of a carbonyl group at C_4 position, followed by hydroxyl groups at positions C_2 and C_3, and then by the presence of glycosides. A loss of polar hydroxyl groups and/or the addition of methoxyl groups can reduce the polarity within each class of phenolic compounds and flavonoids (Lee, 2000).

2.1.1.2.1. Catechins (Flavanols)

Flavanols constitute one of the most commonly distributed flavonoid family in nature (Iwashina, 2000; Escarpa and Gonzalez, 2001). They have the typical flavonoids structure, but lack the C_4 carbonyl group (Iwashina, 2000; Escarpa and Gonzalez, 2001).

Within this structural framework, monomer units corresponding to (+)-catechin and (-)-epicatechin structures and oligomer units consisting of dimeric associations of these two compounds (known as procyanins B_1, B_2, B_3, and B_4) can be found (Escarpa and Gonzalez, 2001).

Flavanols are generally distributed in the plant kingdom as aglycons, but can also occur as condensed tannin polymers in fruits, legumes, and grains (King and Young, 1999; Escarpa and Gonzalez, 2001; Nijveldt *et al.*, 2001). Catechin and epicatechin often combine with gallic acid to form compounds such as (-)-epigallocatechin, (-)-epicatechin-3-gallate and (-)-epigallocatechin-3-gallate, are found in green tea, black tea and red wine.

2.1.1.2.2. Flavanones

The flavanones constitute a minority group within the flavonoid family (Escarpa and Gonzalez, 2001). Flavanones correspond to flavones whose bond between carbons 2 and 3 is saturated, hence the name dihydroflavones which is sometimes used. They also have a hydroxyl group in the 3-position and can also be referred to as dihydroflavonols (Bohm, 1998). The majority of naturally occurring flavanones have an α-configuration with the C_2 phenyl group oriented beneath the plane of the paper (Bohm, 1998).

The most frequent type of flavanone aglycons are hesperetin, naringenin and eiodictyol which are found in citrus foods (Bohm, 1998; Escarpa and Gonzalez, 2001). Common glycosylated flavanones include hesperidin, naringin, limonin, narirutin, neoshesperidoside and nomilin (Iwashina, 2000; Escarpa and Gonzalez, 2001).

2.1.1.2.3. Flavones and Flavonols

Flavones and flavonols only differ from flavanones by having a double bond between carbons C_2 and C_3. They usually have no substituent at the carbon C_3, but are characterized by a planar structure because of the double bond present in the central aromatic ring (Bohm, 1998; Iwashina, 2000; Nijveldt *et al.*, 2001). Flavonols can be differentiated from flavones by the presence of a hydroxyl group at C_3 position (Hertog *et al.*, 1992).

Flavones are the least representative flavonoid group in food. They are most often encountered bound to sugar molecules to form glycosylated compounds like apigetrin, apiin, isorhoifolin, rhoifolin and diosmin, but the most widely distributed forms of flavone aglycon are apigenin, luteolin and tangeretin. Flavonols, on the contrary, are vastly distributed in the plant kingdom and are an integral part of our diet, with quercetin, kaempferol, myricetin and isorhamnetin being the most common distributed members. In many instances, flavonols are also found glycosylated and form rutin and quercitrin, and less frequently, quercimeritrin (Bohm, 1998; King and Young, 1999; Escarpa and Gonzalez, 2001).

Flavonols are found in abundance in onions, apples, broccoli, berries and tea, whereas Celery is known to contain high concentrations of flavones luteolin and apigenin (King and Young, 1999; Nijveldt *et al.*, 2001).

2.1.2. Biosynthesis

2.1.2.1. Phenolic Compounds

HBA derivatives are biosynthesized via shikimic acid and form an obligate intermediate for the synthesis of HCA and flavonoids (Escarpa and Gonzalez, 2001).

HCA are the product of an enzymatic degradation of the 4 chiral centers of shikimic acid, but they can also be synthesized via the acetate pathway, whereby an irreversible enzymatic reactions with aromatic protein amino acid *L*- phenylalanine and *L*-tyrosine generates their $C_6 - C_3$ structure (Harborne, 1998). Once synthesized, the HCA derivatives are usually incorporated into lignin molecules, combined as ester group (Harborne and Simmonds, 1964), but they are also widely present in nature as glucose esters, glycosylated or linked to flavonoids (Harborne, 1998).

2.1.2.2. Flavonoids

Flavonoids derive their carbon skeleton from two carbohydrate derivatives: malonyl CoA and 4-coumaroyl-CoA, which are formed by the shikimic acid pathway. The central step to flavonoid biosynthesis consists of the interactions of 3 molecules of malonyl CoA with HCA-CoA ester, producing a 15 carbons chalcone: this is the primary

flavonoid biosynthetic intermediate. Under the stereospecific action of the chalcone isomerase, flavanone compounds are obtained and from this compound, isoflavonones, flavones and dihydroflavonol can be derived. Dihydroflavonol is another biosynthetic intermediate, which interactions with specific enzymes produce flavonols catechins, proanthocyanidins and anthocyanidins (Harborne and Williams, 1988; Merken and Beecher, 2000a).

All these compounds can be further modified by hydroxylation of the A- and, in particular of the B-ring, by methylation of hydroxyl groups as well as by glycosylation and acylation reactions, which create an immense diversity of existing flavonoids (Harborne and Williams, 1988; Merken and Beecher, 2000a).

2.1.3. Glycosylation

Formally, dietary phenolic compounds and flavonoids were thought to be poorly absorbed by the intestine because they presented as conjugates of sugars and glycosylation made through a β-glycosidic are very stable and can resist pancreatic enzymes hydrolysis (Kühnau, 1976; Hollman *et al.*, 1999; Zechel and Withers, 2000). For this reason, most studies on polyphenolic compounds in past have focused on the characterization and quantification of the aglycon form in food products. But recently, studies have shown that glycosylated polyphenolic compounds appear not only to be better absorbed than the aglycons (Hollman *et al.*, 1997), but also to form more stable aroma precursors (Mayorga *et al.*, 2001).

2.1.3.1. Phenolic Compounds

In general, phenolic compounds nearly always occur in combined forms, as they are aroma precursors that can be released during the maturation, pre-treatment or processing of plants (Amiot *et al.*, 1997).

Ferulic, *p*-coumaric, caffeic and sinapic acids are often found combined with sugars by means of glycosidic linkage (Harborne, 1964) and common glycosylated HCA include esters *p*-coumaroylglucose and caffeoylglucose (Shahidi and Naczk, 1995).

Moreover, the existence of the glycosides of 4-hydroxybenzoic, protocatechuic, vanillic and syringic acids was also recognized recently (Tomas-Barberan and Clifford, 2000).

2.1.3.2. Flavonoids

Flavonoid compounds can range from free (aglycon) to conjugated structures. Quite frequently, the conjugation implies the participation of a variety of natural sugars, chemically bound by virtue of their molecular size to one or several flavonoid functional groups forming a glycosylated compound (Escarpa and Gonzalez, 2001). Glycosylation of these compounds appears to be an important part of the plants economy if only inasmuch as the glycosylated compounds have greater sap solubility and more mobility than the parent flavonoids (Harborne, 1964). Most flavonoids are found glycosylated in their natural dietary form, with the exception being of the flavanols (catechins), which are usually found, in a free form (Rice-Evans, 2001).

Flavonoids can be found bound to one or more sugars; the glycoside usually consist of glucose, galactose, rhamnose, arabinose, xylose, rutinose, glucoronic acid and galacturonic acid, and disaccharide such as sophorose, rutinose, and sambubiose (Shahidi and Naczk, 1995).

The stereochemistry of the glycosidic linkages is an important characteristic of glycosylated flavonoid. The linkage can either be *O*-glycosyl or *C*-glycosyl but most glycosylated flavonoids usually occur as *O*-glycosyl. The *C*-glycosylflavonoids are characterized by having one or two sugar units directly linked to the aromatic nucleus through carbon-carbon bonds (Bohm, 1998), whereas the *O*-Glycosylation can occur at any hydroxyl groups although certain ones, such as 3-*O*-glycosyl and 7-*O*-glycosyl seem favored (Stafford, 1990; Bohm, 1998).

Numerous glycosylated flavones and flavonols known to occur in the plant kingdom are naturally acylated derivatives, involving linkages between aliphatic and aromatic acids (hydroxybenzoic, and gallic, *p*-coumaric, caffeic, ferulic, and sinapic acids) and the sugar hydroxyls (Stafford, 1990; Bohm, 1998). Common position for the sugar substitution in glycosylated flavones is the 7-hydroxyl, although 3-hydroxyl has also been reported (Harborne and Williams, 1988).

For flavanols, the majority of them accumulate as aglycons since, in contrast to other flavonoids; they are soluble enough to be maintained in the vacuole without needing glycosylation. However, *O*- and *C*-glycosyl with sugars and other *C*- substitutions do occur (Stafford, 1990).

2.1.4. Role in Nature

2.1.4.1. Phenolic Compounds

Phenolic compounds are commonly associated with nutritional and organoleptic (sensory) qualities of foods derived of plant origin. Certain ones, such as ellagic acid, are responsible for the astringency and bitterness of foods (Shahidi and Naczk, 1995).

Plants use HBA derivatives for seeds germination and to support growth and HCA derivatives as a defense mechanism for their antifeedant, antipathogen, antibiotic, growth inhibition, antioxidant and germination stimulant properties (Escarpa and Gonzalez, 2001).

When present in low concentrations, humans can also benefit from the consumption of these phenolic compounds to protect themselves against free radical oxidation, but at high concentrations, these compounds cause undesirable discoloration and interact with the foods carbohydrate or protein components, making the food less appealing. The discoloration, also called enzymatic browning, results from an initial oxidation of phenols to quinones which is followed by the formation of undesirable colored pigments (Shahidi and Naczk, 1995; Lee, 2000).

2.1.4.2. Flavonoids

Flavonoids have diverse biological properties. They are involved in the plants antioxidant, anti-inflammatory, antiallergic, and anticarcinogenic activities, and acts as markers in taxonomic studies (Escarpa and Gonzalez, 2001). They participate in the plant metabolism, being responsible for their growth and exhibiting determined interactions with other live organisms. Among other functions, they safeguard plant against infections and aggression by other microorganisms, they can serve as screens against UV radiation, act as growth hormone carriers, hence supporting pollen tubes growth and pollen

germination. Finally, by contributing to the plants wide variety of colors, flavonoids indirectly contribute to the fecundity by helping to attract potential pollinators (Bohm, 1998).

Dietary flavonoids, mainly occurring in fruit and vegetables, also have a strong effect on the mammalian biology (Kren and Martinkova, 2001). *In vitro* experimental systems show that flavonoids possess antioxidant and free radical scavenging activities, metal chelation, antiproliferative, anticarcinogenic, antibacterial, anti-inflammatory, antiallergenic, and antiviral properties (Lee, 2000; Merken and Beecher, 2000b; Nijveldt *et al.*, 2001). Moreover, some flavonoid compounds can mimic animal hormones such as estrogens (Bohm, 1998). All these properties can help prevent many chronic illnesses, such as cardiovascular diseases, cancers, rheumatism, etc. Finally, flavonoids also contribute to the immense variety of flavors and colors of foods of plant origin, essential organoleptic qualities for consumers.

2.2. Phenolic Compounds and Flavonoids in Maple Products

2.2.1. Maple Products

Maple products are produced with the sweet sap collected from North America maple trees: the hard or rock maples (*Acer saccharum*), the soft, white or silver maple (*Acer saccharinum*), the red maple (*Acer rubrum*), and the black maple (*Acer nigrum*). Because the hard maple can produce more sap, 75% of commercial maple syrup and sugar comes from sap extracted from that maple tree (USDA, 1982). 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 (Dumont *et al.*, 1993).

2.2.2. Chemical Composition

The sap produced by maple trees which is used for the production of maple syrup is a sterile liquid that provides the maple tree with water and nutrients prior to buds opening and leaf appearance (Koelling and Heiligmann, 1996a).

Maple sap mainly consists of a diluted solution of sucrose, which appears in xylem water of maple tree from late falls through early spring. The average total sugar

content of sap is 3%, but can vary between 1 to 12% (USDA, 1982). Sucrose is the most prevalent sugar in maple sap; it comprises 98-99% of the dry matter (Koelling and Heiligmann, 1996c).

Maple sap also contains a small percentage (2% or less) of amino acids (aspartic acid, asparagine, glutamine, ammonia, threonine, serine, glutamic acid, alanine, proline, carline, valise, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine and urea), organic acids (oxalic, succinic, fumaric, 1-malic, tartaric, *cis*-aconitic and citric acids), phenolic compounds, hormones, minerals (calcium, potassium, sodium, magnesium and manganese), salts, and other components originating from the roots. These dissolved compounds are responsible for helping initiate growth within the tree (USDA, 1982; Ali *et al.*, 1992, Kermasha *et al.*, 1995; Koelling and Heiligmann, 1996c).

The profile of phenolic compounds in maple sap may vary according to the period in the maple sap season and to gradation of tree health (Koelling and Heiligmann, 1996a). High-performance liquid chromatographic (HPLC) analyses of maple sap extracts have revealed the presence of many lignin derivatives: vanillic, syringic, homovanillic, *p*-coumaric, sinapic, and ferulic acids, coniferol, vanillin, coumarin, syringaldehyde, coniferaldehyde, and 2,6-dimethoxylquinone (Philipic *et al.*, 1964; Belford *et al.*, 1992; Potter *et al.*, 1992; Kermasha *et al.*, 1995). Recently, the presence of flavanols (+)-catechin, (-)-epicatechin, procyanidins and dihydroflavonols related compounds in maple sap extracts was reported (Deslauriers, 2000). The majority of these compounds appear to be heat resistant as their presence was also reported in maple syrup (Philipic *et al.*, 1964; Belford *et al.*, 1992; Potter *et al.*, 1992; Kermasha *et al.*, 1995; Deslauriers, 2000).

The chemical development of maple flavors during the sap boiling process is not clearly understood. However, research on sap biochemistry points to an involvement of the amino acids (Koelling and Heiligmann, 1996c). The typical "caramel" flavor of maple syrup could be attributed to the browning reaction of the invert sugars and contributes to the common "dark amber" color of syrup (USDA, 1982). Hydroxymethylfurfural (HMF), a compound formed by the Maillard reaction between the sugars and the amino acids

and/or the acid-catalyzed sugar degradation reaction, also contributes to the dark brown color of this product (Kermasha *et al.*, 1995).

2.2.3. Maple Sap Collection

The sap collected from maple trees is usually thin, barely sweet, and colorless. It is collected at anytime, from the first spring thaw until the leaf buds burst. Therefore, the period of maple syrup production is direct proportion to the length of time between the first thaw and the swelling of the leaf buds: the longer the time, the more sap is collected and consequently the more syrup is produced (USDA, 1982; Koelling and Heiligmann, 1996b).

As the sap season advances toward maximum flow, either in March or April, the sweetness of the sap increases. Undesirable fermentation occurs as the snow melts, nights get warmer, and days get hotter; micro-organisms develop in the sap spouts and buckets, and the slow dripping sap begins to ferment almost as soon as it leaves the tree. Certain changes in the tree's metabolism, at the turn of warm weather, may release some compounds seemingly necessary for the buds, but which are not conducive to finely flavored syrup production (USDA, 1982; Koelling and Heiligmann, 1996b). The best syrup is made when the sap is collected while the ground is still covered with snow or when the day and night temperatures are low enough to prevent sap fermentation.

Sap collection was traditionally, and is still widely carried out with the use of buckets. A more recent and innovative sap collection system, the plastic pipeline, is gradually replacing this tradition. This system has the advantage of making higher yields during sugaring sap season by decreasing infections in tap holes caused by airborne microorganisms.

2.2.4. Maple Syrup Production

The process of converting maple sap to syrup is essentially by boiling the sap at 93-110° C for a period of time, to allow the water to evaporate, and thereafter a concentrated sap sugar is obtained. During this heating process, chemical changes occur and sugars, amino acids, organic acids, phenolic compounds, minerals and salts interact

together to create the unique color and flavor to maple syrup (Ali *et al.*, 1992; Willits and Hills, 1996). Maple syrup should contain not less than 66% by weight of soluble solids and derive solely from maple tree sap; these requirements are universal in United States and Canada (USDA, 1982; Koelling and Heiligmann, 1996b).

The various degrees of maple syrup quality are based on its color. Color standards for syrup were first developed in 1910 and have ever since evolved. Today, several color grading systems exist for pure maple syrup: extra light, light, medium, amber and dark amber which have degree of transmittance varying between 27 to 75%.

2.3. Extraction and Analysis of Phenolic Compounds and Flavonoids

2.3.1. Extraction of Phenolic Compounds and Flavonoids

Isolation of phenolic compounds and flavonoids from a sample matrix is generally a prerequisite to any comprehensive analysis scheme. Extraction methods can vary from exhaustive extraction and pre-concentration procedures to simple liquid-liquid extraction or filtration (Antolovich *et al.*, 2000).

The fidelity between the phenolic compound and flavonoid profile of the starting material and that of the isolated extract provides the theoretical basis for judging analytical techniques. Hence extraction conditions employed should be as mild as possible to avoid oxidation, thermal degradation and other chemical and biochemical changes in the sample (Antolovich *et al.*, 2000).

The choice of an extraction method for phenolic compounds and flavonoids depends on their solubility properties. This property is influenced to a large degree by whether they occur as glycosylated, which renders them highly polar, or whether they occur in the free form, in which case they are much less polar (Bohm, 1998). Glycosylated phenolic compounds and flavonoids are more soluble in water and the aglycons more soluble in methanol. Extraction efficiency will thus depend on the water/methanol ratio (Hertog *et al.*, 1992). If one is dealing with vacuole components of the plant then the phenolic compounds and flavonoids will most likely exist in a glycosylated form and a very polar solvent will be required for efficient extraction. In this

case, a solvent composed of different percentage of aqueous acetone, ethanol or methanol is required (Antolovich *et al.*, 2000).

A common extraction method for phenolic compounds and flavonoids is a liquid-liquid extraction done with 50% methanol and variable concentrations of HCl (0.6 or 1.2 M) (Hertog *et al.*, 1992; Hakkinen *et al.*, 1998 and 1999). Other extraction procedures were reported using methanol with 1% 2,6-di-*tert*-butyl-4-methylphenol for phenolic compounds and flavonoids extraction from apples and pears (Escarpa and Gonzalez, 1998 and 1999).

Kermasha *et al.* (1995) investigated different extraction methods for the characterization of phenolic and furfural compounds in maple products. The best recovery was obtained with ethyl acetate, with a mean percentage recovery for all phenolic and furfural compounds of 87.6%. The mean percentage recovery with the other methods of extraction was 82.2% with Sep-Pak, 62.9% with lyophilization, 44.3% with diethyl ether and 41.8% with SupelClean.

2.3.2. Separation of Phenolic Compounds and Flavonoids

The isolation of glycosylated phenolic compounds and flavonoids from different food products by selective retention of the compounds on a solid-phase adsorbent is a commonly used technique (Mateo and Jimenez, 2000). By washing the adsorbent with aqueous organic solvent following the adsorption step, polar glycosylated constituents can be eluted while the less polar aglycons are retained.

A method with glass column chromatography containing C_{18} reversed-phase adsorbent was developed. After washing with water and eluting glycosylated compounds with 20% aqueous acetic acid, the free ones were eluted in two fractions with 30% aqueous acetic acid and methanol. Using 1g solid-phase extraction (SPE) cartridges ameliorated the methodology; hydrophilic compounds were eluted with water, free compounds with dichloromethane and glycosylated ones with methanol. This method has been improved few years later, but it has a disadvantage because separation is different depending on the commercial origin of the cartridges (Mateo and Jimenez, 2000).

Montpellier researchers who were trying to recover free terpenols from grape juice (Guanta et al., 1985, 1988 and 1990), proposed a second approach to the problem using Amberlite XAD-2 resin.

Amberlite XAD-2 resin consists of a macroreticular, styrene-divinylbenzene copolymer made into nonionic beads that displays extraction capacities similar to those of coated octadodecyl silica. It has the advantage being sold in large particle sizes; it is thus possible to use it in a wide variety of preparative column at atmospheric pressure. It also allows use of sample without prior extraction method because it can eliminate numerous interfering substances such as sugars and acids by simple washing with water without any loss compounds of interests, which are fixed entirely onto the column (Gunata *et al.*, 1985; Mateo and Jimenez, 2000).

When in contact with the Amberlite XAD-2 resin, the hydrophobic portion of the adsorbate molecule (aglycon compounds) are preferentially adsorbed on the hydrophobic polystyrene surface. The hydrophilic section of the adsorbate (glycosylated compounds) remains oriented in the aqueous phase. Compounds being adsorbed onto this resin ordinarily do not penetrate substantially into the microsphere phase, but remain adsorbed at the surface.

Amberlite XAD-2 resin was previously used to isolate naringin and limonin from grapes juices. It was then used extensively to recover glycosylated monoterpene from grape, grape juice and wine (Guanata *et al.*, 1985 and 1988; Voirin *et al.*, 1992a and 1992b; Mateo and Jimenez, 2000). For the elution of free terpenoids, pentane was used, and after the glycosylated terpenoids were eluted with ethyl acetate. Later, modifications to this method were suggested and free compounds were eluted with pentane:dichloromethane (2:1, v/v) to improve extraction.

This technique is still frequently employed for the separation of bound and free flavonoids in passion and Amazonian fruits, grape juice and wines (Chassagne *et al.*, 1996; Boulanger *et al.*, 1999; Boulanger and Crouzet, 2000; Mateo and Jimenez, 2000; Morales *et al.*, 2000). Amberlite XAD-2 resin was also used for the separation of glycosylated monoterpene from green and black tea (Morita *et al.* 1994; Nishikitani *et al.*,

1996) and ginger (Sekiwa *et al.*, 1999). Glycosylated alcoholic aroma precursors from apples, African mango and Japanese pepper were also separated with this resin (Roberts, *et al.*, 1994; Sakho *et al.*, 1997; Jiang *et al.*, 2001) and the extraction of phenolic acids and flavonoids compounds from apples and pears (Andrade *et al.*, 1998), quince jam and puree (Silva *et al.*, 2000) and from honey (Ferreres *et al.*, 1994) was also performed using this resin has (see Table 1).

2.3.3. Hydrolysis of Glycosylated Phenolic Compounds and Flavonoids

Hydrolysis is often performed in order to characterize the nature of glycosylated phenolic compounds and flavonoids, their sugar moiety and the stereochemistry of the glycosidic linkages.

2.3.3.1. Acid Hydrolysis

Phenolic and flavonoid aglycons can be relieved from of their glycosidic fractions by chemical hydrolysis. To differentiate between *O*- and *C*-glycosyl phenolic compounds, an acid hydrolysis performed after the extraction, is very convenient. In presence of an acid, the sugar moiety from the *O*-glycosyl is removed, while the *C*-glycosyl molecules will remain unchanged. The chromatographic analysis before and after an acid hydrolysis can allow the identification of *O*-glycosyl; if there is no change in the chromatographic behavior, the original compound may be considered to contain only *C*-glycosyl or an *O*-glucoronide. The rate of hydrolysis will vary according to both, the nature of the sugar and the position of attachment of the sugar to the compounds skeleton (Bohm, 1998).

Although the use of chemical hydrolyses is very convenient, there are some attendant problems. First of all, molecular rearrangement reactions can occur under acid conditions. This may cause a false reflection of the true phenolic or flavonoid synthetic potential of the plant (Amiot *et al.*, 1997; Bohm, 1998). Moreover, partial hydrolysis may occur, giving misleading results. Finally, prolonged heating in the presence of strong acid may result in serious degradations of certain phenolic or flavonoid compounds, such as anthocyanins and catechins (Bohm, 1998, Hakkinen *et al.*, 1999; Merken and Beecher, 2000a).

Table 1. Separation of aromatic compounds using Amberlite XAD-2 resin.

Food	Compounds of Interest	Eluents[a]	Order of Elution[b]	References
Grape, grape juice, wine	terpenoids	A: pentane G: ethyl acetate	A→G	Gunata *et al.* (1985,1988)
Grape, wine	terpenoids	A: pentane:dichloro[c] (2:1) G: ethyl acetate	A→G	Voirin *et al.* (1992a,b)
Apple juice	damascenone	A: pentane G: ethyl acetate →metOH	A→G	Roberts *et al.* (1994)
Honey	flavonoids	A: metOH G: water	G→A	Ferreres *et al.* (1994)
Green and black tea	monoterpenic alcohols	A: pentane G: metOH	A→G	Morita *et al.* (1994)
Passion fruit, muscat wine	monoterpenic, aromatic, aliphatic alcohols and phenols	A: pentane:dichloro[c] (2:1) G: metOH	A→G	Chassagne *et al.* (1995)
Green tea	monoterpene alcohols	A: pentane G: metOH	A→G	Nishikitani *et al.* (1996)
Passion fruit	hydrogen cyanide	A: pentane:dichloro[c] (2:1) G: metOH	A→G	Chassagne *et al.* (1996)
Mango pulp	aromatic compounds	A: pentane G: metOH	A→G	Sakho *et al.* (1997)
Quince, apple and pear puree	phenolic compounds	A: metOH G: water	G→A	Andrade *et al.* (1998)
Amazonian fruit	aromatic compounds	A: pentane:dichloro[c] (2:1) G: metOH	A→G	Boulanger *et al.* (1999,2000)
Ginger	aromatic alcohols	A: pentane:ether (4:6) G: ethyl acetate →metOH	A→G	Sekiwa *et al.* (1999)
Quince jam	phenolic compounds	A: metOH G: water	G→A	Silva *et al.* (2000)
Japanese pepper leaves	alcoholic aromatic compounds	A: pentane:ether (2:3) G: ethyl acetate →metOH	A→G	Jiang *et al.* (2001)

[a] Solvents chosen for the elution of aglycone (A) and glycosylated (G) compounds.
[b]Order of elution: eluting aglycone before glycosylated compounds (A→G) or eluting glycosylated before aglycone compounds (G→A).
[c]Dichloromethane

Results reported by Hertog *et al.* (1992) illustrated well these problems. In this study, it was shown that sub-optimal acid hydrolysis conditions lead to an underestimation of up to 50% of the true levels of phenolic compounds and flavonoids in food. Also, in samples with glycosylated flavonoids, an optimum between flavonol yield and degradation was observed as prolonged extraction and hydrolysis showed a decrease in the measured flavonol content. Despite these significant disadvantages, phenolic extraction combined with an acid hydrolysis is still the most reported and commonly used method in the literature (Harborne, 1998).

2.3.3.2. Enzymatic Hydrolysis

Enzymes may be used instead of an acid, for the catalysis of the phenolic compounds and flavonoids hydrolytic reaction. Enzymes are generally highly specific, active at very low concentrations, and the undesirable side reactions associated with chemical hydrolysis is significantly minimized because of the mild conditions of pH and temperatures (Abdelrahim, 1990). However, the drawback of enzymatic hydrolysis is that other hydroxyl containing substrates present in the phenolic and flavonoid extract (such as free phenolic compounds) can react with the newly released aglycon to form new phenolic compounds (Abdelrahim, 1990).

Enzymatic hydrolysis of glycosylated phenolic compounds and flavonoids appears to occur in two sequential steps. First, regardless of the structure of the aglycon moiety, the enzyme cleaves the inter-sugar linkage and a corresponding β-D-glycoside is released. Liberation of the aglycon moiety takes place during the second step, when the enzyme acts on the previous phenolic β-D-glycosides (Reyne *et al.*, 1992; Mateo and Di Stefano, 1997). Hence, it is necessary to know the structure of glycosydically bound components and the kinetic characteristics of the enzyme to maximize the liberation of phenolic or flavonoid aglycons from their glycosylated forms.

The rate and product formed during the hydrolysis varies considerably, depending on the source and purity of the enzyme. Enzymes can be isolated and purified from fungal preparations or vegetal extracts of synthetic culture media inoculated with fungal cultures; unpurified enzymatic preparations are also available. Use of isolated β-

glucosidase is common, although this enzyme is not substrate specific. It can hydrolyze most β-D-glycosylated compounds and it is recognized to remove monosaccharide faster than disaccharide (Harborne, 1964). β-Glucosidase usually hydrolyzes glycosidic bonds of aryl and alkyl β-D-glycosides as well as glycosides of single carbohydrate residue (Zheng and Shetty, 2000). Unpurified enzymatic preparations such as Pectinol C, Rohapect C, pectinase *Aspergillus niger,* sweet almond glucosidase and hemocellulase REG-2 appear best suited for the hydrolysis of glycosylated phenolic compounds (Gunata *et al.*, 1985 and 1988; Reyne *et al.*, 1992; Boulanger and Crouzet, 2000). The success of these enzymatic preparations may be attributed to the impurity of the crude enzyme solutions which contains other enzymes such as esterase α-L-arabinofuranosidase, α-L-rhamnosidase or β-D-apiosidase, in plus of the expected β-D-glucosidase (Zheng and Shetty, 1999; Mateo and Jimenez, 2000).

In general, the rate of hydrolysis of glycosylated compounds appears to be influenced by the polarity the phenolic compound and flavonoids aglycon: it increases with the number of axial hydroxyl groups (Namchuck *et al.*, 2000). This phenomenon may be a consequence of a deoxygenation reaction, as the replacement of an electron-withdrawing hydroxyl group with hydrogen stabilizes the electron-deficient transition state. In the studies carried out to date, the relative rates of hydrolysis of monodeoxygenated glycosylated compounds is 2-deoxy > 4-deoxy > 3-deoxy > 6-deoxy > parent (Namchuck *et al.*, 2000).

2.3.4. HPLC Analysis of Phenolic Compounds, Flavonoids and Their Sugar Components

Although many modern separation systems may be used for the measurement of phenolic compounds and flavonoids in foods (gas chromatography, capillary zone electrophoresis and micellar electrokinetic capillary chromatography), by far, the most widely employed technique has been HPLC (Merken and Beecher, 2000a; Rodriguez-Delgado *et al.*, 2000).

RP HPLC coupled with DAD constitutes a crucial and utterly reliable technique for the analysis of phenolic compounds and flavonoids (Escarpa and Gonzalez, 2000a).

Indeed, the distinctive UV/Vis spectra of these compounds and the wide spread availability of HPLC systems with multiple wavelength capacity, now makes HPLC the method of choice for phenolic compounds and flavonoid analysis (Bohm, 1998; Bloor, 2001).

2.3.4.1. HPLC Analysis of Phenolic Compounds and Flavonoids

2.3.4.1.1. Reverse-Phase Chromatography

RP HPLC is the most popular and suited mode of analytical liquid chromatography for the analysis of polar and ionogenic analytes such as phenolic compounds and flavonoids (Lee, 2000; Merken and Beecher, 2000a; Escarpa and Gonzalez, 2001). The separation of these compounds is usually done on a silica-based C_{18} bound-phase column, the nature of the silica being one of the largest variables of the stationary phase (Lee, 2000; Escarpa and Gonzalez, 2001). Dimensions of the column usually varies between 100 and 300 mm in length and the internal diameter is usually 4.6 mm (Merken and Beecher, 2000a; Escarpa and Gonzalez, 2001).

The mobile phase usually consists of a mixture of water (the diluent) and an organic modifier such as methanol, acetonitrile or tetrahydrofurane (Lee, 2000). Compared to acetonitrile, higher percentage of methanol can be used without disrupting the column packing because of its non-toxic nature (Dondi *et al.*, 1988 and 1989; Pietrogrande and Kahie, 1994). Despite this apparent advantage, use of a water-acetonitrile gradient is still more common, because it reduces the elution time, sharpens peaks shape of late-eluting compounds and provides a more precise quantification at low concentrations (Lee, 2000; Merken and Beecher, 2000a; Escarpa and Gonzalez, 2001).

The majority of phenolic compounds and flavonoids are ionizable and this tends to reduce their adsorption onto the stationary phase. To circumvent this problem, the chromatographic separation of phenolic compounds and flavonoids is often performed using a low pH mobile phase (pH 2 – 4). The addition of small amount of acid, such as acetic (2-5%), phosphoric, formic or trifluoroacetic acids (TFA) (0.1%), suppresses ionization and hence improve the resolution and reproducibility of successive analysis

(Dalluge *et al.*, 1998; Merken and Beecher, 2000a; Bloor, 2001; Escarpa and Gonzalez, 2001).

The choice of elution modality usually depends on the type phenolic compounds and flavonoids present in the sample to be analyzed. Isocratic elution can be used for the study of samples whose compounds are of the same family group or structure; its use has been reported for the determination of flavonols, methoxylated flavones and flavonoids in apples, vegetables, orange an fruit juices, Brazilian beverages, and edible tropical plants (Amakura *et al.*, 2000a and b; Awad *et al.*, 2000; Careri *et al.*, 2000; Lugasi and Hovari, 2000; Escarpa and Gonzalez, 2001; Koo Hui and Suhaila, 2001; Leite *et al.*, 2001;). Yet, gradient elution system is used in the majority of studies: it starts with a predominantly aqueous phase and an increasing proportion of organic solvent (methanol or acetonitrile) is gradually added to the solvent system (Lee, 2000; Merken and Beecher, 2000a; Bloor, 2001). Binary gradient elution systems have also shown good resolution for the separation of phenolic compounds and flavonoids in cranberry juice, apples, pears, raisins, litchi fruit, and green beans (Suarez *et al.*, 1996; Escarpa and Gonzalez, 1998, 1999, 2000a and 2001; Karadeniz *et al.*, 2000; Lee, 2000; Merken and Beecher, 2000a; Sarni-Manchado *et al.*, 2000; Alonso-Salces *et al.*, 2001; Chen *et al.*, 2001; Schieber *et al.*, 2001; Zafrilla *et al.*, 2001). Tertiary solvent system has been used for the analysis of phenolic compounds and flavonoids in berries, tea, parsley, orange and pomegranate juice (Hakkinen *et al*, 1999; Gil *et al.*, 2000; Merken and Beecher, 2000b).

HPLC runs generally last one hour maximum, including the equilibration phase between runs. Flow rates used are usually 1.0 or 1.5 mL/min. Thermostatically controlled columns are normally kept at ambient or slightly above ambient temperatures. Injection volumes generally range from 1 to 100 μL (Merken and Beecher, 2000a).

2.3.4.1.2. Diode-Array Detection

The DAD allows the collection of data for an entire spectrum (190 to 600 nm) in approximately one second, which provides the unique scan for specific compounds and their maximum wavelength (Siouffi, 2000). Phenolic compounds and flavonoids have distinctive UV or UV/Vis spectra where minor differences in structure are often seen as

significant differences in their UV spectra. This enables rapid diagnosis of certain structural features of each eluted band (Escarpa and Gonzalez, 2000b; Bloor, 2001; Escarpa and Gonzalez, 2001).

Most HBA display their maxima at 246-262 nm, with a shoulder at 290-315 nm, except gallic and syringic acids, which have absorbance maxima at 271 and 275 nm, respectively. HCA absorb in two regions in the UV, one maximum occurring in the range 225-235 nm and the other between 290 and 330 nm. The four commonly occurring HCA derivatives - ferulic sinapic, caffeic and *p*-coumaric acids- have absorbance maxima at 300 nm (Ibrahim and Barron, 1989; Van Sumere 1989; Lee, 2000).

Flavonoids typically exhibit two major absorption bands in the UV region. Flavonoids band I absorption occurs in the 320-380 nm range and presumably arises from the B-ring. Band II, which occurs in the 240-285 nm range, is believed to arise from the A-ring. Addition of substituents to an aromatic ring capable of electron donation, such as hydroxyl and methoxyl groups, usually induce a strong (10-15 nm) bathochromic shifts for the absorption band associated with that ring (Lee, 2000; Merken and Beecher, 2000a; Escarpa and Gonzalez, 2001).

Because there is little or no conjugation between the A- and B-rings, the UV spectra of flavanones usually have intense band II peak and a small band I peak. The flavanol catechins, which also lack the conjugation, have a small band I peak. In flavones, band I is most intense, occurring at 320-350 nm and their absorption spectra may have subsidiary peak in the form of a shoulder at 300-310 nm, which may be useful for their identification. Like flavones, the flavonols are known to have their UV spectra composed of a band II peak around 240-280 nm and a band I peak around 300-380 nm (Lee, 2000; Merken and Beecher, 2000a; Bloor, 2001; Escarpa and Gonzalez, 2001). *O*-Methylation, *O*-glycosylation and acylation also affect the band positions in that all three tend to produce shifts to lower wavelengths (hypsochomic). *C*-Glycosylation, however, has a negligible effect on the spectrum (Bloor, 2001).

Typical absorbance wavelength for the analysis of catechins (flavanols) is 280 nm; flavanones and their glycosylated counterparts are generally detected at 280 nm; and

glycosylated flavones and flavonols are usually detected at 270 and 370 nm wavelength such as (Merken and Beecher, 2000a; Escarpa and Gonzalez, 2001) although their maximal absorbance is at 365-370 nm (Bloor, 2001).

Identification and quantification using HPLC-DAD has provided reliable results for phenolic compounds and flavonoids extracted from berries, apples, pears, orange and cranberry juices, litchi fruit, pomegranate, grapes, onion, leek, sweet pepper, tomato, cucumber, green beans, lentils and Malaysian edible plants (Suarez *et al.*, 1996; Escarpa and Gonzalez, 1998, 1999; Hakkinen *et al.*, 1998 and 1999; Careri *et al.*, 2000; Escarpa and Gonzalez, 2000a; Gil *et al.*, 2000; Karadeniz *et al.*, 2000; Lugasi and Hovari, 2000; Sarni-Manchado *et al.*, 2000; Alonso-Salces *et al.*, 2001; Chen *et al.*, 2001; Koo Hui and Suhaila, 2001; Zafrilla *et al.*, 2001). This system offers limits of detection (LOD) ranging between 3 to 57 ng/mL at 280 nm wavelength, however, for highly absorbing species, a detection limit of 1 ng is feasible (White, 1984).

2.3.4.2. HPLC Analysis of Sugar Components

Following the separation and isolation of glycosylated phenolic acid and flavonoid from the aglycons, the glycosylated compounds can be hydrolyzed and the liberated sugar components are identified and quantified using various methods.

2.3.4.2.1. Mode of Separation

The main chromatographic systems used for the separation of underivatized carbohydrates can be generalized as follow: (1) anion-exchange column with water containing bases or salts as the eluent; (2) cation-exchange column with water as the eluent; (3) alkyl-bonded silica gel column with water as the eluent; and (4) amine-bonded silica gel column with water acetonitrile as the eluent. Of these systems, amine modified silica columns are becoming more widely used for the separation of sugars in food (Lee and Coates, 2000; Wei and Ding, 2000).

The amino column is operated with a mixture of water and acetonitrile: 80%, 83%, 76%, and 75% acetonitrile. Since the amino-bonded column operates under normal phase elution conditions, water, which is a solvent more polar than acetonitrile, is the

more concentrated solvent. However, selection of mobile phase composition depends on the resolution, as well as molecular weight range of sugars eluting (Lee and Coates, 2000; Wei and Ding, 2000).

Normal-phase (NP) chromatography is best suited for low molecular weight sugars such a mono- and disaccharides. However, with traditional monomeric amino-bonded columns, retention times shorten over time due to Schiff's base formation between carbonyl groups and the amino groups on the column as well as low of amino functional groups lead to poor resolution of sugars. The loss of resolution between fructose and glucose was particularly noticeable (Lee and Coates, 2000; Wei and Ding, 2000).

Recently, a polyamine column was developed, which incorporates primary amine functionality into the polymer resin (polystyrene) coating, providing a selectivity that is identical to conventional propylamine-bounded column, but has improved stability and longer column life. The polyamine column is more stable over time, allowing reproducible retention times and analysis (Lee and Coates, 2000).

2.3.4.2.2. Laser Detection

Carbohydrates lack chromophoric and fluorophoric groups, which are necessary for their UV and fluorescence detection (Lee and Coates, 2000; Peris-Tortajada, 2000; Wei and Ding, 2000). Refractive index (RI) measurement is the most popular detection method for carbohydrates. However, it has many disadvantages, such as lacking sensitivity, being dependent on temperature and flow-rate and being incompatible with gradient elution (Wei and Ding, 2000).

Laser light scattering detection (LLSD) is now widely used as a semi-universal mass detector for HPLC analysis of sugars. Its detection is based on the detection of solute molecules by light scattering after nebulization and evaporation of the mobile phase, so it is suitable to detect non volatile compounds such as carbohydrates. The effluent from the chromatographic column is converted to a very fine mist by passage through a nebulizer into a stream of nebulizer gas. The fine droplets are then carried

through a temperature-controlled drift tube where the volatile mobile phase is vaporized, leaving very fine particles of solute in the nebulizer gas stream. The particles then pass through a light beam. The light that is scattered by these particles is detected and measured by a sensitive photodetector system. LLSD hence measures the amount of scattered radiation, which is proportional to the momentary concentration of the solute in the flow cell or more precisely to the momentary concentration of the solute in the light beam.

Use of LLSD for the HPLC analysis of carbohydrates is quite recent and offers many advantages; it is more sensitive than the RI, as the limit of detection can go up to a few tens of nanograms injected. Moreover, the LLSD response varies very little with the nature of the carbohydrate being studied, the temperature changes or to the variation in the mobile phase flow. Additionally, it is straight forward, with a great flexibility of use, given the absence of baseline drift and its rapid equilibration (Peris-Tortajada, 2000).

Pena *et al.* (2000) reported using LLSD to determine the partial solubility parameters of selected sugars. Its use was also reported for the analysis of high molecular weight carbohydrates (Bento and Sa, 1998) and carbohydrates in soft drink, where LOD ranging from 10 – 60 μg/mL for fructose, glucose and sucrose were obtained (Wei and Ding, 2000). However, Wallage et al. (2000) which also used LLSD for HPLC analysis of sugars stored in insects obtained the following LOD: 900 μg/mL for fructose and glucose and 171 μg/mL for sucrose standards.

3. MATERIAL AND METHODS

3.1. Materials

3.1.1. Reagents and Standards

Methanol and acetonitrile of HPLC grade and ethyl acetate, dichloromethane and pentane of ACS grade, as well as TFA were purchased from Fisher Scientific Ltd. (Negean, CA).

A wide range of SPE cartridges, including C18 Extract-Clean (Alltech Associates, Inc., Deerfield, IL), DSC-18 and Discovery DPA-6S (Sigma-Chemical Co., St-Louis, MO), Oasis HLB (Waters Associates, Milford, MA) and Amberlite XAD-2 resin (20-60 mesh) (Röhm and Haas, Philadelphia, PA) were used throughout this study.

The phenolic acid and flavonoid standards, the sugar standards and the commercial enzymatic preparations of β-glucosidase, β-galactosidase and hesperinidase were purchased from Sigma Chemical Co.

3.2. Methods

3.2.1. Preparation of the Maple Sap and Maple Syrup Samples

3.2.1.1. Maple Product Samples

Maple sap and maple syrup samples were obtained from the Centre de Recherche, de Développement et de Transfert Technologique en Acériculture (*ACER*, St-Hyacinthe, Québec). Maple sap and syrup samples obtained at different time periods during the 2002 maple sap season were categorized as 0, 25, 50, 75 and 100% of the maple sap season. The maple sap (2°Brix) was stored in a 4-L container at -18° C and samples were prepared by rapidly thawing it under running tap water at 25° C, followed by vaccum filtration on Whatman filter paper No. 1 (Whatman International Ltd., Maidstone, U.K.) to remove undesirable residues.

The maple syrup samples were adjusted from 66° to 2°Brix with deionized water by diluting 15 mL of maple syrup in a total volume of 500 mL, thereby diluting the maple syrup 33 times.

3.2.1.2. Extraction of the Phenolic Compounds and Flavonoids

Extraction of the phenolic compounds and flavonoids from the maple sap and syrup samples was carried out according to a modification of the methods reported by Kermasha *et al.* (1995), as well as by Dawes and Keene (1999) and Deslauriers (2000). Maple sap and maple syrup samples (500 mL) were adjusted to pH 7 with 1 N HCl. Subsequently, the samples were extracted three times for 10 min with 500, 250 and 250 mL of ethyl acetate. The upper organic phase, containing the phenolic compounds and flavonoids, was recovered and washed with deionized water (100 mL) for 10 min to remove any traces of sugars. The ethyl acetate extract was dried using anhydrous sodium sulfate (Na_2SO_4) and concentrated to dryness using the automatic environmental SpeedVac system (System Savant, Holbrook, N.Y.).

Each extract was the redissolved in methanol to obtain a concentration of 2.5 mg/mL solution, centrifuged (3,000xg, 10 min) and stored under nitrogen at -18° C for further analyses.

3.2.2. Separation of the Phenolic Compounds and Flavonoids From Maple Products

3.2.2.1. Development of a Methodology

In order to recover and characterize glycosylated phenolic compounds and flavonoids from maple products, a separation method was developed and optimized using different pre-packed SPE cartridges, including C_{18} Extract-clean, DSC-18, Discovery DPS-6S, Oasis HLB and Amberlite XAD-2 resin. A mixture of rutin and quercetin standards, prepared in methanol, was applied onto the SPE cartridges attached on a Visiprep vacuum manifold (Röhm and Haas, Philadelphia, PA, USA) set at 5 mm Hg.

Different solvent combinations were assayed for the separation of rutin and quercetin. The eluted fractions were concentrated to dryness using the automatic environmental SpeedVac system, redissolved in methanol, centrifuged (3,000xg, 10 min) and subjected to HPLC analysis.

3.2.2.2. Separation of the Phenolic Compounds and Flavonoids From Maple Products

The maple sap and syrup extracts obtained at 0, 25, 50, 75 and 100% of the maple season, were applied to a column packed with Amberlite XAD-2 resin. Approximately 5 mg of each maple extract was dissolved in 1 mL of methanol and applied onto the Amberlite XAD-2 column (30 x 1 cm) pre-conditioned with 60% aqueous methanol. At flow rate of 0.5 mL/min, the glycosylated compounds eluted with 198 mL of the 60% aqueous methanol solution, whereas the aglycons eluted using 282 mL of a methanol:acetonitrile mixture (1:1, v/v).

The collected fractions were concentrated to dryness using the automatic environmental SpeedVac system, redissolved in methanol to approximatly 2.5 mg/mL, centrifuged (3,000xg, 10 min) and subjected to HPLC analysis.

3.2.3. Hydrolysis of the Phenolic Compounds, Flavonoids and Their Sugar Components From Glycosylated Fractions of Maple Products

3.2.3.1. Optimization of Hydrolysis Using a Glycosylated Flavonoid Model

3.2.3.1.1. Selection of Hydrolytic Enzyme

A series of commercial enzymes, including β-glucosidase, β-galactosidase and hesperinidase, were assayed for their hydrolytic activity on glycosylated phenolic compounds and flavonoids. The rutin standard (1 mg/mL) was used as a model substrate. One mL of rutin was incubated with an equal volume of a 0.5 U suspension of the enzyme. A control trial was run in tandem with the assay containing everything in the reaction assay except the enzyme preparation. After vigorous agitation, the mixture was placed in a water-bath at 40°C for 24 and 48 h. The enzymatic reaction assay was halted by subjecting the mixture to a thermal treatment at 80°C for 10 min, centrifuged (3,000xg, 10 min) and subjected to HPLC analysis.

3.2.3.1.2. Determination of Optimum Incubation Time

The assays were carried out at 40°C for 12, 24, 36, 48, 60, and 72 h, subsequently halted by thermal treatment at 80°C for 10 min, centrifuged (3,000xg, 10 min) and subjected to HPLC analysis.

3.2.3.1.3. Determination of Optimum Enzyme Concentration

A wide range of concentrations (1.0, 1.5, 2.0, 2.5 and 3.0 U) of the hesperinidase preparation was assayed with a maple sap extract (1 mg/mL). The assays were conducted at 40°C for 48 h, halted by subjecting the mixture to a thermal treatment (80°C, 10 min), centrifuged (3,000xg, 10 min) and subjected to HPLC analysis.

3.2.3.2. Hydrolysis of the Phenolic Compounds, Flavonoids and Their Sugar Components From Glycosylated Fractions of Maple Products

The glycosylated fractions (2.5 mg/mL) of the maple sap and syrup extracts obtained at 0, 25, 50, 75 and 100% of the maple sap season, were hydrolyzed with the commercial hesperinidase solution (0.5 U). A control trial was run in tandem with the assay containing everything in the reaction assay except the enzyme preparation. After vigorous agitation, the mixture was placed in a water-bath at 40°C for 36 h. The enzymatic reaction was halted by subjecting the mixture to a thermal treatment at 80°C for 10 min, centrifuged (3,000xg, 10 min) and subjected to HPLC analysis.

3.2.4. HPLC Analysis of the Maple Sap and Syrup Extracts

3.2.4.1. HPLC Analysis of the Standards of Phenolic Compounds and Flavonoids

Standards of the phenolic compounds and flavonoids were dissolved in methanol, centrifuged (3,000xg, 10 min), and subjected to HPLC analysis according to a modification of the method described by Deslauriers (2000).

A HPLC system (Model 126, Beckman Instrument Inc., San Ramon, CA) equipped with a DAD (Beckman Model 168) and a computerized data handling system was used. UV detection was performed at 280 and 365 nm. Scanning of the analytes was also performed from 190 to 400 nm with monitoring at 1-second intervals. A RP Zorbax SB-C_{18} column (250 x 4.6 mm, 5 μm) (Chromatographic Specialties Inc., Brockville, ON) was used at a flow rate of 0.75 mL/min, using an aqueous solution of 0.2% TFA as solvent A and methanol as solvent B, with a linear gradient from 5 to 80% methanol in 50 min. Total elution time was 70 min.

Standards of the phenolic compounds and flavonoids were individually injected to determine their exact retention times and maximum absorbances and were then injected in a mixture.

3.2.4.2. HPLC Analysis of the Phenolic Compounds and Flavonoids From Maple Sap and Maple Syrup

HPLC analysis of the phenolic compounds and flavonoids present in the different maple sap and syrup extracts was performed according to the method described above.

3.2.4.3. HPLC Analysis of Sugar Standards

Standards of monosaccharides (rhamnose, D-xylose, L-arabinose, fructose, mannose, D-glucose, galactose) and disaccharides (sucrose, sophorose) were prepared in 10% acetonitrile solution as indicated by Cheng and Kaplan (2001). Dilutions of the sugar standards ranging from 0 to 500 mg/mL in concentrations were prepared and subjected individually and as a mixture to HPLC analysis according to a modification of the methodology reported by Wallage (2000) and Lee (2002).

HPLC analysis of the sugar standards was performed using a YMC-Pak PA column (4.6 x 250 mm, 5 μm) heated at 40°C and protected with a guard column of the same material (Waters, Milford, MA). An isocratic acetonitrile:water mixture (80:20, v/v) was used at a flow rate of 1 mL/min. Detection was achieved using an LLSD (LLSD II Varex, Burstonville, MD), set at 135°C in the presence of a flow of nitrogen at a rate of 55 mL/min. The total elution time was 50 min.

3.2.4.4. HPLC Analysis of the Sugar Components of the Glycosylated Fractions of Maple Products

For analysis of the sugar components, the hydrolyzed samples were dried completely under a stream of nitrogen and redissolved in 70 μL of 10% acetonitrile solution, to obtain approximatly 7 mg of glycosylated material per mL of solution. The samples were then centrifuged (3,000xg, 10 min) and subjected to HPLC-LLSD analyses according to the method described above

3.2.5. Characterization of the Phenolic Compounds, Flavonoids and Sugar Components

3.2.5.1. Phenolic Compounds and Flavonoids

Tentative characterization of the phenolic compounds and flavonoids in the glycosylated fractions of maple sap and maple syrup was based on comparing retention time data obtained with the DAD for the standard compounds and sample analytes. Comparisons of maximum absorbances, obtained from scans ranging from 200 to 400 nm, of the standards and sample was used as confirmation of the presence of phenolic compounds and flavonoids in maple sap and syrup.

3.2.5.2. Sugar Components

Characterization of sugar components in the glycosylated fractions of maple sap and maple syrup was mainly based on the comparison of their retention times in relation to those of the standard compounds.

4. RESULTS AND DISCUSSION

4.1. Characterization of Phenolic Compounds and Flavonoids from Maple Products

Characterization of the phenolic compounds and flavonoids in maple products was performed using HPLC analysis coupled with DAD. Comparison of the retention times and maximum absorbance spectra of the standards and those of the phenolic compounds and flavonoids present in maple sap and maple syrup allowed a tentative characterization of these compounds.

4.1.1. HPLC Analysis of Standards of Phenolic Compounds and Flavonoids

A typical HPLC elution profile for a mixture of selected standards of phenolic compounds and flavonoids is shown in Figure 1. With the use of RP chromatography, the most polar phenolic compounds and flavanols eluted early, whereas the less polar flavonoids including flavanones, flavonols and flavones, eluted later.

The retention times in RP chromatography are dependent upon the relative affinities of the compounds for the stationary and mobile phases (Markham and Bloor, 1998). The parts of the flavonoid molecule capable of forming hydrogen-bonds such as the C-4 carbonyl and hydroxyl groups will therefore have a greater affinity for the more polar mobile phase and will elute earlier.

The results (Table 2) demonstrate that all HBAs and HCAs eluted before 38 min, whereas most of the flavonoids eluted later, with the exception of (+)-catechin and (-)-epicatechin. This order of elution may be due to differences in the molecular structures of these two classes of compounds. Phenolic compounds have a molecular structure consisting of a single phenolic ring, while flavonoids possess three phenolic rings; this difference in structure can facilitate the hydrophobic interactions of the flavonoids with the C18 stationary phase (Markham and Bloor, 1998). The present results indicate that most of the flavonoids were retained on the column until the concentration of methanol in the mobile phase became greater than 45%.

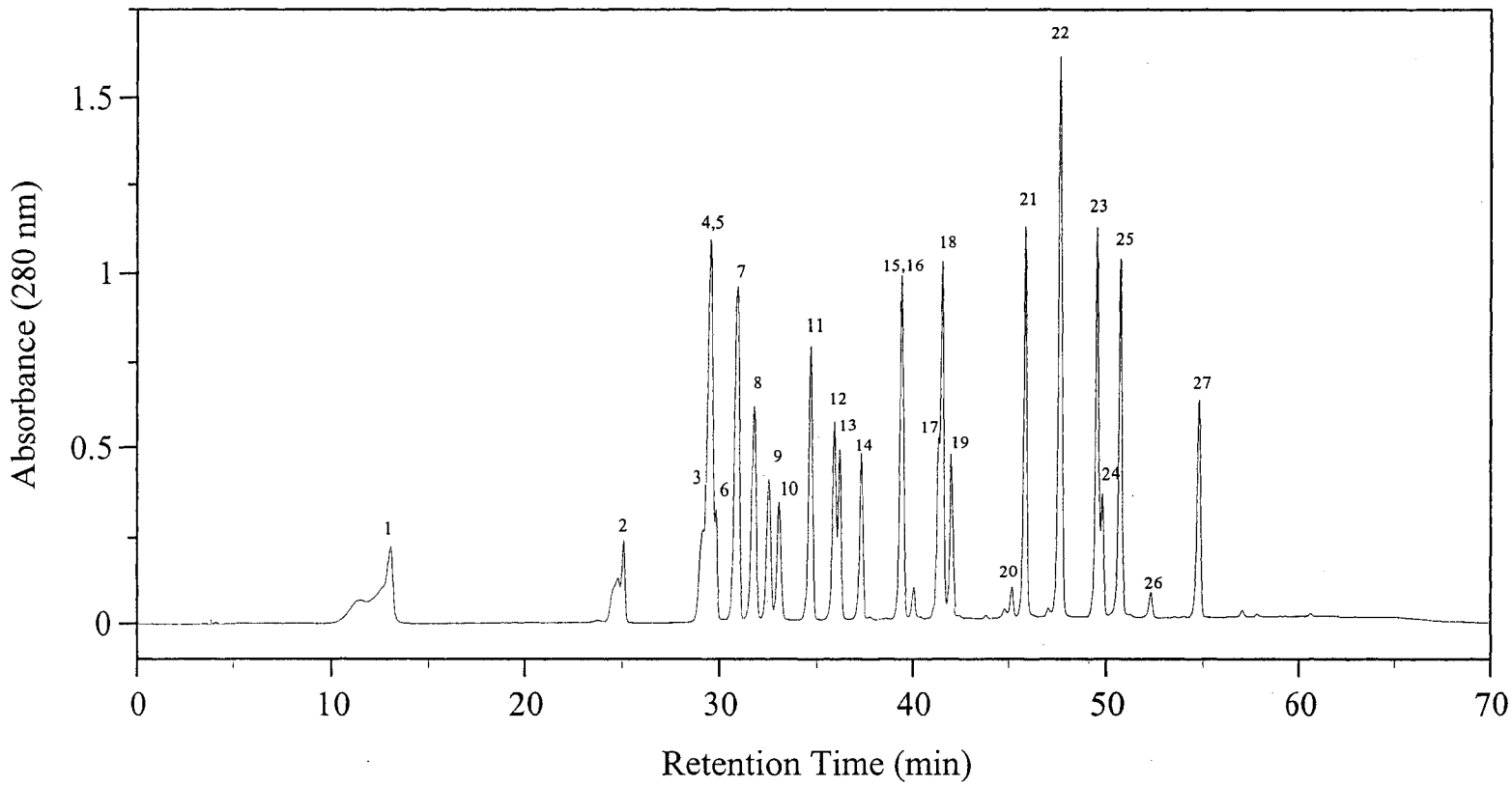


Figure 1. HPLC chromatogram, at 280 nm, of the standards of the phenolic compounds and flavonoids: gallic acid (1), (+)-catechin (2), vanillic acid (3), caffeic and homovanillic acids (4 and 5), (-)-epicatechin (6), syringic acid (7), vanillin (8), coniferol (9), syringaldehyde (10), *p*-coumaric acid (11), ferulic acid (12), sinapic acid (13), coniferal (14), rutin and hesperidin (15 and 16), rhoifolin (17), myricetin (18), quercetrin (19), quercetin (20), hesperitin (21), luteolin (22), kaempferol (23), 4-hydroxyflavanone (24), apigenin (25), 6-hydroxyflavanone (26) and 2-hydroxyflavanone (27).

Table 2. HPLC analysis of the standards of the phenolic compounds and flavonoids, using UV/Vis DAD.

Peak No[a]	Standard	Retention Time (min)[b]		Detection Response
		Individual	Mixture	λ_{max} (nm)[c]
1	Gallic acid	12.55	12.93	215, 225, 260, 280
2	(+)-Catechin	24.97	25.15	205, 280
3	Vanillic acid	29.03	29.20	207, 215, 260, 293
4	Caffeic acid	29.47	29.58	202, 240, 290, 325
5	Homovanillic acid	29.55	29.58	207, 230, 281
6	(-)-Epicatechin	29.70	29.83	205, 227, 280
7	Syringic acid	30.82	30.95	218, 276
8	Vanillin	31.58	31.83	230, 260, 285, 320
9	Coniferol	32.58	32.60	202, 265, 300
10	Syringaldehyde	32.97	33.12	217, 230, 307
11	*p*-Coumaric acid	34.43	34.77	210, 227, 293, 310
12	Ferulic acid	35.73	35.98	202, 215, 235, 287, 320
13	Sinapic acid	35.88	36.27	202, 235, 320
14	Coniferal	37.22	37.38	202, 223, 243, 300, 340
15	Hesperidin	38.93	39.48	205, 216, 227, 283, 337
16	Rutin	38.95	39.48	205, 227, 255, 267, 297, 357
17	Rhoifolin	40.77	41.35	203, 266, 336
18	Myricetin	40.92	41.57	210, 223, 255, 304, 370
19	Quercitrin	41.37	42.03	205, 227, 255, 267, 297, 350
20	Quercetin	45.10	45.18	205, 227, 255, 297, 371
21	Hesperitin	47.23	45.87	205, 216, 227, 286, 337
22	Luteolin	47.27	47.68	207, 252, 267, 292, 347
23	Kaempferol	48.88	49.60	203, 223, 246, 265, 294, 321, 367
24	4'-Hydroxyflavanone	49.08	49.85	202, 225, 255, 280, 322
25	Apigenin	50.65	50.78	205, 223, 267, 337
26	6-Hydroxyflavanone	51.62	52.32	202, 225, 255, 359
27	2'-Hydroxyflavanone	53.97	54.80	202, 217, 255, 280, 322

[a]Peak number with respect to Figure 1.
[b]Average retention time, calculated from triplicates, with a relative standard deviation of less than 5%.
[c]Maximum absorbance wavelengths.

Most of the phenolic compounds and flavonoids had a peak-to-peak separation of 0.5 min or greater (Table 2). However, vanillic, caffeic and homovanillic acids (peaks 3, 4 and 5), ferulic and sinapic acids (peaks 12 and 13), hesperidin and rutin (peaks 15 and 16), rhoifolin and myricetin (peaks 17 and 18), as well as kaempferol and 4-hydroxyflavanone (peaks 23 and 24) were only partially resolved with the current chromatographic system.

The elution profile of the standards of the phenolic compounds and flavonoids (Fig. 1) was similar to that reported by Deslauriers (2000), where gallic acid, catechin, vanillic acid, caffeic acid, homovanillic acid, epicatechin, syringic acid, vanillin, coniferol, syringaldehyde, *p*-coumaric acid, coniferal, quercetrin, quercetin, hesperitin and kaempferol eluted in the same order. However, the order of elution (Fig. 1) for ferulic and sinapic acids, as well for hesperidin and rutin, was opposite to that reported by Deslauriers (2000). However, the order of elution of hesperinidin and rutin (Fig. 1) was similar to that reported by Wulf and Nagel (1976).

4.1.1.1. Phenolic Compounds

Table 2 shows that in general, the HBAs showed less affinity towards the stationary phase compared to that observed with the HCAs. This difference in the adsorption behaviors between these two groups of phenolic compounds may be due to their structural differences, as the presence of an extra ethylenic chain in the cinnamic acids could render these molecules more hydrophobic. Wulf and Nagel (1976) reported that the relative retention on a column between two compounds that differ only in their degree of hydroxylation was independent of the methanol content of the mobile phase used for separation.

The results show that the HBAs that possessed a greater number of hydroxyl groups and a lower degree of methoxyl substituents eluted earlier (Fig. 1, Table 2), while the compounds with an aldehyde group attached onto the aromatic ring, such as vanillin (peak 8) and syringaldehyde (peak 10) eluted last. In the HCA family, the results (Fig. 1, Table 2) indicated that ferulic acid eluted after *p*-coumaric acid which may be due to differences in the substituents attached onto the phenol ring. Ferulic acid has a non-polar

methoxyl substituent and tended to be retained longer on the column, whereas *p*-coumaric acid has a hydrophilic hydroxyl substituent and therefore eluted earlier.

Although most of the HBAs eluted before the HCAs, caffeic acid which is an HCA, eluted before homovanillic acid, an HBA, which may be due to the fact that caffeic acid possesses an additional hydroxyl group. Moreover, coniferol, an HCA, eluted before syringaldehyde, an HBA, thereby suggesting that the presence of an aldehyde group and an additional methoxyl substituent can prolong the retention time on the RP stationary phase.

4.1.1.2. Aglycon Flavonoids

Table 2 indicates that the retention times of the flavonoids on the RP column were greater than those found for the phenolic compounds. However, the flavonols (+)-catechin and (-)-epicatechin that possessed a C3-OH group in the place of the hydrophobic C4-carbonyl group, had sufficient polarity to be eluted at a methanol concentration less than 45%. All the other flavonoids, such as flavonol, flavanone and flavone, eluted at a methanol concentration greater than 45% which may be due to the presence of the C4-carbonyl group that increased their hydrophobicity and affinity towards the C18 stationary phase.

When comparing the elution profile of the flavonoids which possess a 4-carbonyl group, in particular the flavonols, the flavanones and the flavones, separation can be expected to be based on the degree of hydroxylation and the occurrence of hydrogen bonding between the C4-carbonyl group and the C3-OH group and of double bonding between C2 and C3, as these factors can influence the retention times of the flavonoids on a C18 column (Wulf and Nagel, 1976; Bloor, 2001). Moreover, methylation of the hydroxyl groups appears to lower the polarity of the flavonoids, resulting in increased elution times (Bloor, 2001).

The results (Table 2) indicate that the degree of hydroxylation was the most important factor influencing the order of elution of the flavonoids. Indeed, despite the lowering effect in polarity due to hydrogen bond formation between the C4-carbonyl

group and C3-OH group as well as the additional insaturation between C2 and C3 (Bloor, 2001), the flavonols myricetin and quercetin, which have a higher degree of hydroxylation, eluted before the flavanones and flavones.

In addition to the degree of hydroxylation, the formation of hydrogen bonding between the C4-carbonyl groups and the *peri*-hydroxyl (*peri*-OH) groups may have an effect on the elution profile of flavonoids. Wulf and Nagel (1976) indicated that dut to the occurrence of resonance structures, classes of flavonoids with a C4-carbonyl group can assume a negative charge which provides a larger electron density around the oxygen atom of the C4-carbonyl group and may strengthen hydrogen bonding between the *peri*-OH groups and the C4-carbonyl groups so that both functional groups appear less polar to the solvent.

Flavanones are known to have the C4-carbonyl group positioned out of the plane of the adjacent phloroglucinol ring which may increase their polarity, as weaker hydrogen bond will form with the *peri*-OH group and in turn may expose both C4-carbonyl and *peri*-OH groups to stronger interactions with the mobile phase (Wulf and Nagel, 1976); this particularity in structure could explain the elution of the flavanone hesperitin, despite its low hydroxylation degree, before most of the other flavones (luteolin and apigenin). The flavanones 4'-hydroxyflavanone, 6-hydroxyflavanone and 2'-hydroxyflavanone, however, lacked the *peri*-OH group and thus could not form hydrogen bonds with their C4-carbonyl group, which may explain why they eluted last (Table 2).

The experimental results (Table 2) indicate that flavonoids within each family eluted according to their degree and position of hydroxylation, where their retention times decreased with an increase in hydroxyl number. These findings may explain why the flavonol myricetin possessing 5 hydroxyl groups eluted before quercetin, a flavonol with only 4 hydroxyl group. The order of elution of flavanones with equal degrees of hydroxylation, however, was dictated by the position of the hydroxyl group where the hydroxyflavanone with a hydroxyl group at the C-4' position, eluted before the one with a hydroxyl group either at the C-6 or C-2' position. Bloor (2001) indicated that both the degree and position of hydroxylation tend to influence compound mobility.

4.1.1.3. Glycosylated Flavonoids

The results (Table 2) indicate that the glycosylated flavonoids were more polar than the parent aglycons and hence eluted earlier. However, the degree of separation between the glycosylated and aglycon compounds was not extensive. The glycosylation of quercetin with rhamnose to produce quercitrin, for example, resulted in only a small separation factor between quercitrin and quercetin, as the latter eluted only 3.15 min after the former.

Moreover, when comparing the elution order of two glycosylated flavonols, the results (Table 2, Fig. 1) indicate that the sugar component attached to the flavonol influences its retention time on the column. Indeed, rutin, a flavonol that is glycosylated with rutinose (a disaccharide) eluted before quercitrin, a flavonol glycosylated with rhamnose (a monosaccharide). However, the degree of separation between rutin and quercitrin (2.55 min) was less significant than that between quecitrin and quercetin (3.15 min). Table 2 also shows that myricetin and quercitrin closely eluted, within 0.5 min, while quercetin eluted more than 3.5 min after myricetin. These findings are in agreement with those of Wulf and Nagel (1976), who suggested that the glycosylation of quercetin with rhamnose at position C-3, does not result in an increase in polarity greater than what could be achieved by the addition of a hydroxyl group.

4.1.2. Characterization of Phenolic Compounds and Flavonoids From Maple Products

HPLC analysis of the phenolic compounds and flavonoids, recovered from maple products, was performed using a DAD at 280 nm.

Figure 2 shows the HPLC chromatograms of the phenolic compounds and flavonoids from maple products obtained at 50% of the maple sap season. The results indicate the presence of 24 major peaks. Peaks 9, 13, 17', 27, 28, 29, 30, 33, 34, 36, 37, 38, 44, 48 and 49 were present in both the maple sap and maple syrup extracts. In addition, the maple sap extract contained peaks 15, 26 and 26, whereas peaks 16, 19, 20, 21, 33' and 54 were predominant only in the maple syrup extract.

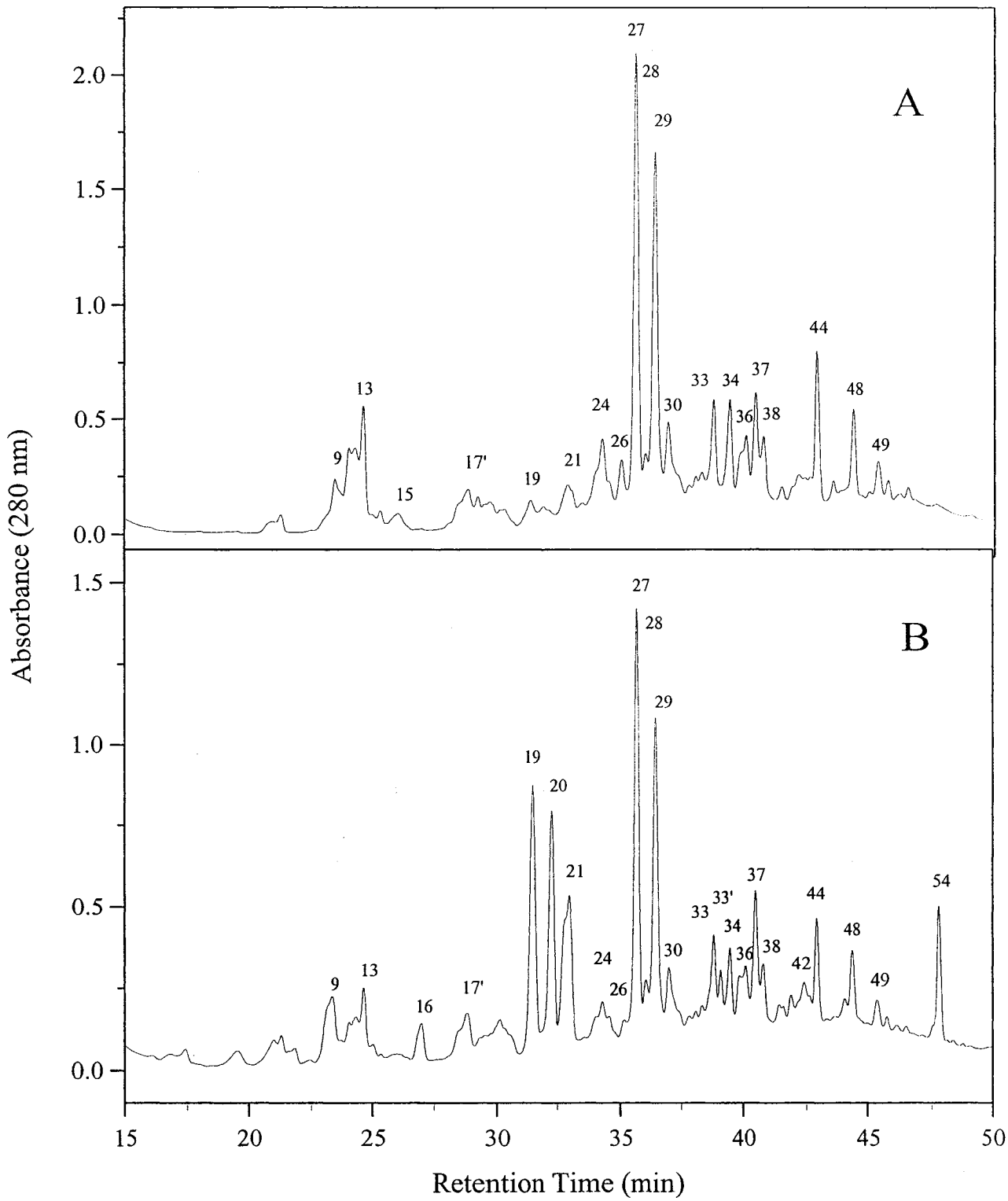


Figure 2. HPLC chromatograms, at 280 nm, of the phenolic compounds and flavonoids in (A) maple sap and (B) maple syrup obtained at 50% of the maple sap season.

The phenolic compounds and flavonoids in maple products harvested at 50% of the maple sap season were characterized by their UV spectra by comparison with standard compounds. Since the order of elution of phenolic compounds and flavonoids was not influenced by minor variations in the solvent system (Markham and Bloor, 1998), it was possible to confirm the identification of the separated compounds by comparing their relative retention time with those of known standards.

Tables 3 and 4 present the tentative identification of the predominant phenolic compounds and flavonoids found in the maple sap and maple syrup extracts obtained at 50% of the maple sap season. Spectral characteristics of peaks 9, 15 and 16, which eluted at 23.42, 25.91 and 26.92 min, respectively, suggested the presence of an HCA derivative due to absorbances in the range of 225 to 235 and 290 to 330 nm. Lee (2000) reported that most HCAs absorb in two UV regions, with one absorbance maximum in the range of 225 to 235 nm and the other one between 290 and 330 nm. The identification of peaks 9, 15 and 16 (Tables 3 and 4) is in agreement with that reported previously by Deslauriers (2000).

Peak 19 (Tables 3 and 4), which has an absorbance maximum in the range of 250 to 280 nm, was tentatively identified as a HBA derivative according to Harborne (1998) and Lee (2000) who reported that most HBAs display their absorbance maximum at 246 to 262 nm, with a shoulder peak at 290 to 315 nm. With our experimental conditions, the HBAs were expected to elute before the HCAs due to the absence of the hydrophobic ethylenic group (Wulf an Nagel, 1976); however, our findings with standard compounds (Table 2) indicated that the presence of an aldehyde group and an additional methoxyl substituent prolonged the retention of HBAs onto the stationary phase. Hence, peak 19 may correspond to an HBA derivative which has either an aldehyde or a methoxyl group attached onto its phenol ring.

The results (Tables 3 and 4) also indicate that peak 13, which eluted at 24.59 min with absorbance maxima at 205, 231 and 280 nm, may correspond to (+)-catechin, whereas peak 17', at 28.77 min, may correspond to (-)-epicatechin. Individual properties of the standards catechin and (-)-epicatechin (Table 2), eluted at 24.97 and 29.70 min,

Table 3. Tentative HPLC identification of the phenolic compounds and flavonoids from maple sap obtained at 50% of the maple sap season, using UV-Vis DAD.

Peak No[a]	Compounds	Retention Time (min)[b]	Detection Response λ_{max} (nm)[c]
9	HCA[d] derivative	23.42	206, 232, 281, 312
13	Catechin	24.59	205, 231, 280
15	HCA[d] derivative	25.91	204, 290
17'	Epicatechin	28.77	207, 227, 283
19	HBA[e] derivative	31.26	205, 276
21	Syringaldehyde	32.84	207, 230, 310
24	Vanillin derivative	34.22	208, 229, 260, 295, 345
26	Flavanol derivative	35.05	206, 231, 280
27	Flavanol derivative	35.64	206, 226, 281
28	Flavanol derivative	35.98	209, 227, 280
29	Flavanol derivative	36.42	204, 226, 280'
30	Flavone derivative	36.94	209, 229, 281, 348
33	Flavanol derivative	38.71	207, 233, 280
34	Flavanol derivative	39.35	207, 233, 280
36	Flavanol derivative	40.10	207, 227, 279
37	Flavanol derivative	40.44	207, 235, 282
38	Flavanol derivative	40.77	207, 235, 282
44	Flavanol derivative	42.85	207, 234, 280
48	Flavone derivative	44.40	207, 230, 281, 344
49	Flavanol derivative	45.20	210, 233, 279, 318

[a]Peak number with respect to Figure 2A.

[b]Average retention time, calculated from triplicates, with a relative standard deviation of less than 5%.

[c]Maximum absorbance wavelengths.

[d]Hydroxycinnamic acid.

[e]Hydroxybenzoic acid.

Table 4. Tentative HPLC identification of the phenolic compounds and flavonoids from maple syrup obtained at 50% of the maple sap season, using UV/Vis DAD.

Peak No[a]	Compounds	Retention Time (min)[b]	Detection Response λ_{max} (nm)[c]
9	HCA[d] derivative	23.42	206, 232, 281, 312
13	Catechin	24.59	205, 231, 280
16	HCA[d] derivative	26.92	205, 289
17'	Epicatechin	28.77	207, 227, 283
19	HBA[e] derivative	31.26	205, 276
20	Coniferol	32.06	211, 264, 301
21	Syringaldehyde	32.84	207, 230, 310
24	Vanillin derivative	34.22	208, 229, 260, 295, 345
26	Flavanol derivative	35.05	206, 231, 280
27	Flavanol derivative	35.64	206, 226, 281
28	Flavanol derivative	35.98	209, 227, 280
29	Flavanol derivative	36.42	204, 226, 280
30	Flavone derivative	36.94	209, 229, 281, 348
33	Flavanol derivative	38.71	207, 233, 280
33'	Dihydroflavonol derivative	39.21	207, 233, 280, 309
34	Flavanol derivative	39.35	207, 233, 280
36	Flavanol derivative	40.10	207, 227, 279
37	Flavanol derivative	40.44	207, 235, 282
38	Flavanol derivative	40.77	207, 235, 282
42	Flavanol derivative	42.35	205, 231, 281, 314
44	Flavanol derivative	42.85	207, 234, 280
48	Flavone derivative	44.40	207, 230, 281, 344
49	Flavanol derivative	45.20	210, 233, 279, 318
54	Flavanol derivative	47.51	205, 230, 280

[a]Peak number with respect to Figure 2A.

[b]Average retention time, calculated from triplicates, with a relative standard deviation of less than 5%.

[c]Maximum absorbance wavelengths.

[d]Hydroxycinnamic acid.

[e]Hydroxybenzoic acid.

respectively, indicate absorbance maxima at 230 and 280 nm; these retention times and spectral characteristics correspond to those of peaks 13 and 17', respectively. Moreover, peaks 24, 26, 27, 28, 29, 33, 34, 36, 37, 38, 44, 49 and 54 (Tables 3 and 4) showed absorbance maxima at 200, 230 and 280 nm and displayed identical spectra to those of (+)-catechin and (-)-epicatechin; hence, these peaks were tentatively assigned as flavanol related compounds. The overall results (Tables 3 and 4) are in agreement with those reported by Deslauriers (2000).

A comparison of the HPLC analysis between the phenolic compounds and flavonoids in maple products and the respective standards indicated that peak 20, which eluted at 32.06 min, had spectral characteristics similar to that of the coniferol standard; peak 20 had absorbance maxima at 211, 264 and 301 nm, whereas coniferol, at 32.58 min, absorbed at 202, 265 and 300 nm. In addition, scan spectrum comparisons of the coniferol standard with peak 20 resulted in a positive match. Peak 21, which eluted at 32.84 min with absorbance maxima at 207, 230 and 310 nm, was analyzed to be syringaldehyde; a comparison of the scan spectrum of the syringaldehyde standard with peak 21 was made and a positive match was obtained. These results are in agreement with those reported by Deslauriers (2000).

Based on their absorbance spectra, peaks 30 and 48 (Tables 3 and 4) which eluted at 36.94 and 44.40 min, respectively, with absorbance maxima at 209, 229, 281 and 348 nm, were found to be flavone derivatives. Markham and Bloor (1998) reported that within the range of 210 to 600 nm, most flavonoids exhibit absorption peaks in these two regions, 210 to 290 nm (band II) and 320 to 380 nm (band II); the exact position of band I could give a reasonable indication of the type of flavonoid since flavones are known to have their absorbance maxima in the range of 320 to 355 nm, but also absorb in the 250 to 270 nm region.

The results (Table 4) indicate that peak 33', which eluted at 39.21 min, pisplayed absorbance maxima at 207, 233 and 280 nm and a lower intensity shoulder at 309 nm and was hence characterized as dihydroflavonol. Markham and Bloor (1998) reported that the wavelength of band I for dihydroflavonols was in the 310 to 330 nm region, with a low-

intensity shoulder since their maximum absorbance was in the 275 to 290 nm region; this low-intensity band I absorption, which often appears as a shoulder to band II, can be attributed to the lack of all conjugation between the A- and B-rings of dihydroflavonols (Markham and Mabry, 1975).

4.2. Separation and Characterization of Phenolic Compounds and Flavonoids From Maple Products

In order to optimize the separation, recovery and characterization of glycosylated phenolic compounds and flavonoids in maple products, the efficiency of the separation method was evaluated according to certain criteria, including selective recovery and permanent retention of compounds. Based on the literature, a variety of techniques have been used to isolate these hydrophilic compounds, but the most commonly used for the isolation of glycosylated compounds was selective retention of compounds in a solid phase adsorbent (Mateo and Jimenez, 2000).

4.2.1. Methodology for the Separation of a Mixture of Flavonoid Standards

The separation of a mixture of standards composed of rutin, a glycosylated flavonoid and quercetin, an aglycon flavonoid, was based on the concept that glycosylated phenolic compounds and flavonoids have an additional glycosylated moiety attached to their structure which makes them more hydrophilic than the free aglycons. This difference in polarity could allow the separation and the recovery of glycosylated and aglycon compounds. Hence, a mixture of the standards was applied onto selected pre-packed SPE cartridges, including C_{18} Extract-clean, DSC-18, Discovery DPS-6S, Oasis HLB and Amberlite XAD-2, and elution were performed consecutively with different solvent combinations. By washing the adsorbent with an aqueous based solvent following the adsorption step, polar constituents could be recovered while the less polar aglycons were retained; the elution with a more organic solvent could give the aglycon fractions. The fractions collected from each SPE cartridge were analyzed by HPLC.

The SPE cartridges including, C_{18} Extract-clean, DSC-18, Discovery DPS-6S and Oasis HLB were assayed, using a mixture of water:methanol (20:1, v/v) for the elution of rutin and 100% methanol for the elution of quercetin. The results (Table 5) indicate that

Table 5. HPLC analysis, at 280 nm, of the degree of separation of a standard mixture of rutin and quercetin using selected solid phase extraction (SPE) cartridges and mobile phases.

Compounds	Mobile Phases	Relative Peak Area (%)[a,b]				
		SPE Cartridges[c]				
		Extract-Clean C18	DSC18	Discovery DPA	Oasis HLB	Amberlite XAD-2
Rutin	water:methanol (20:1, v/v)	34.38	40.21	97.77	80.60	-[d]
Quercetin	methanol	68.12	75.44	17.60	82.69	-[d]
Rutin	methanol:water (7:3, v/v)	-[d]	-[d]	-[d]	50.21	-[d]
Quercetin	dichloromethane:acetonitrile (2:1, v/v)	-[d]	-[d]	-[d]	19.14	-[d]
Rutin	methanol:water (3:1, v/v)	-[d]	-[d]	-[d]	32.55	-[d]
Quercetin	methanol:acetonitrile (1:3, v/v)	-[d]	-[d]	-[d]	41.57	-[d]
Rutin	methanol:water (4:1, v/v)	-[d]	-[d]	-[d]	39.83	-[d]
Quercetin	methanol:acetonitrile (1:3, v/v)	-[d]	-[d]	-[d]	13.03	-[d]
Rutin	methanol:water (3:2, v/v)	-[d]	-[d]	-[d]	-[d]	57.37
Quercetin	methanol:acetonitrile (1:1, v/v)	-[d]	-[d]	-[d]	-[d]	69.79

[a]Relative percent area, calculated by dividing the area of each peak by the sum of the peak areas of the sample, and then multiplying by 100.
[b]Average relative peak area, calculated from triplicates, with a relative standard deviation of less than 5%.
[c]Solid- phase extraction cartridges including Extract-Clean C18, Oasis HLB, DSC-C18, Discovery DPA-6S and Amberlite XAD-2.
[d]Trials were not performed with this SPE cartridge.

the Oasis HLB SPE cartridge could be a suitable support for the separation of the two compounds. The relative peak areas (Table 5) indicated that rutin was the predominant compound eluted by the mixture of water:methanol (20:1, v/v), whereas quercetin was the predominant one eluted by methanol; the use of the other solvent combinations with the Oasis HLB SPE cartridge did not improve the degree of separation (Table 5).

However, the experimental findings (not shown) also indicated that the Oasis HLB cartridge and the other SPE cartridges tended to retain certain amounts of rutin and quercetin on the column. The maximum amount of rutin and quercetin recovered were 20 and 46% of the original amounts applied, respectively.

Since the use of Amberlite XAD-2 resin has been extensively reported for the separation of glycosylated and aglycon terpenoids in wine (Table 1), it was subsequently assayed for the recovery of the components of the standard mixture. Comparisons of the relative peak areas (Table 5) indicated that rutin and quercetin were partially separated after their application onto the Amberlite XAD-2 SPE cartridge. Rutin was the predominant compound recovered by the methanol:water mixture (3:2, v/v), but certain amounts of quercetin also eluted. In addition, quercetin was the main compound recovered with the mixture of methanol:acetonitrile (1:1, v/v), but certain amounts of rutin were also present.

Regarding the different SPE cartridges assayed, the experimental results (not shown) indicated that certain amounts of rutin and quercetin were retained permanently on the supports, but that lesser amounts were retained onto the Amberlite XAD-2 SPE cartridge. The amounts of rutin and quercetin recovered from the Amberlite XAD-2 SPE cartridge were 55 and 48% of the original amounts applied, respectively, whereas with the other SPE cartridges, only between 6.7 and 20% and 10.2 and 45.87% of the rutin and quercetin amounts, respectively, were recovered. On the basis of these results, the Amberlite XAD-2 resin was selected for the separation and recovery of the glycosylated phenolic compounds and flavonoids from maple products.

4.2.2. Separation and Characterization of Phenolic Compounds and Flavonoids From Maple Sap

4.2.2.1. Separation of Phenolic Compounds and Flavonoids From Maple Sap

Figures 3 and 4 show the HPLC chromatograms of the phenolic compounds and flavonoids recovered from maple sap, harvested at 0% and 100% of the maple sap season, respectively, and separated by Amberlite XAD-2 column. The results (Fig. 3A and 4A) show the profile of phenolic compounds and flavonoids before their separation on the Amberlite XAD-2 column, whereas Figures 3B, 4B and 3C, 4C demonstrate their profiles after elution with 60% aqueous methanol and a mixture of methanol:acetonitrile (1:1, v/v), respectively.

When comparing the profiles of the phenolic compounds and flavonoids from maple sap extracts before and after separation on the Amberlite XAD-2 column, several differences were observed. Certain compounds, for instance, were selectively recovered from the column by successive elution using 60% aqueous methanol and the methanol:acetonitrile mixture, whereas others were not separated. Among the compounds present in the maple sap obtained at 0% of the maple sap season (Fig. 3), peaks 9, 11, 12, 13, 19, 21, 24, 27, 28, 29, 33 and 35 were only found in the glycosylated fraction eluted with the 60% methanol, whereas peaks 40, 43, 44, 46, 47, 48, 49 and 50 were only found in the aglycon fraction eluted with the methanol:acetonitrile mixture. Peaks 34 and 37 were not totally recovered with 60% methanol, since they were also present in the aglycon fractions.

For maple sap obtained at 100% of the maple sap season (Fig. 4), peaks 12, 19, 25, 27, 28, 29, 30, 33, 36, 37 and 38 were only found in the glycosylated fraction eluted with 60% methanol solution, whereas peaks 41, 43, 44, 46, 47, 48, 49, 50 and 52 were only found in the aglycon fraction with the methanol:acetonitrile mixture. Some compounds present in maple sap obtained at 100% of the maple sap season, however, appeared to be permanently retained on the Amberlite column, since they did not appear in any of the eluted fractions. For peaks 11, 12, 13, 20 and 34, the results (Fig. 4) indicated that these compounds tended to be either partially or totally permanently retained.

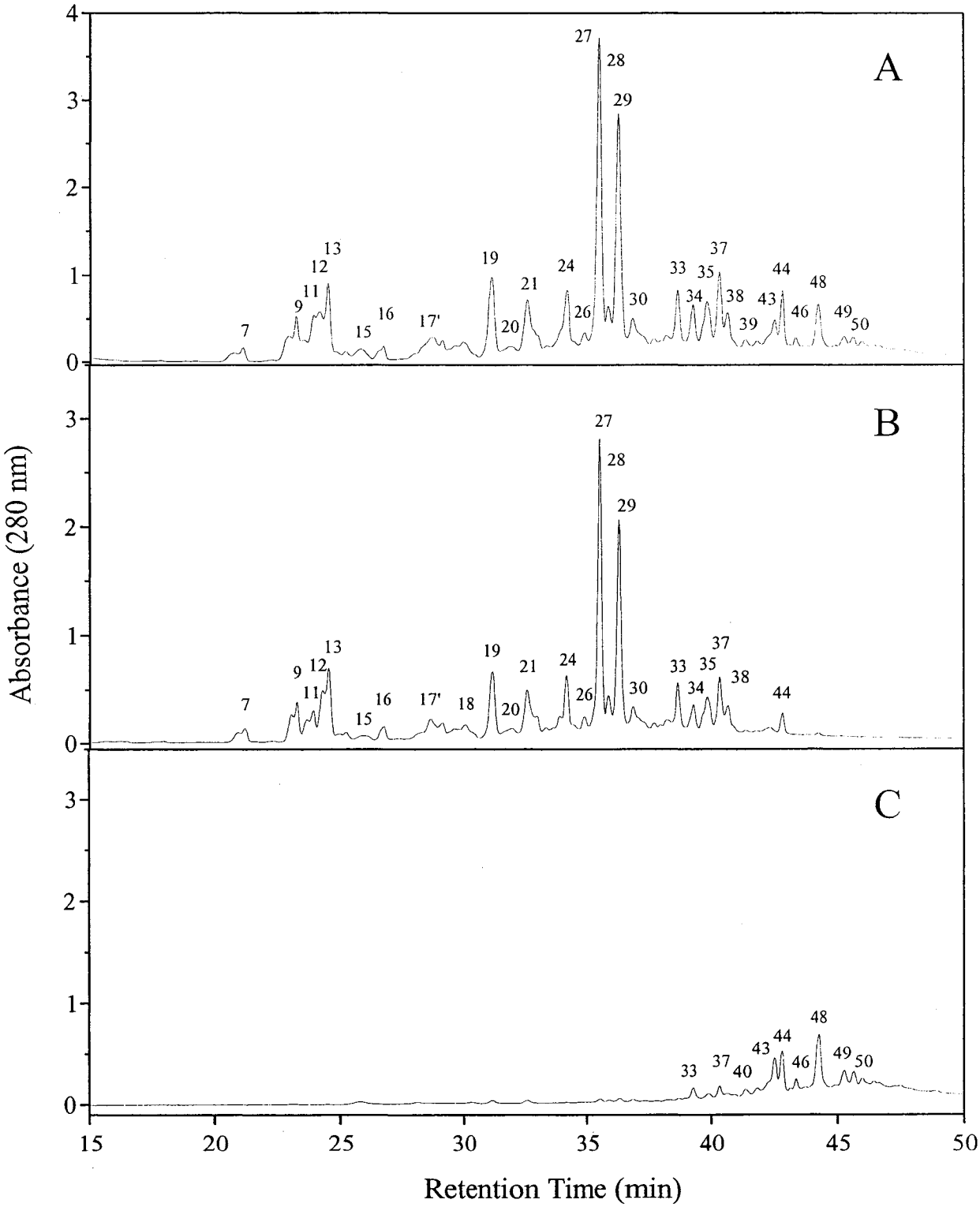


Figure 3. HPLC chromatograms, at 280 nm, of the phenolic compounds and flavonoids from maple sap obtained at 0% of the maple sap season and recovered using Amberlite XAD-2 column: (A) before separation, (B) elution with 60% aqueous methanol and (C) elution with a mixture of methanol:acetonitrile (1:1, v/v).

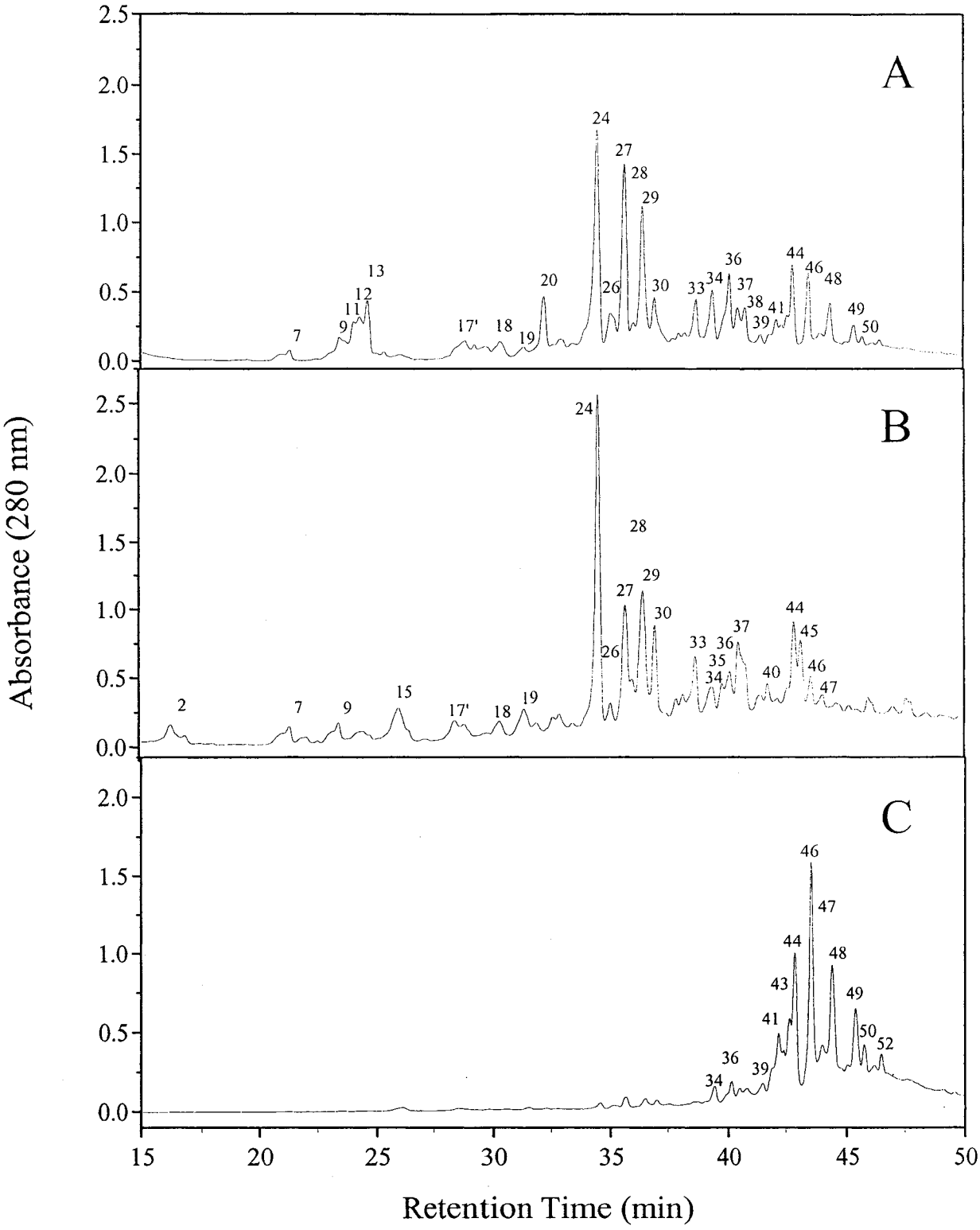


Figure 4. HPLC chromatograms, at 280 nm, of the phenolic compounds and flavonoids from maple sap obtained at 100% of the maple sap season and recovered using Amberlite XAD-2 column: (A) before separation, (B) elution with 60% aqueous methanol and (C) elution with a mixture of methanol:acetonitrile (1:1, v/v).

Moreover, separation by Amberlite XAD-2 allowed the detection of compounds that could hardly be detected earlier by UV at 280 nm because of their low concentration; peaks 43, 49 and 50 in the aglycon fraction of maple sap obtained at 0% of the maple sap season (Fig. 3) and peaks 40, 41, 43, 45, 49 and 50 in the glycosylated and aglycon fractions of maple sap obtained at 100% of the maple sap season (Fig. 4) could be detected.

Certain compounds in maple sap that previously co-eluted, were properly separated and integrated when recovered from the Amberlite column after elution with the 60% methanol or the methanol:acetonitrile mixture. Peaks 11, 12, 23, 24, 43 and 44 in maple sap obtained at 0% of the maple sap season (Fig. 3), as well as peaks 35, 36, 44 and 45 in maple sap obtained at 100% of the maple sap season (Fig. 4), were better resolved after separation.

4.2.2.2. Characterization of Total Phenolic Compounds and Flavonoids From Maple Sap

Figures 3A and 4A show the HPLC chromatograms of the profiles of the total phenolic compounds and flavonoids present in maple sap extracts obtained at 0 and 100% of the maple sap season, respectively. The HPLC analysis indicates the presence of 23 major peaks that occurred during the different periods of the maple sap season, including peaks 11, 12, 13, 15, 19, 21, 24, 25, 26, 27, 28, 29, 30, 33, 34, 35, 36, 37, 38, 44, 46, 48 and 49, which eluted between 24.02 and 45.20 min.

Table 6 shows the HPLC analysis of the total phenolic compounds and flavonoids present in maple sap obtained during different periods of the maple sap season. The results indicate that peaks 9, 11, 12, 13, 19, 21, 24, 27, 28, 29, 30, 33, 34, 35, 37, 44, and 48 were predominant at the beginning of the maple sap season and that peaks 13, 19, 21, 27, 29 and 37 were accounted for more than 4% of the total peak area of the sample. At the end of the maple sap season, the major peaks were 12, 13, 20, 25, 26, 27, 29, 30, 33, 34, 36, 37, 38, 44, 46 and 48, where peaks 20, 25, 27, 29, 34, 36 and 44 accounted for more than 4% of the total peak area of the sample. At the earlier stages of the maple sap

Table 6. HPLC analysis, at 280 nm, of the total phenolic compounds and flavonoids from maple sap obtained at different periods of the maple sap season.

Peak No[a]	Retention Time (min)[b]	Tentative Identification[c]	Relative Peak Area (%)[d,e] Maple Sap Season Periods (%)[f] 0	25	50	75	100
7	21.22	HBA[g] derivative	0.31	0.36	0.31	1.38	0.24
9	23.42	HCA[h] derivative	2.26	0.38	1.52	0.70	0.21
11	24.02	Flavanol derivative	2.14	0.00	2.85	2.03	0.94
12	24.24	Flavanol derivative	5.06	0.32	3.08	1.81	3.07
13	24.59	Catechin	4.04	0.00	3.81	4.16	2.77
15	25.91	HCA[h] derivative	1.18	30.99	0.96	9.27	0.00
17'	28.77	Epicatechin	0.90	0.00	1.06	0.15	0.70
17''	29.10	Flavanol derivative	0.96	0.00	1.00	0.12	0.63
19	31.26	HBA[g] derivative	5.18	4.41	0.94	1.18	0.76
20	32.06	Coniferol	1.21	0.14	0.00	0.18	4.82
21	32.84	Syringaldehyde	4.05	1.97	1.33	0.54	0.74
24	34.22	Flavone derivative	3.72	0.73	3.02	3.89	16.04
26	35.05	Flavanol derivative	1.53	0.00	2.35	0.96	2.04
27	35.64	Flavanol derivative	16.67	11.94	18.91	12.51	12.56
28	35.98	Flavanol derivative	2.33	3.86	1.94	1.41	1.61
29	36.42	Flavanol derivative	12.50	9.94	15.73	10.47	10.86
30	36.94	Flavone derivative	2.01	6.53	3.50	3.12	3.29
33	38.71	Flavanol derivative	3.87	3.70	4.18	3.80	3.27
34	39.35	Flavanol derivative	3.19	2.70	4.88	3.11	4.23
35	39.84	Undetermined	2.81	4.14	2.08	1.60	0.00
36	40.14	Flavanol derivative	0.00	0.00	3.23	4.22	3.99
37	40.44	Flavanol derivative	4.05	6.06	4.49	3.60	2.78
38	40.77	Flavanol derivative	1.92	2.19	2.34	2.66	2.54
39	41.47	Dihydroflanol derivative	0.77	0.20	0.44	1.07	0.84
41	42.00	Dihydroflanol derivative	0.00	0.00	0.00	0.00	1.33
43	42.64	Flavone derivative	1.22	1.39	0.00	0.00	0.00
44	42.85	Flavanol derivative	2.53	3.51	3.91	7.98	5.54
46	43.48	Dihydroflanol derivative	0.55	0.64	0.59	1.38	3.40
48	44.40	Flavone derivative	2.54	4.29	3.06	3.75	2.65
49	45.20	Flavanol derivative	0.70	1.98	1.44	2.11	0.95

[a]Peak number with respect to Figures 3A and 4A.
[b]Average retention time, calculated from triplicates, with a relative standard deviation of less than 5%.
[c]Tentative identification performed by comparing the spectrum of the compound with those of the standard compounds.
[d]Relative percent area for each peak, calculated by dividing the area of each peak by the sum of the areas for each maple extract, and then multiplying by 100.
[e]Average relative peak area, calculated from triplicates, with a relative standard deviation of less than 5%.
[f]Maple sap samples collected at different periods during the maple sap season (0, 25, 50, 75 and 100%).
[g]Hydroxybenzoic acid.
[h]Hydroxycinnamic acid.

season, peaks 20, 25, 26, 36, 38 and 46 were not predominant, whereas peaks 9, 11, 19, 21, 24, 28 and 35 were not predominant at the later stages.

Table 6 shows a tentative identification of the major phenolic compounds and flavonoids, including peaks 11, 12, 13, 15, 19, 21, 24, 25, 26, 27, 28, 29, 30, 32, 33, 34, 35, 36, 37, 38, 44, 46, 48 and 49, from maple sap obtained during the different periods of the maple sap season; the majority of these peaks, 13, 15, 19, 26, 27, 28, 29, 30, 33, 34, 36, 37, 38, 44, 48 and 49, have already been identified in maple sap (Table 3).

Certain peaks present in maple sap samples obtained during other periods of the maple sap season were also tentatively identified by their spectral characteristics. Peaks 11 and 12 (Figs. 3 and 4), which eluted at 24.02 and 24.24 min, respectively, were designated as flavanol derivatives since their maximum absorbance was in the region of 227 to 280 nm. As reported in previous work on maple products (Deslauriers, 2000), peaks 11 and 12 could correspond to either procyanidin B1 or B3, since they eluted before (+)-catechin (peak 13). The elution order reported for this family of compounds was procyanidins B1 < B3 < catechin < B4 < B2 < epicatechin (Jaworski and Lee, 1987; Oszmianski *et al.*, 1988).

From its absorbance spectra, absorbance maxima at 208, 229, 260, 295 and 345 nm, peak 24, which eluted at 34.22 min, was tentatively identified as a flavone derivative. Flavones are known to have their maximum absorbance in the region of 320 to 355 nm, but also absorb in the region of 250 to 280 nm (Bohm, 1998; Markham and Bloor, 1998); the experimental findings indicated that peak 24 also had these spectral characteristics.

4.2.2.3. Characterization of Phenolic Compounds and Flavonoids From Glycosylated Fractions of Maple Sap

Figures 3B and 4B show the HPLC chromatograms of the phenolic compounds and flavonoids present in maple sap, obtained at 0 and 100% of the maple sap season, respectively, after elution from the Amberlite XAD-2 column with 60% methanol. The HPLC analysis indicates the presence of 20 major peaks in maple sap samples obtained during the different periods of the maple sap season, including peaks 3, 12, 15, 18, 19, 21,

24, 25, 27, 28, 29, 30, 33, 35, 36, 37, 38, 39, 44 and 45, which eluted between 16.78 and 43.15 min.

The results (Table 7) show that at the beginning of the maple sap season (0%), the predominant peaks were 9, 11, 12, 13, 19, 21, 24, 27, 28, 29, 33, 34, 35 and 37, where peaks 13, 19, 21, 24, 27, 29 and 37 accounted for more than 4% of the total peak area of the sample. However, at the end of the maple sap season (100%), the major peaks were 12, 19, 25, 27, 28, 29, 30, 33, 36, 37, 38, 44, and 45, where peaks 25, 27, 29, 30, 33, 37 and 44 accounted for more than 4% of the total peak area of the sample. Hence, peaks 9, 11, 13, 21, 24, 34 and 35 were predominant in the earlier stages of the maple sap season, while peaks 25, 30, 36, 38, 44 and 45 were predominant in the later stages of the season.

Table 7 also shows a tentative identification of the major phenolic compounds and flavonoids, including peaks 3, 12, 15, 18, 19, 21, 24, 25, 27, 28, 29, 30, 33, 35, 36, 37, 38, 39, 44 and 45, from the glycosylated fractions of maple sap obtained at different periods of the maple sap season. The majority of these peaks, 12, 15, 19, 21, 24, 27, 28, 29, 30, 33, 36, 37, 38 and 44, were already identified in maple sap (Tables 3 and 6).

Certain peaks (Table 7) in the glycosylated fractions of maple sap were better resolved and could be tentatively identified by their spectral characteristics. It was suggested that peak 3, which eluted at 16.78 min with absorbance maxima at 209 and 271, could be tentatively identified as a HBA derivative as HBAs are known to have their absorbance maximum in the region of 270 to 290 nm (Harborne, 1998). Due to its absorbance in the 300 to 320 nm region, peak 18, which eluted at 30.27 min, was tentatively identified as a HCA derivative. Peak 45, at 43.15 min, showed absorbance maxima at 207, 229 and 280 nm and displayed similar spectral characteristics to those of (+)-catechin and (-)-epicatechin; hence, this peak was tentatively assigned as a flavanol related compound.

4.2.2.4. Characterization of Phenolic Compounds and Flavonoids From Aglycon Fractions of Maple Sap

Figures 3C and 4C show the HPLC chromatograms of the profiles of the phenolic compounds and flavonoids present in maple sap, obtained at 0 and 100% of the maple sap

Table 7. HPLC analysis, at 280 nm, of the phenolic compounds and flavonoids recovered from glycosylated fractions of maple sap obtained at different periods of maple sap season.

Peak No[a]	Retention Time (min)[b]	Tentative Identification[c]	Relative Peak Area (%)[d,e] Maple Sap Season Periods (%)[f] 0	25	50	75	100
2	16.29	HCA[g] derivative	0.00	0.00	1.75	2.20	1.11
3	16.78	HBA[h] derivative	0.00	7.83	1.39	1.98	0.33
9	23.42	HCA[g] derivative	2.48	1.64	0.94	0.72	0.66
11	24.02	Flavanol derivative	2.15	0.00	0.63	0.00	0.00
12	24.24	Flavanol derivative	3.60	0.23	2.75	2.01	0.00
13	24.59	Catechin	8.74	0.00	0.49	0.00	0.00
14	25.28	Undetermined	0.00	0.00	1.78	0.00	0.00
15	25.91	HCA[g] derivative	0.00	13.56	5.78	16.39	1.95
16	26.92	HCA[g] derivative	1.21	0.38	0.00	0.00	0.00
17	28.33	HBA[h] derivative	0.00	0.96	0.93	1.07	1.26
17'	28.77	Epicatechin	1.89	0.43	0.34	0.71	0.84
17''	29.10	Flavanol derivative	1.38	0.00	0.00	0.00	0.00
18	30.27	HCA[g] derivative	1.29	0.47	0.62	3.81	0.00
19	31.26	HBA[h] derivative	5.75	1.50	4.30	2.07	2.04
20	32.06	Coniferol	1.09	0.78	0.83	0.80	0.77
21	32.84	Syringaldehyde	4.58	3.05	2.30	1.12	0.92
24	34.22	Flavone derivative	6.99	2.33	2.34	2.54	16.31
26	35.05	Flavanol derivative	1.61	1.10	1.06	0.34	1.37
27	35.64	Flavanol derivative	19.10	8.19	4.43	4.24	8.73
28	35.98	Flavanol derivative	2.49	3.07	3.41	2.50	2.01
29	36.42	Flavanol derivative	14.19	12.06	6.21	5.43	10.15
30	36.94	Flavone derivative	1.85	1.73	3.49	4.58	5.27
33	38.71	Flavanol derivative	3.79	1.09	0.00	0.51	4.20
34	39.35	Flavanol derivative	2.49	1.34	1.07	1.30	1.25
35	39.84	Undetermined	3.00	1.94	3.57	2.42	1.68
36	40.14	Flavanol derivative	0.00	3.63	4.01	3.86	3.21
37	40.44	Flavanol derivative	3.99	5.17	5.80	5.84	5.28
38	40.77	Flavanol derivative	1.79	1.48	0.00	2.36	2.12
39	41.47	Dihydroflanol der.	0.00	0.70	2.56	2.18	1.76
40	41.80	Dihydroflanol der.	0.00	0.66	2.68	0.80	1.78
41	42.00	Undetermined	0.00	0.59	1.49	1.18	1.06
43	42.64	Flavone derivative	0.23	0.00	0.00	0.00	1.46
44	42.85	Flavanol derivative	1.05	4.89	9.74	7.84	5.83
45	43.15	Flavanol derivative	0.00	3.62	7.86	5.58	3.16
46	43.48	Dihydroflanol der.	0.00	0.00	1.00	0.78	1.78
47	43.90	Flavanol derivative	0.00	0.71	1.43	1.17	1.21
48	44.40	Flavone derivative	0.00	0.65	1.00	1.42	0.34
51	46.02	Undetermined	0.00	0.00	1.15	0.91	0.18
54	47.51	Flavanol derivative	0.00	0.00	1.35	0.92	0.17

[a]Peak number with respect to Figures 3B and 4B.
[b]Average retention time, calculated from triplicates, with a relative standard deviation of less than 5%.
[c]Tentative identification performed by comparing the spectrum of the compound with those of the standard compounds.
[d]Relative percent area for each peak, calculated by dividing the area of each peak by the sum of the areas for each maple extract, and then multiplying by 100.
[e]Average relative peak area, calculated from triplicates, with a relative standard deviation of less than 5%.
[f]Maple sap samples collected at different periods during the maple sap season (0, 25, 50, 75 and 100%).
[g]Hydroxycinnamic acid.
[h]Hydroxybenzoic acid.

season, respectively, after elution from Amberlite XAD-2 column with a mixture of methanol:acetonitrile. The HPLC analysis indicates the presence of 13 major peaks in maple sap samples obtained during the different periods of the maple sap season, including peaks 35, 37, 40, 41, 42, 43, 44, 46, 47, 48, 49, 50 and 52, which eluted between 39.35 and 47.51 min.

The results (Table 8) show that at the beginning of the maple sap season (0%), the predominant peaks were 35, 37, 40, 43, 44, 46, 47, 48, 49, 50, 51 and 52, where peaks 43, 44, 46, 48, 49, 50 and 51 accounted for more than 4% of the total peak area of the sample. At the end of the maple sap season (100%), the major peaks were 40, 41, 42, 43, 44, 46, 47, 48, 49 and 50, where peaks 41, 43, 44, 46, 47, 48 and 49 accounted for more than 4% of the total peak area of the sample. Peaks 34, 37, 51 and 52 were predominant only in the earlier stages of the maple sap season, whereas peaks 41 and 42 were predominant only in the later stages.

Table 8 indicates a tentative identification of the major phenolic compounds and flavonoids, including peaks 35, 37, 40, 41, 42, 43, 44, 46, 47, 48, 49, 50 and 52, from the aglycon fractions of maple sap. Certain peaks, 35, 37, 44 and 48, were already characterized in maple sap extracts and in the glycosylated fractions of maple sap (Tables 3, 4, 6 and 7), while others were better resolved after separation on the Amberlite XAD-2 column with mixture of methanol:acetonitrile. Peaks 42, 47 and 52, which eluted between 42 and 47 min, were designated as flavanols because of their maximum absorbance in the region of 230 to 280 nm. The spectral properties of the standards (Table 1) show that (+)-catechin and (-)-epicatechin absorbed mainly at 280 nm; the experimental findings (Table 8) indicate that peaks 42, 47 and 52 also have these characteristics.

The results (Table 8) indicate that peak 40, which eluted at 41.80 min with absorbance maxima at 207, 228, 280 and a lower intensity shoulder at 311 nm, was found to be a dihydroflavonol derivative; the presence of this type of flavonoid was already reported in maple syrup (peak 33' which eluted at 39.21 min, Table 4). Since this dihydroflavonol derivative eluted from the Amberlite XAD-2 column in the presence of the less polar mixture of methanol:acetonitrile, this compound might be a dihydroflavonol

Table 8. HPLC analysis, at 280 nm, of the phenolic compounds and flavonoids recovered from the aglycon fractions of maple sap obtained at different periods of the maple sap season.

Peak No[a]	Retention Time (min)[b]	Tentative Identification[c]	Relative Peak Area (%)[d,e] Maple Sap Season Period (%)[f] 0	25	50	75	100
35	39.84	Undetermined	2.98	12.68	1.98	1.59	1.35
36	40.14	Flavanol derivative	0.00	7.86	0.00	0.71	1.71
37	40.44	Flavanol derivative	2.56	17.87	2.85	2.66	1.04
38	40.77	Flavanol derivative	0.00	0.00	5.09	0.81	1.12
39	41.47	Dihydroflavonol derivative	1.90	1.84	0.00	0.72	1.28
40	41.8	Dihydroflavonol derivative	2.34	0.00	0.00	0.00	2.31
41	42.00	Undetermined	0.00	3.12	6.00	3.33	8.73
42	42.35	Flavanol derivative	0.00	0.00	2.86	2.44	2.64
43	42.64	Flavone derivative	11.14	4.39	8.68	4.14	8.87
44	42.85	Flavanol derivative	12.37	11.61	14.61	26.87	12.04
45	43.15	Flavanol derivative	1.11	0.00	0.00	0.00	0.00
46	43.48	Dihydroflavonol derivative	5.20	1.80	4.55	5.52	21.01
47	43.90	Flavanol derivative	1.91	2.86	5.49	4.60	5.31
48	44.40	Flavone derivative	27.82	15.80	27.53	20.15	12.70
49	45.20	Flavanol derivative	9.58	5.19	13.37	14.28	9.30
50	45.70	Flavone derivative	5.96	3.13	5.53	3.74	3.28
51	46.02	Undetermined	4.44	1.42	1.26	1.67	1.27
52	46.47	Flavanol derivative	2.11	0.91	2.28	1.67	1.67
53	46.63	Flavanol derivative	1.36	0.00	0.00	0.00	0.00
54	47.51	Flavanol derivative	0.30	0.00	0.00	0.00	0.00

[a]Peak number with respect to Figures 3B and 4B.
[b]Average retention time, calculated from triplicates, with a relative standard deviation of less than 5%.
[c]Tentative identification performed by comparing the spectrum of the compound with those of the standard compounds.
[d]Relative percent area for each peak, calculated by dividing the area of each peak by the sum of the areas for each maple extract, and then multiplying by 100.
[e]Average relative peak area, calculated from triplicates, with a relative standard deviation of less than 5%.
[f]Maple sap samples collected at different periods during the maple sap season (0, 25, 50, 75 and 100%).

aglycon. Peaks 43 and 50, eluted at 42.64 and 45.70 min, respectively, were tentatively identified as a flavone derivatives due to their maximum absorbance in the region 340 nm and shorter absorption band in the region of 230 to 280 nm.

4.2.3. Separation and Characterization of Phenolic Compounds and Flavonoids From Maple Syrup

4.2.3.1. Separation of Phenolic Compounds and Flavonoids From Maple Syrup

Figures 5 and 6 illustrate the HPLC chromatograms of the phenolic compounds and flavonoids, from maple syrup, obtained at 0 and 100% of the maple sap season, respectively, and separated by Amberlite XAD-2 column. The results (Figs. 5A and 6A) show the profiles of the phenolic compounds and flavonoids before their separation on the Amberlite XAD-2 column, whereas Figures 5B, 6B and 5C, 6C show their profiles after elution with 60% methanol and a mixture of methanol: acetonitrile (1:1, v/v), respectively.

When comparing the profiles of the phenolic compounds and flavonoids from maple syrup extracts, before and after separation on the Amberlite XAD-2 column, several differences can be observed. Certain compounds, for instance, were selectively recovered from the column by successive elutions using 60% methanol and the methanol:acetonitrile mixture, whereas others could not be separated. Of the compounds present in maple syrup obtained at 0% of the maple sap season (Fig. 5), peaks 19, 20, 21, 27, 29, 30, 33, 33', 34 and 37 were only found in the glycosylated fraction eluted with 60% methanol, whereas peaks 39, 40, 42, 43, 44, 45, 48, 49, 50, 51 and 54 could only be found in the aglycon fraction eluted with the methanol:acetonitrile mixture. Peak 19 was not totally recovered using 60% methanol, since it was present in both fractions. A proportion of peak 20, present in the total maple sap extract, appeared to have been permanently retained on the Amberlite column, since its relative area significantly decreased after its elution with 60% methanol.

For maple syrup obtained at 100% of the maple sap season (Fig. 6), peaks 2, 9, 11, 13, 20, 25, 27, 29 and 36 were only found in the glycosylated fraction eluted with 60% methanol, whereas peaks 42, 43, 44, 45, 46, 47, 48, 49, 50 and 53 could only be found in

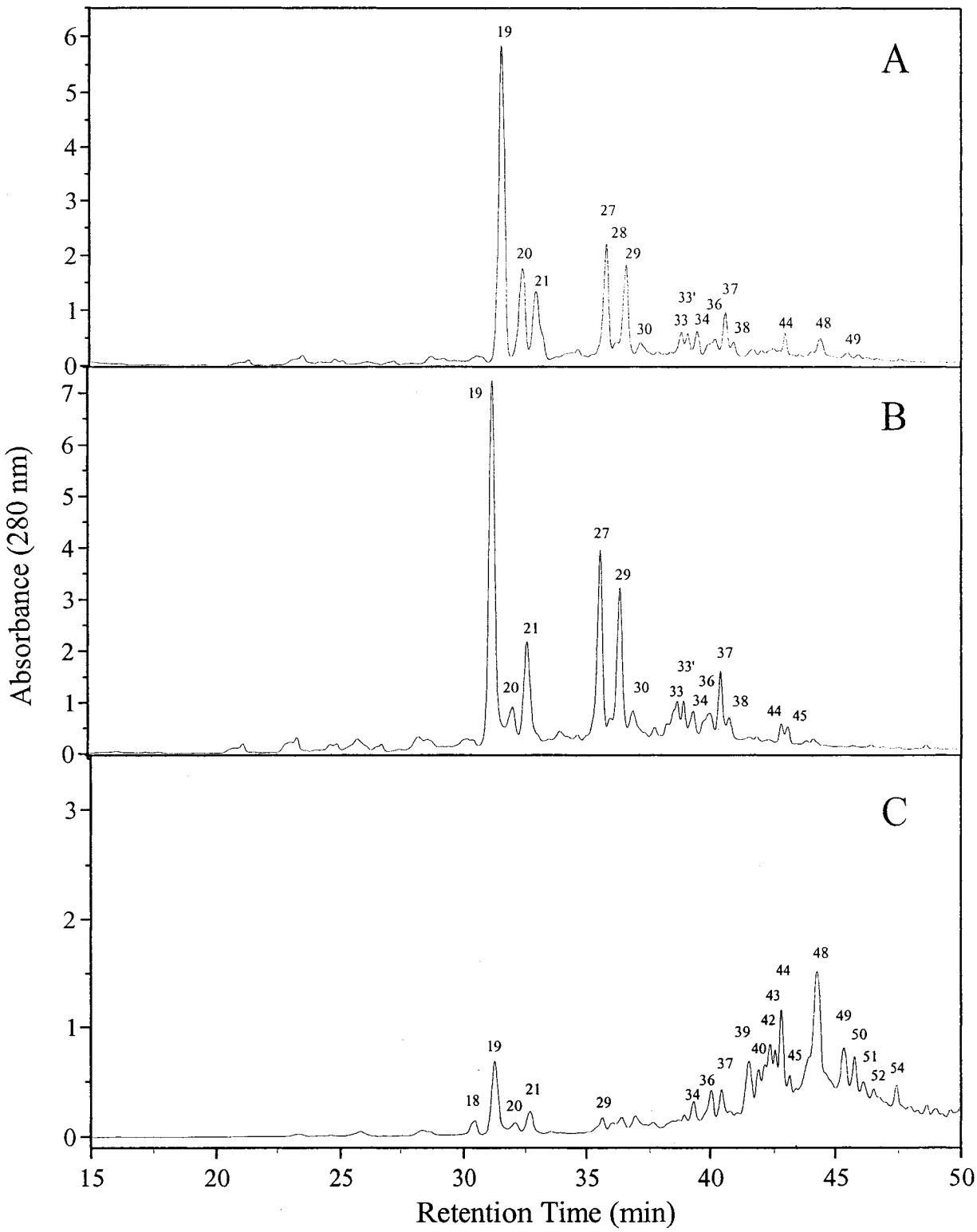


Figure 5. HPLC chromatograms, at 280 nm, of the phenolic compounds and flavonoids from maple syrup obtained at 0% of the maple sap season and recovered using Amberlite XAD-2 column: (A) before separation, (B) elution with 60% aqueous methanol and (C) elution with a mixture of methanol:acetonitrile (1:1, v/v).

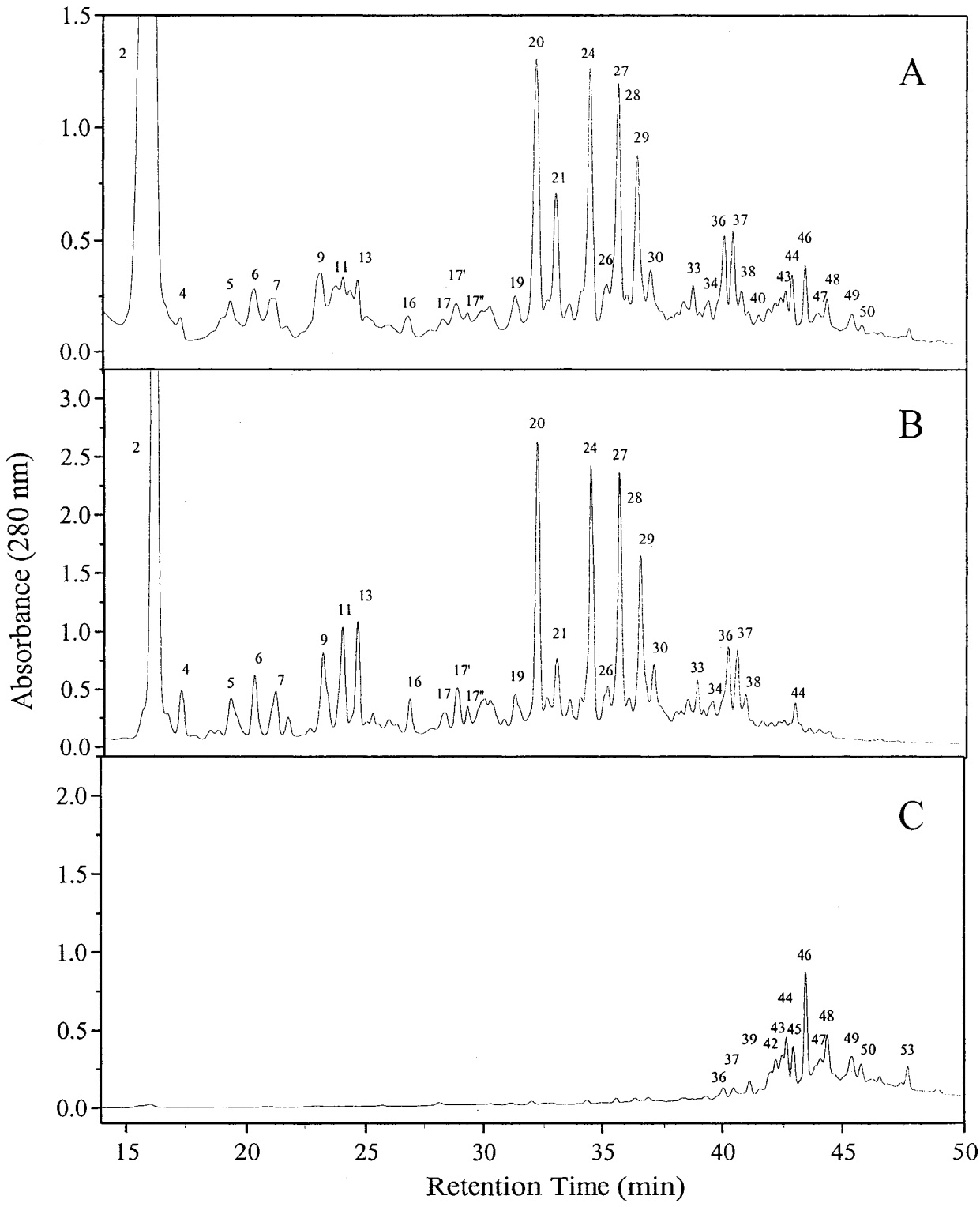


Figure 6. HPLC chromatograms, at 280 nm, of the phenolic compounds and flavonoids from maple syrup obtained at 100% of the maple sap season and recovered using Amberlite XAD-2 column: (A) before separation, (B) elution with 60% aqueous methanol and (C) elution with a mixture of methanol:acetonitrile (1:1, v/v).

the aglycon fraction eluted with the methanol:acetonitrile mixture. Other compounds, present in maple syrup obtained at 100% of the maple sap season (peaks 2, 21), however, appeared to have been permanently retained on the Amberlite column, as their relative peak area in the glycosylated fractions of maple syrup was lower than in the maple syrup extract before separation.

Moreover, separation with Amberlite XAD-2 allowed the detection of compounds that could hardly be detected previously by UV at 280 nm in maple syrup due to low concentrations. Peaks 4, 16, 17, 17' and 17'' were thus integrated when present in the glycosylated fraction of maple syrup obtained at 100% of the maple sap season (Fig. 5) as well as peaks 39, 42, 43, 48, 49, 50 and 54 in the aglycon fractions of maple syrup obtained at 0 and 100% of the maple sap season (Figs. 5 and 6).

Certain compounds that co-eluted in maple syrup extract were separated and integrated when recovered from the Amberlite column after elution with 60% methanol or the methanol:acetonitrile mixture. Peaks 44 and 45 in maple syrup obtained at 0% of the maple sap season (Fig. 5) and peaks 2, 4, 5, 6, 7, 16, 17, 17', 17'', 44 and 45 in maple syrup obtained at 100% of the maple sap season (Fig. 6) were better resolved.

4.2.3.2. Characterization of Total Phenolic Compounds and Flavonoids From Maple Syrup

Figures 5A and 6A illustrate HPLC chromatograms of the profiles of the phenolic compounds and flavonoids present in maple syrup obtained at 0 and 100% of the maple sap season, respectively. HPLC analysis indicates the presence of 14 major peaks in the maple syrup extracts obtained during the different periods of the maple sap season, including peaks 9, 19, 20, 21, 27, 29, 30, 33, 33', 34, 36, 37, 44 and 48, which eluted between 16.29 and 47.51 min.

The results (Table 9) show that at the beginning of the maple sap season (0%), the predominant peaks were 19, 20, 21, 27, 29, 33, 34, 37 and 44, where peaks 19, 20, 21, 27 and 29 accounted for more than 4% of the total peak area of the sample. At the end of the maple sap season (100%), the major peaks were 2, 6, 9, 11, 20, 21, 25, 27 and 29, where peaks 2, 20, 25 and 27 accounted for more than 4% of the total peak area of the sample.

Table 9. HPLC analysis, at 280 nm, of the total phenolic compounds and flavonoids from maple syrup obtained at different periods of the maple sap season.

Peak No[a]	Retention Time (min)[b]	Tentative Identification[c]	Relative Peak Area (%)[d,e] Maple Sap Season Periods (%)[f]				
			0	25	50	75	100
2	16.29	HCA[g] derivative	0.00	0.00	0.00	0.00	37.05
5	19.33	HBA[h] derivative	0.00	0.00	0.00	0.62	1.03
6	20.25	HBA[h] derivative	0.00	0.00	0.00	0.00	2.02
9	23.42	HCA[g] derivative	0.29	1.30	3.18	0.00	2.37
11	24.02	Flavanol derivative	0.00	0.00	0.00	0.00	2.49
13	24.59	Catechin	0.00	0.00	5.35	1.03	0.82
16	26.92	HCA[g] derivative	0.00	0.00	1.55	2.50	0.47
17	28.77	HBA[h] derivative	0.74	0.81	0.69	1.09	0.89
18	30.27	HCA[g] derivative	0.00	0.00	0.00	2.27	0.83
19	31.26	HBA[h] derivative	31.58	23.16	10.52	4.07	1.03
20	32.06	Coniferol	10.10	8.17	9.53	11.55	5.78
21	32.84	Syringaldehyde	7.35	7.84	4.68	8.85	3.26
24	34.22	Flavone derivative	0.00	1.60	1.41	1.55	5.72
25	34.55	Undetermined	0.00	0.00	0.91	1.60	0.00
27	35.64	Flavanol derivative	10.29	10.33	14.14	10.60	4.90
28	35.98	Flavanol derivative	1.26	1.64	1.93	1.87	0.38
29	36.42	Flavanol derivative	9.52	9.81	11.33	8.70	3.28
30	36.94	Flavone derivative	1.41	1.12	2.31	2.39	0.84
33	38.71	Flavanol derivative	2.31	2.44	2.63	2.38	0.56
33'	39.21	Dihydroflavonol derivative	1.73	2.49	2.83	2.54	0.00
34	39.35	Flavanol derivative	2.08	2.77	0.00	1.72	0.35
36	40.14	Flavanol derivative	1.28	2.01	3.86	4.39	1.71
37	40.44	Flavanol derivative	3.26	5.27	4.58	4.11	1.64
38	40.77	Flavanol derivative	0.95	1.41	1.84	1.94	0.56
39	41.47	Dihydroflavonol derivative	0.69	1.45	0.81	0.93	0.00
40	41.80	Dihydroflavonol derivative	0.00	0.82	0.84	1.33	0.32
42	42.35	Flavanol derivative	1.05	2.14	1.25	0.00	0.94
44	42.85	Flavanol derivative	2.12	1.95	2.64	3.14	0.80
46	43.48	Dihydroflavonol derivative	0.00	0.00	0.00	0.00	1.00
47	43.90	Flavanol derivative	0.67	0.00	0.00	1.20	0.44
48	44.40	Flavone derivative	1.96	2.10	2.11	2.29	1.00
49	45.20	Flavanol derivative	0.62	0.83	0.86	1.34	0.33
54	47.51	Flavanol derivative	0.00	0.38	2.73	2.79	0.12

[a]Peak number with respect to Figures 5A and 6A.
[b]Average retention time, calculated from triplicates, with a relative standard deviation of less than 5%.
[c]Tentative identification performed by comparing the spectrum of the compound with those of the standard compounds.
[d]Relative percent area for each peak, calculated by dividing the area of each peak by the sum of the areas for each maple extract, and then multiplying by 100.
[e]Average relative peak area, calculated from triplicates, with a relative standard deviation of less than 5%.
[f]Maple syrup samples collected at different periods during the maple sap season (0, 25, 50, 75 and 100%).
[g]Hydroxycinnamic acid.
[h]Hydroxybenzoic acid.

Hence, peaks 19, 33, 34, 37 and 44 were present in the earlier stages of the maple sap season, while peaks 2, 6, 9, 11 and 25 were present in the later stages.

Table 9 shows a tentative identification of the major phenolic compounds and flavonoids, including peaks 9, 19, 20, 21, 27, 29, 30, 33, 33', 34, 36, 37, 44 and 48, from maple syrup obtained during the different periods of the maple sap season; most of these peaks were already characterized (Table 4). Peak 9 was found to be a HCA derivative, whereas peak 19 was a HBA derivative. The presence of coniferol and syringaldehyde (peaks 20 and 21, respectively) in maple syrup was also confirmed; these experimental findings are in accordance with previous analyses reported for maple products (Potter and Fagerson, 1992; Kermasha *et al.*, 1995; Deslauriers, 2000). In addition, peaks 27, 29, 33, 34, 36, 37 and 44 were characterized as being flavanol derivatives, while peaks 30 and 48 were tentatively identified as flavone derivatives. The presence of a dihydroflavonol (peak 33') was also found to be a predominant compound of maple syrup.

4.2.3.3. Characterization of Phenolic Compounds and Flavonoids From Glycosylated Fractions of Maple Syrup

Figures 5B and 6B illustrate the HPLC chromatograms of the phenolic compounds and flavonoids present in maple syrup, obtained at 0 and 100% of the maple sap season, respectively, after elution from the Amberlite XAD-2 column with 60% methanol. The HPLC analysis indicates the presence of 16 major peaks in maple syrup samples obtained during the different periods of the maple sap season, including peaks 9, 13, 19, 20, 21, 25, 27, 29, 30, 33, 33', 34, 36, 37 and 38, which eluted between 21.22 and 43.15 min.

The results (Table10) show that at the beginning of the maple sap season (0%), the predominant peaks were 19, 20, 21, 27, 29, 30, 33, 33', 34 and 37, where peaks 19, 21, 27, 29 and 37 accounted for more than 4% of the total peak area of the sample. At the end of the maple sap season (100%), however, the results indicate that the major peaks were 2, 9, 11, 13, 20, 25, 27, 29 and 36, where peaks 2, 20, 25, 27 and 29 accounted for more than 4% of the total peak area of the sample. Hence, peaks 19, 21, 30, 33, 33', 34

Table 10. HPLC analysis, at 280 nm, of the phenolic compounds and flavonoids recovered from the glycosylated fractions of maple syrup obtained at different periods of the maple sap season.

Peak No[a]	Retention Time (min)[b]	Tentative Identification[c]	Relative Peak Area (%)[d,e] Maple Sap Season Periods (%)[f] 0	25	50	75	100
2	16.29	HCA[g] derivative	0.00	0.00	0.00	0.00	25.78
4	17.28	HBA[h] derivative	0.00	0.00	0.25	0.30	1.18
5	19.33	HBA[h] derivative	0.00	0.00	0.00	1.07	1.52
6	20.25	HBA[h] derivative	0.00	0.00	1.12	0.99	1.56
7	21.22	HBA[h] derivative	0.23	0.16	0.84	0.87	1.47
9	23.42	HCA[g] derivative	0.26	0.81	2.52	2.62	2.44
11	24.02	Flavanol derivative	0.00	0.00	1.24	1.07	2.54
13	24.59	Catechin	0.26	0.33	2.30	1.64	2.51
15	25.91	HCA[g] derivative	1.06	0.64	0.46	0.00	0.00
16	26.92	HCA[g] derivative	0.00	0.34	0.59	2.99	0.92
17	28.33	HBA[h] derivative	1.18	1.48	0.87	1.19	0.89
17'	28.77	Epicatechin	0.77	0.50	1.81	3.21	1.32
18	30.27	HCA[g] derivative	0.71	0.42	0.00	3.11	0.67
19	31.26	HBA[h] derivative	28.25	28.05	5.49	5.45	1.21
20	32.06	Coniferol	3.59	2.58	7.70	10.36	6.02
21	32.84	Syringaldehyde	8.12	7.41	4.04	5.78	1.95
24	34.22	Flavone derivative	0.38	1.50	3.18	1.39	6.42
25	34.55	Undetermined	0.00	0.55	0.88	2.10	0.00
27	35.64	Flavanol derivative	12.53	11.99	14.78	10.97	5.74
28	35.98	Flavanol derivative	1.55	1.36	2.01	2.22	0.90
29	36.42	Flavanol derivative	11.28	11.16	11.80	8.84	4.54
30	36.94	Flavone derivative	2.69	2.21	3.05	2.76	1.82
33	38.71	Flavanol derivative	2.07	2.06	1.95	2.63	1.23
33'	39.21	Dihydroflavonol derivative	2.45	2.56	2.96	2.42	0.00
34	39.35	Flavanol derivative	2.62	2.17	2.31	2.45	0.77
36	40.14	Flavanol derivative	1.63	2.40	2.71	2.33	2.20
37	40.44	Flavanol derivative	4.33	5.39	4.09	3.56	1.80
38	40.77	Flavanol derivative	1.63	1.86	2.22	2.29	0.98
39	41.47	Dihydroflavonol derivative	0.72	0.90	1.24	0.00	0.50
42	42.35	Flavanol derivative	0.68	0.92	1.35	0.00	0.69
44	42.85	Flavanol derivative	1.20	1.51	1.70	1.47	0.67
45	43.15	Flavanol derivative	1.07	0.71	0.41	0.34	0.26

[a]Peak number with respect to Figures 5B and 6B.
[b]Average retention time, calculated from triplicates, with a relative standard deviation of less than 5%.
[c]Tentative identification performed by comparing the spectrum of the compound with those of the standard compounds.
[d]Relative percent area for each peak, calculated by dividing the area of each peak by the sum of the areas for each maple extract, and then multiplying by 100.
[e]Average relative peak area, calculated from triplicates, with a relative standard deviation of less than 5%.
[f]Maple syrup samples collected at different periods during the maple sap season (0, 25, 50, 75 and 100%).
[g] Hydroxycinnamic acid.
[h] Hydroxybenzoic acid.

and 37 were only present in the earlier stages of the maple sap season, while peaks 2, 9, 11, 13 and 25 were only present in the later stages.

Table 10 shows a tentative identification of the major phenolic compounds and flavonoids, including peaks 9, 13, 19, 20, 21, 25, 27, 29, 30, 33, 33', 34, 36, 37 and 38, from the glycosylated fractions of maple syrup obtained during the different periods of the maple sap season. These peaks correspond to compounds that were already identified in maple syrup (Tables 4 and 9).

4.2.3.4. Characterization of Phenolic Compounds and Flavonoids From Aglycon Fractions of Maple Syrup

Figures 5C and 6C illustrate the HPLC chromatograms of the profiles of the phenolic compounds and flavonoids present in maple syrup, obtained at 0 and 100% of the maple sap season, respectively, after elution from Amberlite XAD-2 column with a methanol:acetonitrile mixture. The HPLC analysis indicates the presence of 17 major peaks in maple syrup samples obtained during the different periods of the maple sap season, including peaks 19, 37, 39, 40, 41, 42, 43, 44, 45, 47, 48, 49, 50, 51, 52, 53 and 54, which eluted between 31.26 and 47.51 min.

The results (Table 11) show that at the beginning of the maple sap season (0%), the predominant peaks were 19, 39, 40, 42, 43, 44, 45, 48, 49, 50, 51 and 54, where peaks 19, 39, 42, 44, 48, 49 and 50 accounted for more than 4% of the total peak area of the sample. The results also indicate that at the end of the maple sap season (100%), the major peaks 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 and 53, where peaks 42, 43, 44, 45, 46, 47, 48 and 49 accounted for more than 4% of the total peak area of the sample. Hence, peaks 19, 39, 40, 51, 52 and 54 were predominant only in the earlier stages of the maple sap season, while peaks 46, 47 and 53 were predominant only in the later stages.

Table 11 shows a tentative identification of the major phenolic compounds and flavonoids, including peaks 19, 37, 39, 40, 41, 42, 43, 44, 45, 47, 48, 49, 50, 51, 52, 53 and 54, present in the aglycon fractions of maple syrup obtained at the different periods of the maple sap season; most of these peaks were already characterized (Tables 4, 9 and 10), with the exception of peaks 53 and 54, which eluted between 46 and 48 min, and

Table 11. HPLC analysis, at 280 nm, of the phenolic compounds and flavonoids recovered from the aglycon fractions of maple syrup obtained at different periods of the maple sap season.

Peak No[a]	Retention Time (min)[b]	Tentative Identification[c]	Relative Peak Area (%)[d,e] Maple Sap Season Periods (%)[f] 0	25	50	75	100
18	30.27	HCA[g] derivative	1.01	0.88	1.34	1.02	0.00
19	31.26	HBA[h] derivative	6.45	2.09	1.78	0.00	0.00
20	32.06	Coniferol	1.03	0.00	0.00	0.00	0.00
21	32.84	Syringaldehyde	1.83	0.65	0.00	0.00	0.00
29	36.42	Flavanol derivative	1.14	0.00	0.00	0.00	0.42
34	39.35	Flavanol derivative	1.04	1.50	0.45	1.10	1.19
36	40.14	Flavanol derivative	1.92	2.13	0.85	1.17	1.61
37	40.44	Flavanol derivative	1.96	2.19	0.79	2.28	1.91
38	40.77	Flavanol derivative	0.56	0.65	2.09	1.50	0.00
39	41.47	Dihydroflavonol der.	5.19	5.59	5.27	1.72	0.72
40	41.80	Dihydroflavonol der.	3.28	2.81	4.34	2.93	0.00
41	42.00	Undetermined	2.76	4.27	9.85	6.60	3.25
42	42.35	Flavanol derivative	7.46	4.05	6.79	4.58	8.53
43	42.64	Flavonol derivative	3.69	5.32	6.82	2.95	4.64
44	42.85	Flavanol derivative	7.45	5.36	8.98	11.67	12.07
45	43.15	Flavanol derivative	2.79	2.83	0.00	0.58	4.85
46	43.48	Dihydroflavonol der.	1.71	0.00	0.88	0.00	14.28
47	43.90	Flavanol derivative	0.00	0.00	5.62	4.98	5.38
48	44.40	Flavone derivative	14.50	16.48	14.38	10.58	6.40
49	45.20	Flavanol derivative	7.63	7.58	7.56	5.61	4.27
50	45.70	Flavone derivative	4.86	4.75	2.78	1.95	2.24
51	46.02	Undetermined	3.52	4.19	1.64	1.85	0.00
52	46.47	Flavanol derivative	2.21	2.33	1.40	1.32	0.00
53	46.63	Flavanol derivative	1.62	3.21	0.00	1.36	2.86
54	47.51	Flavanol derivative	2.95	2.93	11.44	29.94	0.00
55	48.36	Dihydroflavonol der.	1.57	1.88	0.00	0.79	0.00
56	49.06	Dihydroflavonol der.	1.27	1.75	0.00	0.00	0.00

[a]Peak number with respect to Figures 5C and 6C.
[b]Average retention time, calculated from triplicates, with a relative standard deviation of less than 5%.
[c]Tentative identification performed by comparing the spectrum of the compound with those of the standard compounds.
[d]Relative percent area for each peak, calculated by dividing the area of each peak by the sum of the areas for each maple extract, and then multiplying by 100.
[e]Average relative peak area, calculated from triplicates, with a relative standard deviation of less than 5%.
[f]Maple syrup samples collected at different periods during the maple sap season (0, 25, 50, 75 and 100%).
[g]Hydroxycinnamic acid.
[h]Hydroxybenzoic acid.

were designated as flavanols due to their maximum absorbance in the region of 230 to 280 nm. The spectral properties of standards (Table 1) show that (+)-catechin and (-)-epicatechin absorbed mainly at 280 nm; the experimental findings (Table 11) indicate that peaks 53 and 54 shared these characteristics.

4.3. Hydrolysis and Characterization of Phenolic Compounds, Flavonoids and Their Sugar Components From Glycosylated Fractions of Maple Products

In order to elucidate and characterize the structure of the phenolic compounds and flavonoids present in the glycosylated fractions of maple products, an enzymatic hydrolysis reaction was performed. Hydrolysis of the glycosylated phenolic compounds and flavonoids led to the separation of the aglycons and the sugar moieties, thereby enabling analysis of all the components, including the sugar components. The enzymatic hydrolysis process was initially performed using the standard rutin as a model substrate. The glycosylated fractions of maple sap and maple syrup extracts were hydrolyzed and subjected to HPLC analysis for characterization of the phenolic compounds, flavonoids and their sugar components.

4.3.1. Optimization of Hydrolysis Using a Glycosylated Flavonoid Model

4.3.1.1. Enzyme Selection for the Hydrolysis of a Glycosylated Flavonoid Model

The hydrolytic activities of β-galactosidase, β-glucosidase and hesperinidase on rutin (quercetin 3-β-D-rutinoside), used as a model substrate, was investigated.

Figure 7 shows the HPLC chromatograms of the enzymatic hydrolysis of the rutin substrate (peak 1) and the subsequent formation of quercetin (peak 2), at 280 nm. Chromatograms A1, B1 and C1 correspond to the reaction components before hydrolysis, whereas chromatograms A2, B2, C2 correspond to these components after 48 h incubation.

The experimental results (not shown) indicated that after a period of 48 h of incubation, 14.9, 41.1 and 38.3% of rutin was hydrolyzed by β-galactosidase, β-glucosidase and hesperinidase, respectively. However, the results (Fig. 7) indicate that the hydrolytic activity of β-galactosidase (Fig. 7A) and β-glucosidase (Fig. 7B) did not result

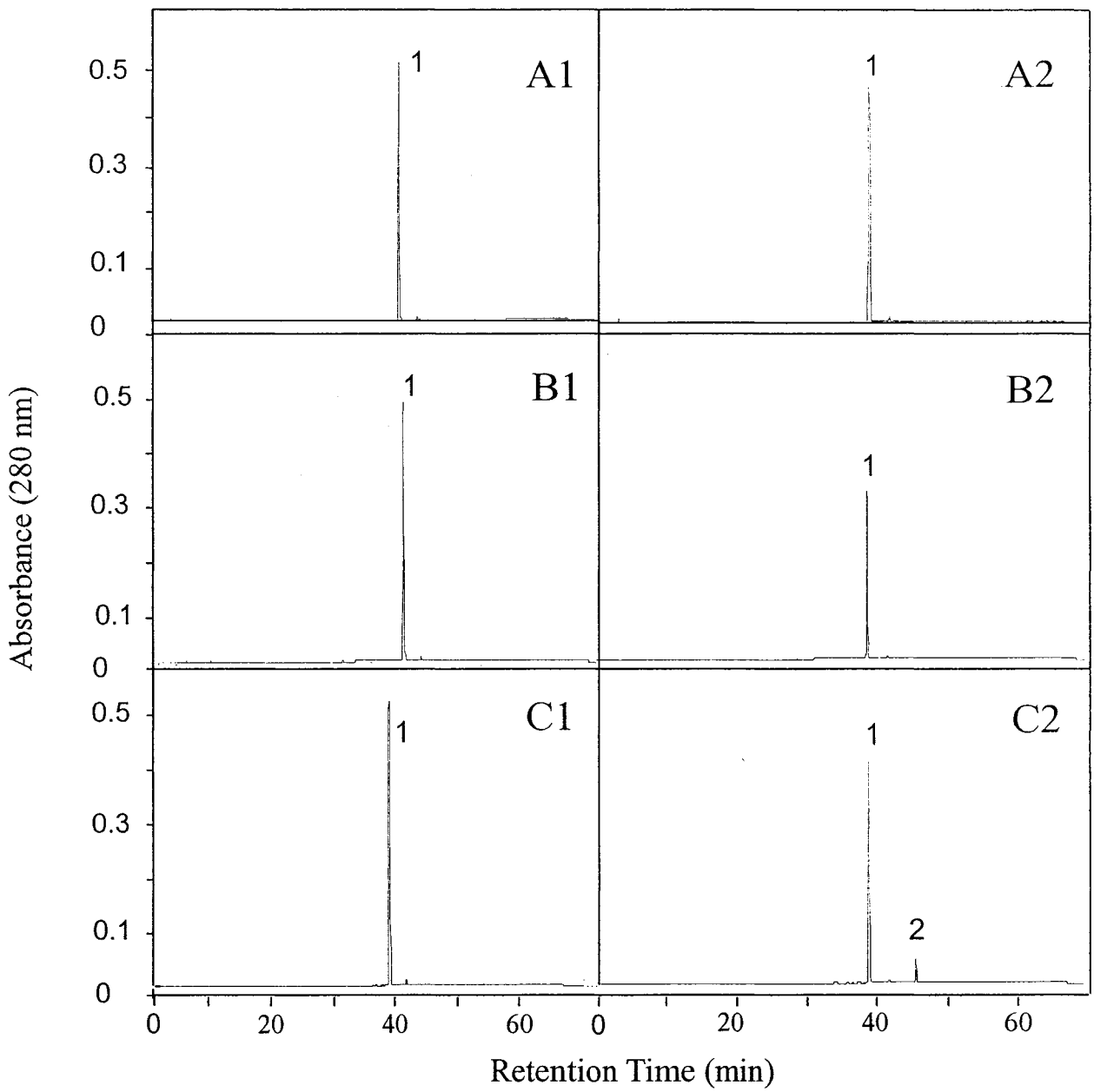


Figure 7. HPLC chromatogram, at 280 nm, obtained (1) before and (2) after enzymatic hydrolysis using (A) ß-galactosidase, (B) ß-glucosidase and (C) hesperinidase with rutin standard (peak 1) to produce quercetin (peak 2).

in the cleavage of quercetin-3-β-D-rutinoside to produce rutinose and quercetin, while the use of hesperinidase (Fig. 7C) resulted in the liberation of quercetin (peak 2), the aglycon part of rutin. On the basis of these results, hesperinidase was selected for the hydrolysis of the glycosylated phenolic compounds and flavonoids from maple products.

4.3.1.2. Effect of Incubation Time on Hydrolysis of a Glycosylated Flavonoid Model

The optimal incubation time for the hydrolysis of the glycosylated substrate model, rutin was investigated. Figure 8 shows the results obtained from the HPLC analysis of the enzymatic reactions at different times. The results indicate that there was a first-order increase in the quercetin peak area, considered to be the product of hydrolysis, after 24 h of incubation, followed by a slower rate of increase up to 36 h before reaching a zero-order rate of reaction. Simultaneously, the peak area of rutin, the substrate, decreased during the incubation time and appeared to stabilize after 36 h of incubation. These results suggest that most of the hydrolytic activity occurred during the first 36 h of incubation.

4.3.1.3. Effect of Enzyme Concentration on Hydrolysis of Maple Sap

To determine the optimal enzyme concentration for the hydrolysis of the multiple substrates consisting of the phenolic compounds and flavonoids from a maple sap extract, different concentrations of hesperinidase enzyme were used. Table12 summarizes the HPLC analysis of the phenolic compounds and flavonoids, in maple sap obtained at 0% of the maple sap season, that were hydrolyzed using a wide range of concentrations of hesperinidase. The experimental findings (Table 12) indicate that the entire enzyme concentrations used resulted in a decrease in the areas of peaks 1, 2, 4, 7, 8, 9, 14, 16, and 17. However, changes in the peak areas were more pronounced when 0.5 U of hesperinidase was used. These decreases in the peak areas of the phenolic compounds and flavonoids from the maple sap extract suggest that these analytes were glycosylated compounds. On the basis of these results, 0.5 U of hesperinidase was selected for the hydrolysis of the glycosylated fractions of maple sap and maple syrup extracts.

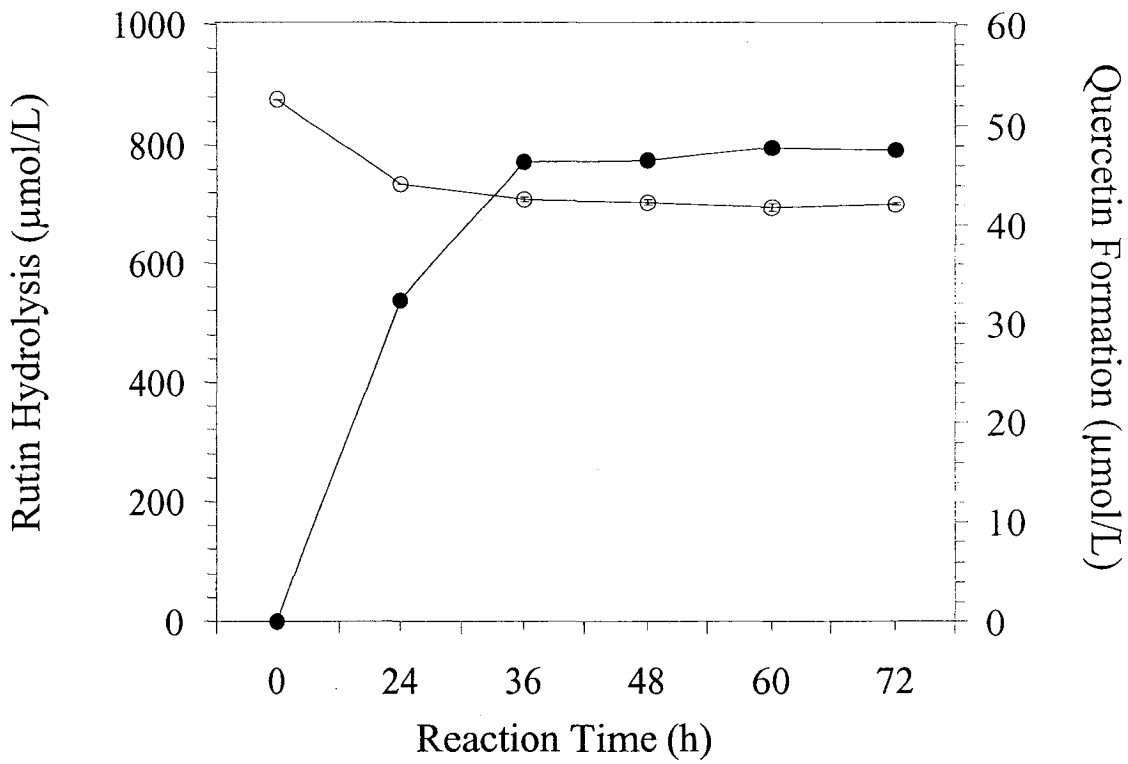


Figure 8. Effect of reaction time on the activity of hesperinidase for the hydrolysis of rutin (○) and formation of quercetin (●), by HPLC analysis at 280 nm.

Table 12. HPLC analysis, at 280 nm, of the phenolic compounds and flavonoids from maple sap obtained at 0% of the maple sap season, after hydrolysis with the hesperinidase enzyme preparation.

Peak No	Retention Time (min)[a]	Tentative Identification[b]	Relative Change in Peak Area (%)[c,d,e] Hesperinidase Concentrations (U)[f]					
			0.5	1.0	1.5	2.0	2.5	3.0
7	21.22	HBA[g] derivative	-23.33	-[i]	-20.00	-16.67	-13.33	-3.33
9	23.42	HCA[h] derivative	-27.42	+1.61	-20.97	-17.74	-16.13	-6.45
12	24.24	Flavanol derivative	-23.19	+13.04	-14.49	-11.59	-7.25	-[i]
13	24.59	Catechin	-33.12	+1.91	-19.75	-15.29	-14.65	-7.01
14	25.28	Undetermined	+1.79	+8.93	-23.21	-23.21	-26.79	-19.64
16	26.92	HCA[h] derivative	-68.75	-[i]	-18.75	-6.25	-[i]	+12.5
17'	28.77	Epicatechin	+30.77	-66.67	-38.46	-25.64	-64.10	-53.85
18	30.27	HCA[h] derivative	-42.22	-37.78	-26.67	-22.22	-48.89	-44.44
19	31.26	HBA[g] derivative	-50.00	-38.46	-30.77	-26.92	-46.15	-34.62
20	32.06	Coniferol	-23.61	+4.17	-16.67	-5.56	-2.78	+13.89
21	32.84	Syringaldehyde	-7.69	+7.69	-9.23	-4.62	-3.08	+33.85
24	34.16	Flavone derivative	-9.76	+4.88	-12.20	-10.98	-9.76	+6.10
27	35.64	Flavanol derivative	-12.95	+0.72	-17.99	-10.07	-9.71	+2.16
29	36.42	Flavanol derivative	-12.23	-0.44	-20.52	-11.79	-11.35	+1.31
30	36.94	Flavone derivative	+16.00	-12.00	+8.00	-4.00	-[i]	+28.00
33	37.71	Flavanol derivative	-20.00	+4.44	-20.00	-8.89	-13.33	-2.22
36	40.14	Flavanol derivative	-1.92	-5.77	-28.85	-26.92	-21.15	-13.46
37	40.44	Flavanol derivative	-3.77	+6.60	-17.92	-15.09	-5.66	+6.60
44	42.85	Flavanol derivative	-11.76	+3.92	-13.73	-9.80	-7.84	+11.76
48	44.40	Flavone derivative	-13.64	+2.27	-15.91	-43.18	-11.36	-[i]
54	47.51	Flavanol derivative	-10.00	+40.00	-20.00	-10.00	-10.00	-[i]

[a]Average retention time, calculated from triplicates, with a relative standard deviation of less than 5%.
[b]Tentative identification performed by comparing the spectrum of the compound with those of the standard compounds.
[c]The (–) sign indicates a decrease in the peak area and the (+) sign is an increase in peak area.
[d]Relative changes in peak area, calculated using this formula: [(Peak area of hydrolyzed maple extract - Peak area of maple extract)/ Peak area of maple extract]*100.
[e]Average peak area, calculated from triplicates, with a relative standard deviation of less than 5%.
[f]One unit of hesperinidase liberates 1.0 µmol of glucose from hesperidin/min.
[g]Hydroxybenzoic acid
[h]Hydroxycinnamic acid
[i]No changes were observed for this peak.

4.3.2. Hydrolysis and Characterization of Phenolic Compounds, Flavonoids and Their Sugar Components From Glycosylated Fractions of Maple Products

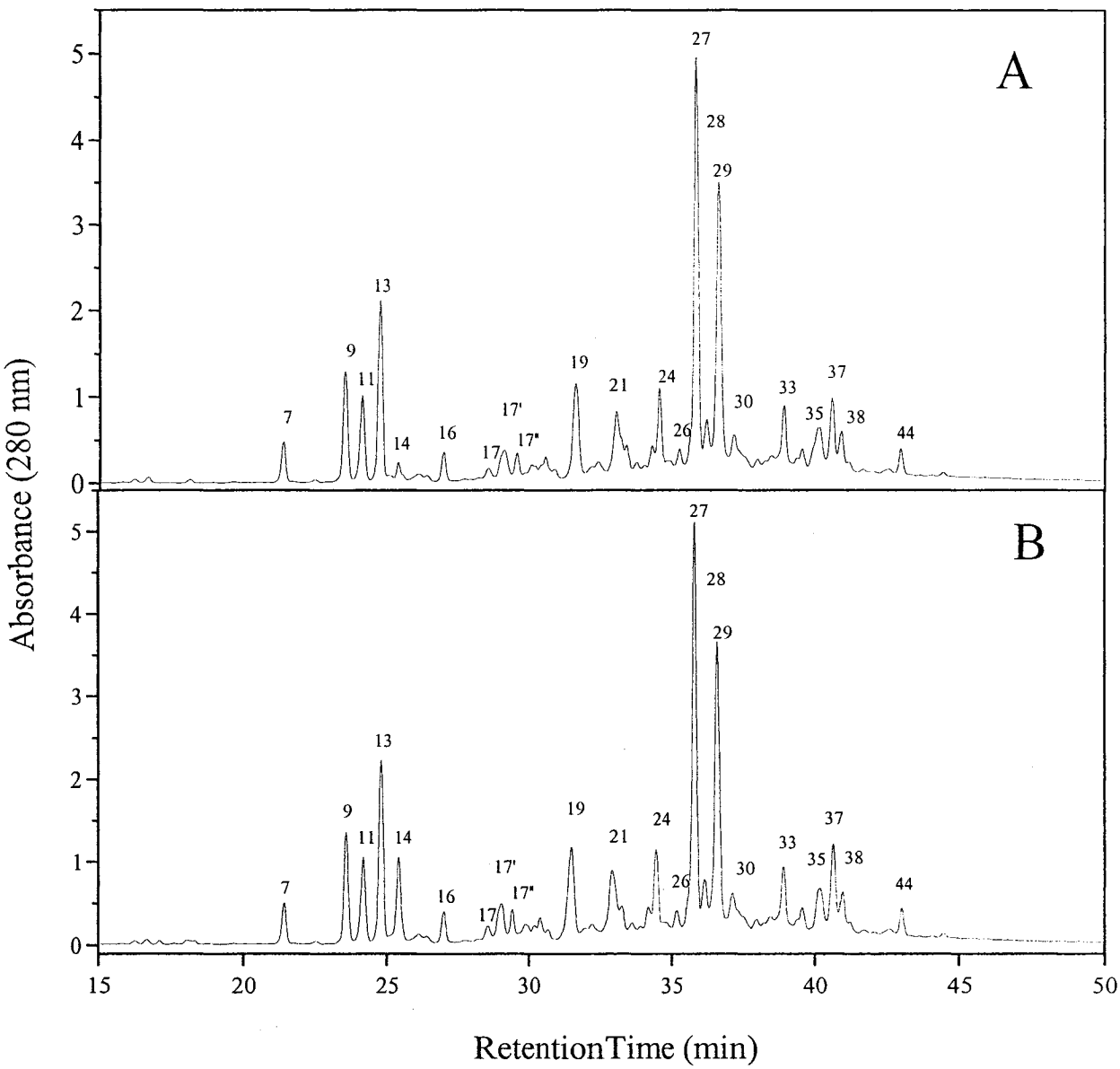
The HPLC analysis of the phenolic compounds and flavonoids aglycons that were released by enzymatic hydrolysis of the glycosylated fractions from maple products could provide interesting information about the possible nature of these glycosydically bound phenolic compounds and flavonoids. However, the nature of the released aglycons is also dependent of the enzyme preparation (Guanata *et al.*, 1988). In the present study, a commercial hesperinidase preparation, containing both α-L-arabinosidase and β-D-glucosidase activities, was used and the reaction was performed for 36 h at 40°C, to obtain adequate hydrolysis conditions. The hydrolyzed glycosylated fractions of maple products were subjected to HPLC for the analysis of phenolic compounds, flavonoids and sugar components.

4.3.2.1. Hydrolysis and Characterization of Phenolic Compounds and Flavonoids From Glycosylated Fractions of Maple Products

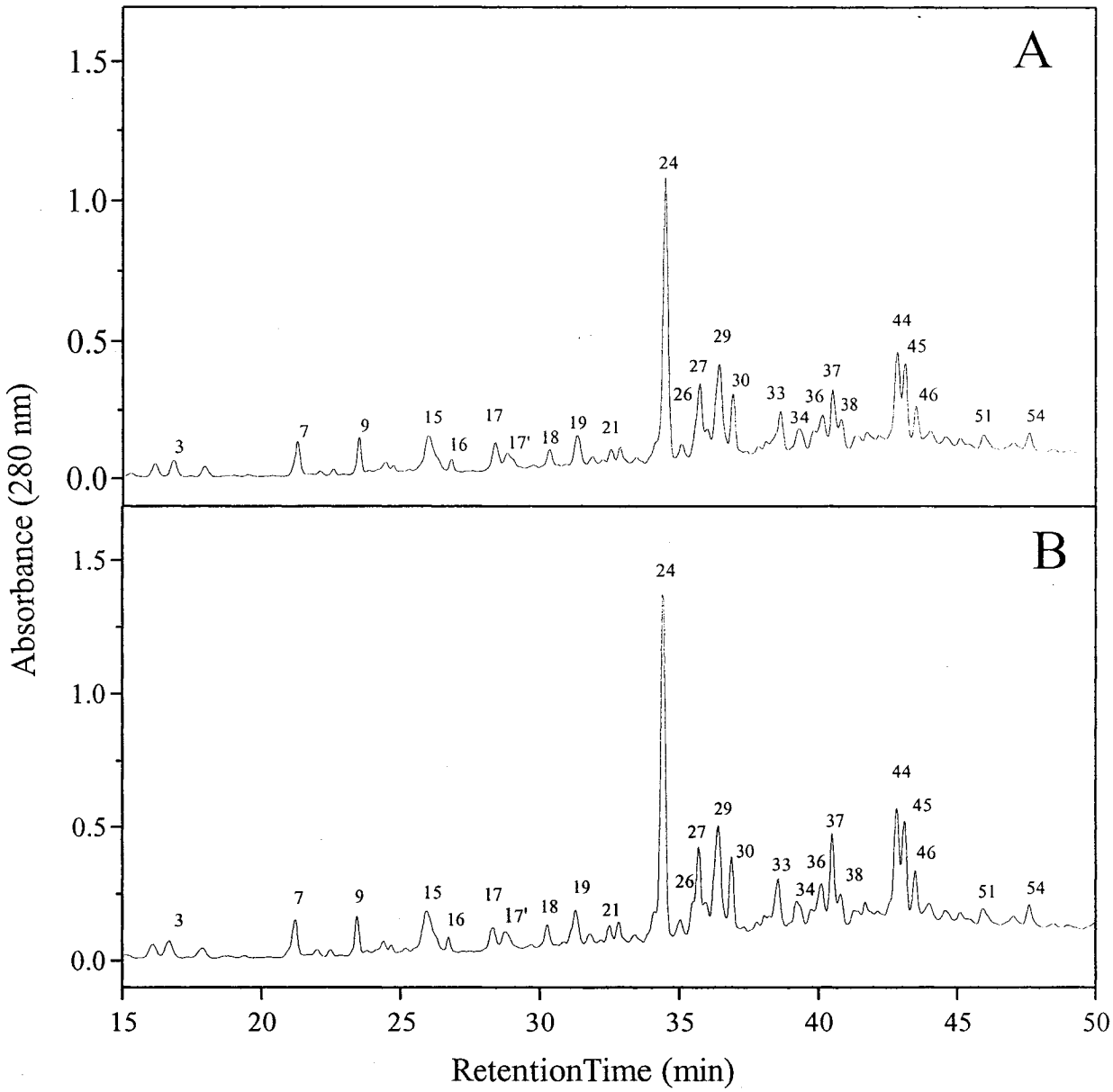
4.3.2.1.1. Hydrolysis and Characterization of Phenolic Compounds and Flavonoids From Glycosylated Fractions of Maple Sap

Figures 9 and 10 illustrate HPLC chromatograms of the phenolic compounds and flavonoids from the glycosylated fractions of maple sap, obtained at 0 and 100% of the maple sap season, respectively, and hydrolyzed by hesperinidase. The results (Fig. 9A and 10A) show the profiles of the phenolic compounds and flavonoids before hydrolysis with hesperinidase and after hydrolysis (Fig. 9B and 10B).

In the hydrolyzed glycosylated fraction of maple sap obtained at 0% of the maple sap season, the results (Fig. 9) illustrate that peak 14, an unidentified compound, may be present in maple sap as an aglycon since its area noticeably increased following enzymatic hydrolysis. In addition, hydrolysis resulted in an increase in the area of peak 37, which suggests the presence of an aglycon flavanol derivative. In the hydrolyzed glycosylated fraction of maple sap obtained at 100% of the maple sap season, the results (Fig. 10) suggest that peak 17 was a glycosylated HBA, since its peak area was reduced by enzymatic hydrolysis, whereas peak 18 could correspond to an aglycon HCA derivative as its peak area increased. Peak 24, a flavone derivative can be tentatively



Figures 9. HPLC chromatograms, at 280 nm, obtained (A) before and (B) after hydrolysis using the hesperinidase preparation, of the phenolic compounds and flavonoids from the glycosylated fraction of maple sap obtained at 0 % of the maple sap season.



Figures 10. HPLC chromatograms, at 280 nm, obtained (A) before and (B) after hydrolysis using the hesperinidase preparation, of the phenolic compounds and flavonoids from the glycosylated fraction of maple sap obtained at 100 % of the maple sap season.

classified as being an aglycon since hydrolysis resulted in an increase in its peak area. Increases in the areas of peaks 27, 33, 37, 44 and 45, considered to be flavanol derivatives, were also apparent after hydrolysis, thereby suggesting their presence as aglycons.

Comparisons of the relative peak areas of the phenolic compounds and flavonoids in the glycosylated fractions of maple sap, before (Table 7) and after hydrolysis (Table 13), indicated that it was possible to determine which phenolic compounds and flavonoids from maple sap were glycosylated or aglycons. The results indicated the presence of 6 glycosylated phenolic compounds and flavonoids and 11 aglycons in the glycosylated fractions of maple sap.

The results (Tables 7 and 13) show that the enzymatic hydrolysis of the glycosylated fractions of maple sap resulted in an increase in the relative peak area of peaks 14, 15, 19, 24, 27, 29, 30, 33, 37, 38 and 48, thereby suggesting that these peaks correspond to aglycon phenolic compounds and flavonoids. Moreover, throughout the maple sap season, a general decrease in the relative peak areas was observed for peaks 3, 9, 28, 36, 44 and 45, after hydrolysis of the glycosylated fractions of maple sap which could indicate the presence of glycosylated phenolic compounds and flavonoids.

4.3.2.1.2. Hydrolysis and Characterization of Phenolic Compounds and Flavonoids From Glycosylated Fractions of Maple Syrup

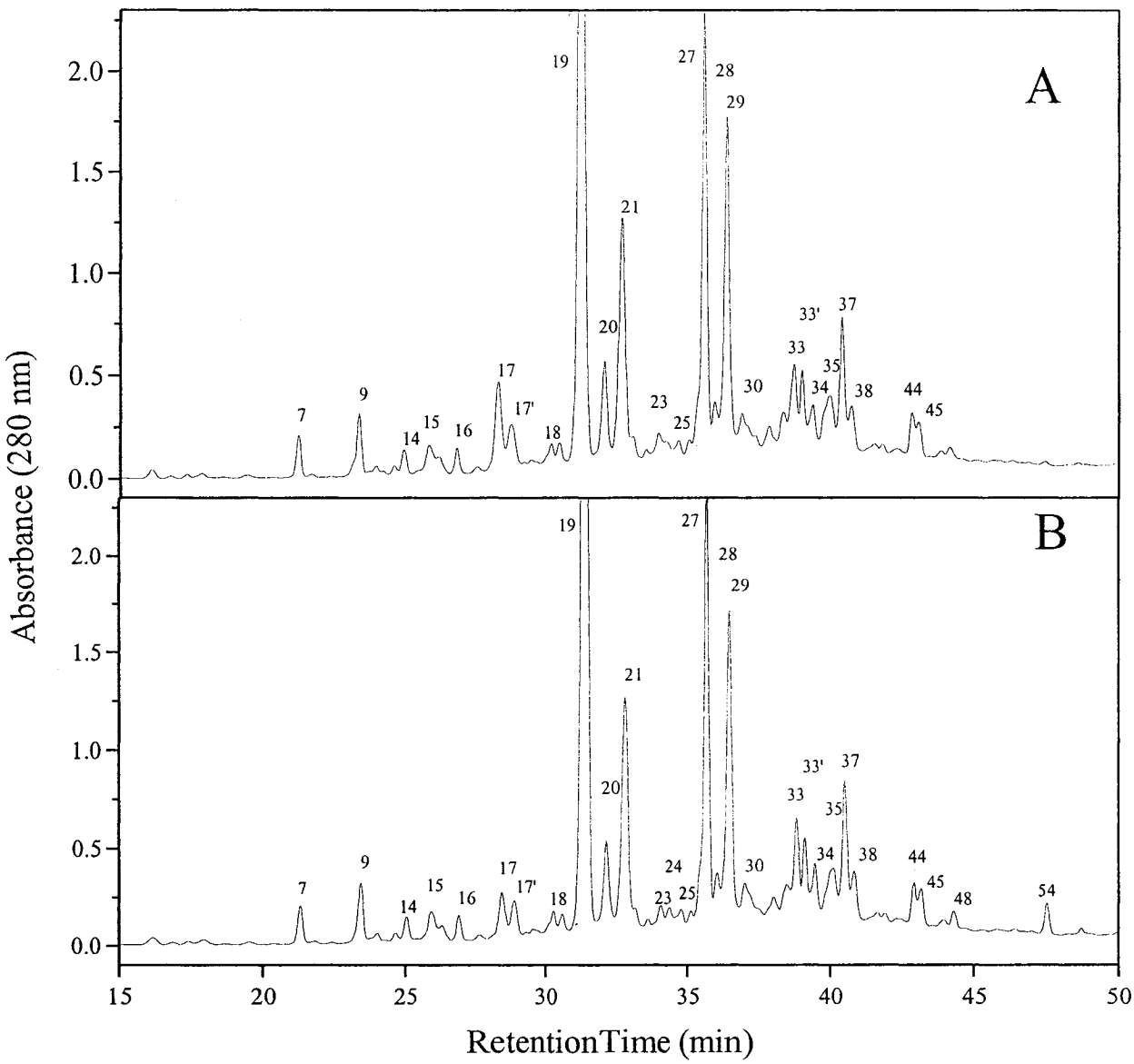
Figures 11 and 12 illustrate HPLC chromatograms of the phenolic compounds and flavonoids from the glycosylated fractions of maple syrup obtained, at 0 and 100% of the maple sap season, respectively, and hydrolyzed by hesperinidase. The results (Fig. 11A and 12A) show the profiles of the phenolic compounds and flavonoids before hydrolysis with hesperinidase and after the hydrolysis (Fig. 11B and 12B). Although the profiles of the phenolic compounds and flavonoids in the glycosylated fractions of maple syrup differed noticeably from those found in maple sap, similar changes could be observed after enzymatic hydrolysis.

In the hydrolyzed glycosylated fraction of maple syrup obtained at 0% of the maple sap season, the results (Fig. 11) illustrate that hydrolysis resulted in an important

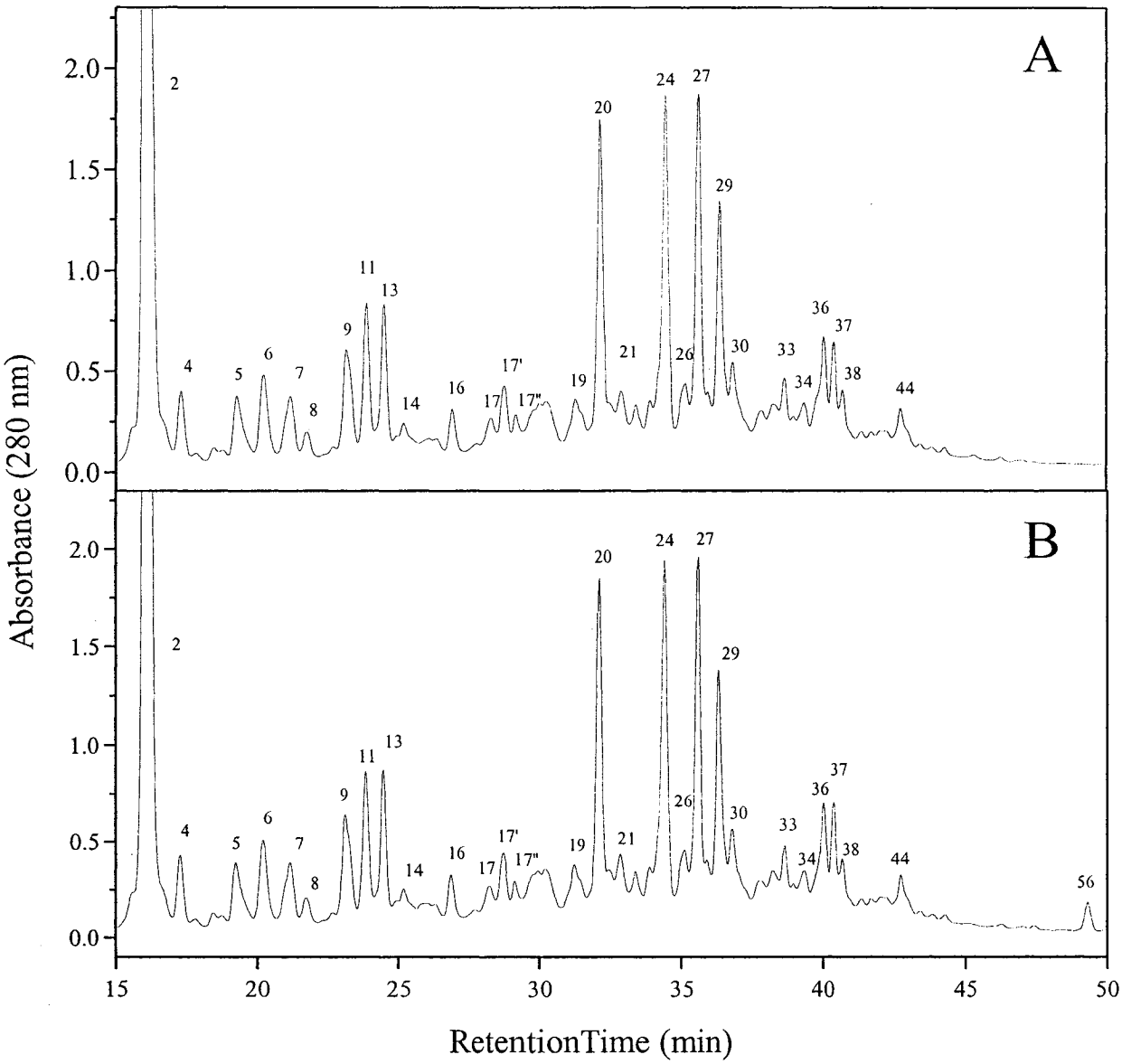
Table 13. HPLC analysis, at 280 nm, of the phenolic compounds and flavonoids recovered from the glycosylated fractions of maple sap obtained at different periods of the maple sap season, after hydrolysis with the hesperinidase enzyme preparation.

Peak No[a]	Retention Time (min)[b]	Tentative Identification[c]	Relative Peak Area (%)[d,e] Maple Sap Season Periods (%)[f] 0	25	50	75	100
3	16.56	HBA[g] derivative	0.18	9.88	4.20	4.66	1.44
4	17.77	HBA[g] derivative	0.13	0.92	1.20	1.26	0.00
7	21.22	HBA[g] derivative	1.55	2.30	1.92	2.93	1.92
8	21.97	HBA[g] derivative	0.00	0.00	1.03	0.00	0.00
9	23.42	HCA[h] derivative	4.26	3.53	1.86	1.94	2.23
11	24.02	Flavanol derivative	3.72	0.51	0.00	0.00	0.00
13	24.59	Catechin	7.42	0.00	0.82	0.00	0.00
14	25.28	Undetermined	4.22	9.43	9.84	0.00	0.00
15	25.91	HCA[h] derivative	0.00	15.91	9.93	21.79	4.94
16	26.92	HCA[h] derivative	1.14	0.00	0.00	1.44	0.00
17	28.33	HBA[g] derivative	0.67	0.79	0.89	1.56	1.49
17'	28.77	Epicatechin	2.10	0.00	0.00	0.00	1.61
17"	29.23	Flavanol derivative	1.29	0.00	0.00	0.00	0.00
18	30.27	HCA[h] derivative	1.56	0.34	0.00	0.00	1.12
19	31.26	HBA[g] derivative	5.42	1.97	2.66	3.25	2.68
20	32.06	Coniferol	0.00	0.74	1.38	1.55	0.00
21	32.84	Syringaldehyde	4.68	1.05	1.37	1.48	1.32
22	33.4	Flavanol derivative	0.00	3.17	1.39	0.00	1.40
23	34.02	Flavanol derivative	1.93	0.00	0.00	0.00	0.00
24	34.22	Flavone derivative	4.04	1.32	2.34	1.79	20.25
25	34.55	Undetermined	1.04	0.00	0.50	2.88	0.00
26	35.05	Flavanol derivative	1.55	0.36	1.01	0.00	1.61
27	35.64	Flavanol derivative	18.27	7.38	5.35	2.10	7.07
28	35.98	Flavanol derivative	2.72	3.44	3.11	2.49	2.25
29	36.42	Flavanol derivative	13.74	12.12	3.98	2.62	11.31
30	36.94	Flavone derivative	2.39	1.32	3.98	5.00	4.64
33	38.71	Flavanol derivative	4.12	0.18	0.80	1.94	3.72
34	39.35	Flavanol derivative	0.00	0.60	0.25	0.00	1.49
35	39.84	Undetermined	3.35	0.00	0.00	1.83	1.98
36	40.14	Flavanol derivative	0.00	2.96	3.29	4.42	3.95
37	40.44	Flavanol derivative	4.30	4.59	8.41	6.01	6.73
38	40.77	Flavanol derivative	2.05	1.99	1.39	2.94	2.02
39	41.47	Dihydroflavonol der.	0.00	0.00	1.34	2.10	0.00
40	41.8	Dihydroflavonol der.	0.00	0.00	0.50	0.00	1.94
44	42.85	Flavanol derivative	1.07	6.00	10.35	10.66	7.21
45	43.15	Flavanol derivative	0.00	4.79	9.88	9.10	6.28
46	43.48	Dihydroflavonol der.	0.00	0.00	0.54	0.00	3.07
47	43.90	Flavanol derivative	0.00	0.27	2.17	2.93	1.59
48	44.4	Flavone derivative	0.00	0.00	0.80	1.12	0.92
51	46.02	Undetermined	0.00	0.00	0.00	0.98	1.25
54	47.51	Flavanol derivative	0.00	0.00	1.13	1.03	1.38

[a]Peak number with respect to Figures 9 and 10.
[b]Average retention time, calculated from triplicates, with a relative standard deviation of less than 5%.
[c]Tentative identification performed by comparing the spectrum of the compound with those of the standard compounds.
[d]Relative percent area for each peak, calculated by dividing the area of each peak by the sum of the areas for each maple extract, and then multiplying by 100.
[e]Average relative peak area, calculated from triplicates, with a relative standard deviation of less than 5%.
[f]Maple sap samples collected at different periods during the maple sap season (0, 25, 50, 75 and 100%).
[g]Hydroxybenzoic acid.
[h]Hydroxycinnamic acid.



Figures 11. HPLC chromatograms, at 280 nm, obtained (A) before and (B) after hydrolysis using the hesperinidase preparation, of the phenolic compounds and flavonoids from the glycosylated fraction of maple syrup obtained at 0 % of the maple sap season.



Figures 12. HPLC chromatograms, at 280 nm, obtained (A) before and (B) after hydrolysis using the hesperinidase preparation, of the phenolic compounds and flavonoids from the glycosylated fraction of maple syrup obtained at 100 % of the maple sap season.

decrease in the peak area of a HBA derivative (peak 17), thereby confirming the results obtained from hydrolysis of the glycosylated fractions of maple sap. In addition, peaks 18, 24, 33, 45 and 54 were tentatively identified as phenolic compound and flavonoid aglycons since their areas noticeably increased following hydrolysis. In the hydrolyzed glycosylated fraction of maple syrup obtained at 100% of the maple sap season, the results (Fig. 12) suggest that peak 36 corresponds to a glycosylated flavanol derivative, since its peak area decreased after hydrolysis, whereas peaks 21 and 56 were tentatively identified as syringaldehyde and dihydroflavonol aglycons, respectively, since their areas increased after hydrolysis.

Comparison of the relative peak areas of the phenolic compounds and flavonoids in the glycosylated fractions of maple sap, before (Table 10) and after hydrolysis (Table 14), allowed the classification of the phenolic compounds and flavonoids from the glycosylated fractions of maple syrup. The results indicate the presence of 4 glycosylated phenolic compounds and flavonoids and 11 aglycons in the glycosylated fractions of the maple syrup extracts.

The results (Tables 10 and 14) show that the enzymatic hydrolysis of the glycosylated fractions of the maple syrup extracts resulted in an increase in the relative area of peaks 13, 19, 21, 24, 27, 29, 30, 33, 33', 34 and 37, suggesting that these peaks correspond to aglycon phenolic compounds and flavonoids. Moreover, throughout the maple sap season, a general decrease in the relative peak areas was observed for peaks 7, 9, 20 and 36, after hydrolysis of the glycosylated fractions of maple sap extracts.

4.3.2.2. Hydrolysis and Characterization of Sugar Components From Glycosylated Fractions of Maple Products

4.3.2.2.1. Elution Profile of Sugar Standards

Figure 13 depicts the HPLC elution profile of the standards of glucose, galactose, xylose, rhamnose, arabinose, fructose, mannose, sucrose and sophorose. All monosaccharides (peaks 1, 2, 3, 4, 5, 6 and 7) eluted within the first 15 min, whereas disaccharides (peaks 8 and 9) eluted just after 15 min.

Table 14. HPLC analysis, at 280 nm, of the phenolic compounds and flavonoids recovered from the glycosylated fractions of maple syrup obtained at different periods of the maple sap season, after hydrolysis with the hesperinidase enzymepreparation.

Peak No[a]	Retention Time (min)[b]	Tentative Identification[c]	Relative Peak Area (%)[d,e] Maple Sap Season Periods (%)[f] 0	25	50	75	100
2	16.29	HCA[g] derivative	0.00	0.00	0.00	0.79	45.72
4	17.28	HBA[h] derivative	0.00	0.00	1.28	1.34	2.03
5	19.33	HBA derivative	0.00	0.00	0.00	1.16	2.61
6	20.25	HBA[h] derivative	0.00	0.00	0.00	0.38	2.75
7	21.22	HBA[h] derivative	1.10	0.85	2.33	1.97	2.32
9	23.42	HCA[g] derivative	1.74	1.58	4.89	2.40	3.97
11	24.02	Flavanol derivative	0.00	0.00	1.50	1.75	4.03
13	24.59	Catechin	0.00	0.58	3.62	3.78	3.58
14	25.28	Undetermined	0.72	0.00	0.32	1.27	0.46
15	25.91	HCA[g] derivative	1.52	1.23	0.66	1.90	0.00
16	26.92	HCA[g] derivative	0.70	0.71	1.79	2.97	1.04
17	28.33	HCA[g] derivative	1.73	1.77	2.23	0.71	1.25
17'	28.77	Epicatechin	1.31	1.08	1.87	1.01	2.15
18	30.27	HCA[g] derivative	0.51	0.57	0.69	2.33	4.80
19	31.26	HBA[h] derivative	33.35	31.84	6.00	6.26	2.10
20	32.06	Coniferol	3.12	2.69	6.84	7.11	9.56
21	32.84	Syringaldehyde	8.37	8.20	4.13	4.22	2.48
24	34.22	Flavone derivative	0.53	1.68	2.79	1.21	10.71
25	34.55	Undetermined	0.51	0.63	1.15	1.10	0.00
26	34.99	Flavanol derivative	0.00	0.51	0.79	0.86	2.71
27	35.64	Flavanol derivative	12.46	10.19	15.14	15.50	9.74
28	35.98	Flavanol derivative	1.25	1.43	1.94	1.90	1.31
29	36.42	Flavanol derivative	9.09	9.04	11.01	11.23	7.59
30	36.94	Flavone derivative	0.82	1.13	2.36	2.34	2.74
33	38.71	Flavanol derivative	3.15	2.79	4.14	3.91	2.10
33'	39.21	Dihydroflavonol der.	2.24	2.59	1.65	1.76	0.78
34	39.35	Flavanol derivative	1.92	1.87	2.47	2.96	1.94
36	40.14	Flavanol derivative	1.42	2.50	3.57	3.22	3.13
37	40.44	Flavanol derivative	4.38	5.32	4.30	4.41	3.20
38	40.77	Flavanol derivative	1.52	1.60	1.73	1.71	1.66
44	42.85	Flavanol derivative	1.61	1.70	1.11	0.62	1.56
45	43.15	Flavanol derivative	1.10	0.91	0.49	0.00	0.00

[a]Peak number with respect to Figures 11 and 12.
[b]Average retention time, calculated from triplicates, with a relative standard deviation of less than 5%.
[c]Tentative identification performed by comparing the spectrum of the compound with those of the standard compounds.
[d]Relative percent area for each peak, calculated by dividing the area of each peak by the sum of the areas for each maple extract, and then multiplying by 100.
[e]Average relative peak area, calculated from triplicates, with a relative standard deviation of less than 5%.
[f]Maple syrup samples collected at different periods during the maple sap season (0, 25, 50, 75 and 100%).
[g]Hydroxycinnamic acid.
[h]Hydroxybenzoic acid.

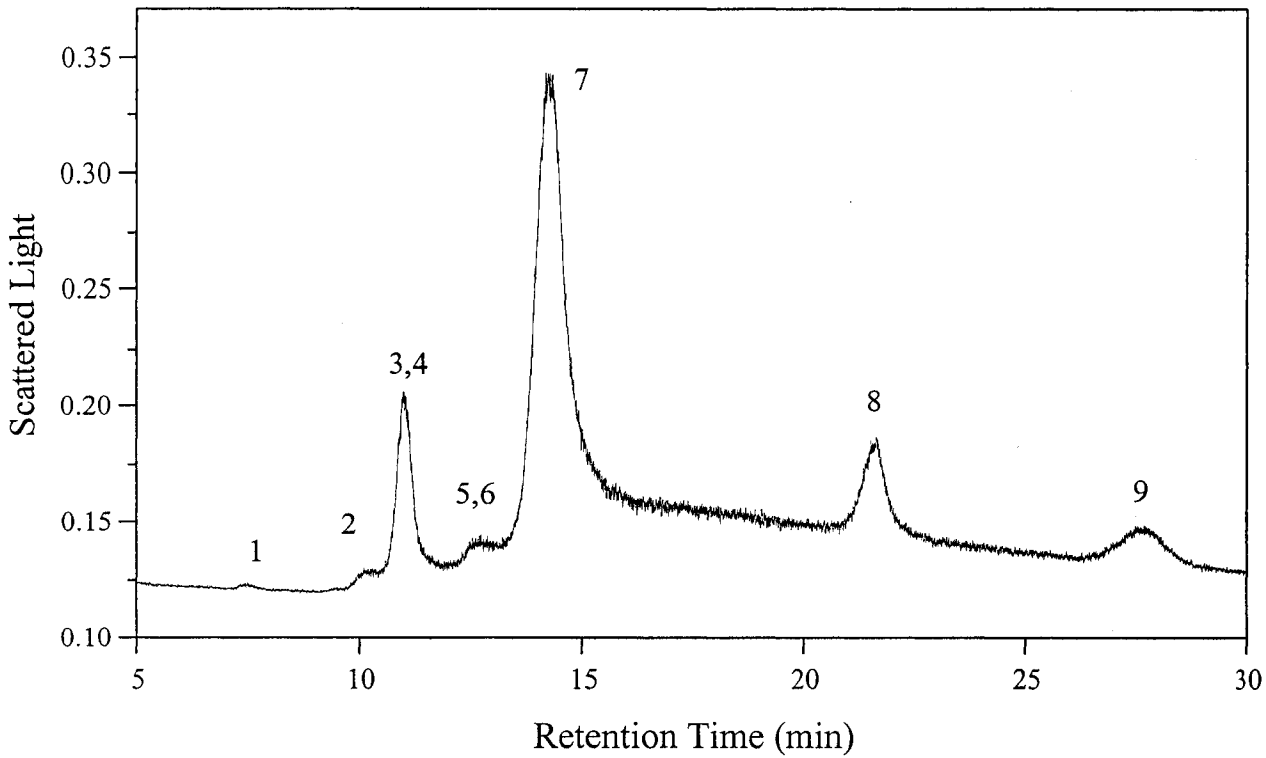


Figure 13. HPLC chromatogram of the sugar standards: rhamnose (1), xylose (2), arabinose and fructose (3 and 4), mannose and glucose (5 and 6), galactose (7), sucrose (8) and sophorose (9), using LLSD.

Table 15 shows the HPLC elution order of the sugar standards. Comparisons between the retention times of the sugar standards injected individually and as a mixture were performed. The results indicate that the average retention time was 7.48 min for rhamnose, 10.59 min for xylose, 14.18 min for galactose, 21.63 min for sucrose and 27.68 min for sophorose. When the sugar standards were injected individually, arabinose and fructose had similar retention times at, 10.00 and 9.99 min, respectively, which explains their co-elution, as peaks 3 and 4 (Fig. 13). The results also indicate that mannose and D-glucose had similar retention times and co-eluted just after fructose.

Although a similar HPLC elution profile was reported by Lee and Coates (2000), the retention times of the injected sugar standards reported were noticeably different. However, Lee and Coates (2000) used a slightly different isocratic elution system composed of 75% acetonitrile instead of 80% acetonitrile (Fig. 13). Nielsen *et al.* (1998) indicated that the sample retention times can be influenced by varying the concentration of the hydrophobic solvent of the mobile phase; a mobile phase with a lower percentage of organic solvent tends to increase the retention time, whereas a mobile phase with a higher percentage of organic solvent decreases it. However, the experimental findings (Table 15) showed the opposite trend, which may suggest that the differences in the mobile phase used were not sufficiently significant to influence the retention times. In addition, the column temperature used by Lee and Coates (2000) was 25°C compared to 40°C used in the present study. The chromatographic resolution of sugars is known to be dependent on the column temperature used for separation as higher temperatures tend to improve the solubility of sugars and hence increase their interactions with the stationary phase (White and Widmer, 1990).

4.3.2.2.2. Characterization of Sugar Components From Glycosylated Fractions of Maple Sap

Figure 14 illustrates the HPLC chromatograms for the sugars recovered from the glycosylated fractions of maple sap extracts obtained at 0 and 100% of the maple sap season. The results indicate the presence of 5 major peaks during the different periods of the maple sap season, including peaks 2, 3, 4, 6 and 7, which eluted between 7.05 and 19.28 min. Comparisons of the retention times of the selected peaks (Table 16), with

Table 15. HPLC analysis of sugar standards, using LLSD.

Peak No[a]	Standards	Retention Time (min)[b]	
		Individual	Mixture
1	Rhamnose	7.20	7.48
2	Xylose	9.15	10.59
3	Arabinose	10.00	11.07
4	Fructose	9.99	11.07
5	Mannose	12.44	12.75
6	Glucose	12.52	12.75
7	Galactose	14.57	14.18
8	Sucrose	17.62	21.63
9	Sophorose	27.45	27.68

[a]Peak number with respect to Figure 13.

[b]Average retention time, calculated from triplicates, with a relative standard deviation of less than 5%.

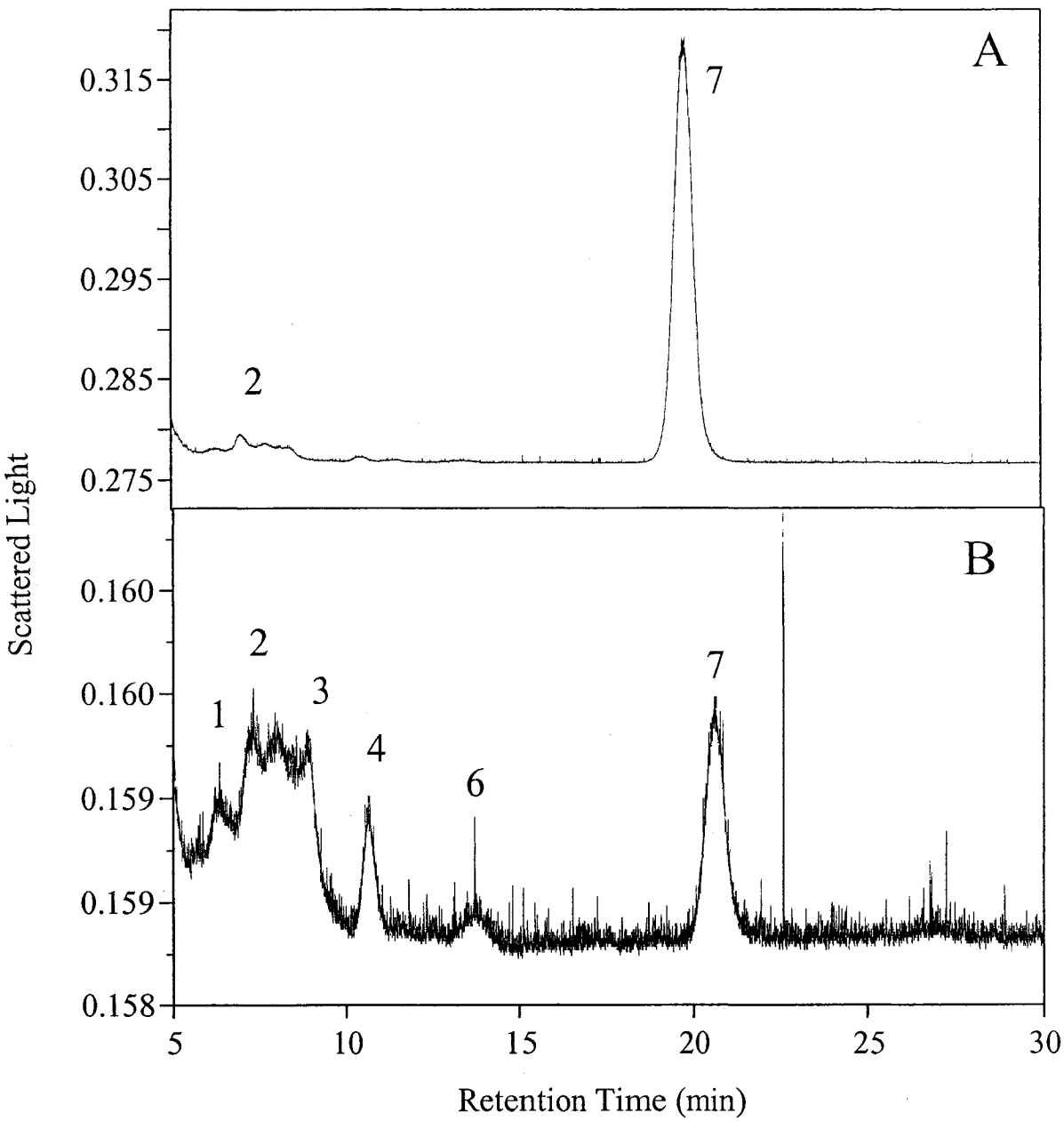


Figure 14. HPLC chromatograms of the sugar components from glycosylated fractions of maple sap obtained at (A) 0 and (B) 100% of the maple sap season, using LLSD.

Table 16. HPLC analysis of the sugar components recovered from the glycosylated fractions of maple sap obtained at different periods of the maple sap season, using LLSD.

Peak No[a]	Retention Time (min)[b]	Tentative Identification[c]	Relative Peak Area (%)[d,e] Maple Sap Season Periods (%)[f] 0	25	50	75	100
2	7.05	Rhamnose	1.34	2.18	1.55	3.78	15.54
3	8.25	Undetermined	0.00	8.13	19.24	41.45	18.99
4	10.37	Arabinose & fructose	0.67	2.05	0.68	8.27	15.46
6	13.19	Glucose & mannose	0.34	1.92	1.25	6.11	4.27
7	19.28	Sucrose	96.76	75.61	51.24	40.39	45.74

[a]Peak number with respect to Figure 14.
[b]Average retention time, calculated from triplicates, with a relative standard deviation of less than 5%.
[c]Tentative identification performed by comparing the retention time of the compound with those of the standard compounds.
[d]Relative percent area for each peak, calculated by dividing the area of each peak by the sum of the areas of each maple extract, and then multiplying by 100.
[e]Average relative peak area, calculated from triplicates, with a relative standard deviation of less than 5%.
[f]Maple sap samples collected at different periods during the maple sap season (0, 25, 50, 75 and 100%).

those of sugar standards (Table 15), suggests the presence of rhamnose (peak 2), arabinose and fructose (peak 4), glucose and mannose (peak 6), and sucrose (peak 7) in the glycosylated fractions of maple sap. Sucrose is known to be the most prevalent sugar in maple sap, comprising 98-99.9% of the dry matter, although the presence of lower concentrations of other sugars, such as fructose and glucose, has also been reported (Koelling and Heiligmann, 1996c; Stuckel and Low, 1996).

At the beginning of the maple sap season, the results (Table 16) indicate that sucrose, which eluted at 19.28 min, was the predominant sugar found in the glycosylated fractions of maple sap. As the sap season evolved, the results show an increase in the relative peak area of an unidentified sugar, peak 3 at 8.25 min, whose presence was predominant at 75% of the maple sap season (Table16). In addition, the results (Table 16) show an increase in the relative peak areas of rhamnose, fructose and glucose. The occurrence of these sugars in the later part of the maple sap season could result from the microbial fermentation of sucrose present in the maple sap, as the temperature increased during the maple sap season (Whalen and Morselli, 1985).

4.3.2.2.3. Characterization of Sugar Components From Glycosylated Fractions of Maple Syrup

Figure 15 shows the HPLC chromatograms of the sugars recovered from the glycosylated fractions of maple syrup obtained at 0 and 100% of the maple sap season. The presence of the same 5 major peaks, during the different periods of the maple sap season, was observed as those found present in maple sap (Fig. 14).

The results (Table 17) show that at the beginning of the maple sap season (0%), sucrose was the predominant sugar found in the glycosylated fractions of maple syrup; however, its presence appeared to be reduced compared to that reported in the glycosylated fractions of maple sap (Table 16). During the evolution of the maple sap season, the results (Table 17) indicate an increase in the relative peak area of an undetermined sugar, peak 3 at 8.25 min, whereas the relative peak areas of rhamnose, fructose and glucose remained approximately the same. The experimental results are in agreement with those reported by Stuckel and Low (1996), who determined that the

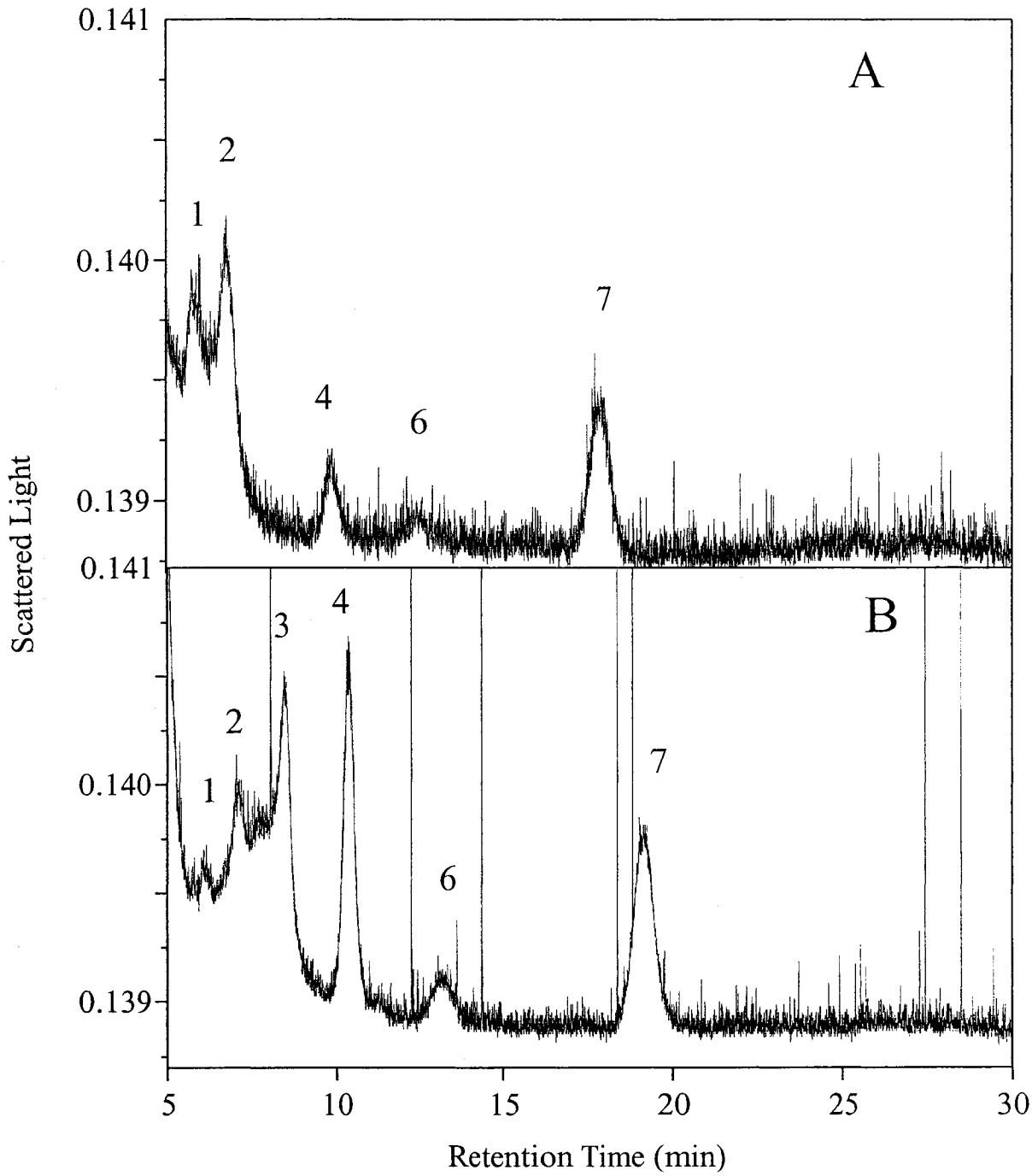


Figure 15. HPLC chromatograms of the sugar components from glycosylated fractions of maple syrup obtained at (A) 0 and (B) 100% of the maple sap season, using LLSD.

Table 17. HPLC analysis of the sugar components recovered from the glycosylated fractions of maple syrup obtained at different periods of the maple sap season, using LLSD.

Peak No[a]	Retention Time (min)[b]	Tentative Identification[c]	Relative Peak Area (%)[d,e] Maple Sap Season Periods (%)[f] 0	25	50	75	100
2	7.05	Rhamnose	7.78	17.05	17.03	8.49	5.76
3	8.25	Undetermined	0.00	0.00	0.00	37.72	20.66
4	10.37	Arabinose & fructose	9.21	13.80	16.26	5.67	32.69
6	13.19	Glucose & mannose	4.50	8.35	9.03	2.84	4.98
7	19.28	Sucrose	79.03	61.85	57.46	47.20	34.45

[a]Peak number with respect to Figure 15.
[b]Average retention time, calculated from triplicates, with a relative standard deviation of less than 5%.
[c]Tentative identification performed by comparing the retention time of the compound with those of the standard compounds.
[d]Relative percent area for each peak, calculated by dividing the area of each peak by the sum of the areas of each maple extract, and then multiplying by 100.
[e]Average relative peak area, calculated from triplicates, with a relative standard deviation of less than 5%.
[f]Maple syrup samples collected at different periods during the maple sap season (0, 25, 50, 75 and 100%).

sucrose content in maple syrup ranged from 51.7 to 75.6%, whereas glucose and fructose contents ranged from 0.00 to 9.59% and 0.00 to 3.95%, respectively.

4.3.2.2.4. Hydrolysis and Characterization of Sugar Components From Glycosylated Fractions of Maple Products

The enzymatic hydrolysis of the glycosylated fractions of both, maple sap (Table 18) and maple syrup (Table 19), indicates a general increase in the peak area of rhamnose. Five sugars have been commonly found as components of the glycosylated phenolic compounds and flavonoids, including glucose, galactose, xylose, rhamnose and arabinose (Duffus and Duffus, 1984, Harborne, 1998). The presence of glycosylated flavonoids bound to rhamnose has also been reported in literature (Harborne, 1998).

The experimental findings (Tables 18 and 19) suggest that rhamnose bound to the phenolic compounds and flavonoids, was liberated by the enzymatic hydrolysis of the glycosylated fractions of the maple products. However, the results (Tables 18 and 19) do not demonstrate an increase in the area of peak 6, tentatively identified as glucose and mannose in the glycosylated fractions of the maple products; on the contrary, the results indicate a decrease in the area of peak 6. Thse experimental findings could be explained by the possibility that the hydrolytic reaction was influenced by the presence of sucrose and the inverted sugars in the commercial enzyme extract. The HPLC analysis of the sugars present in the hesperinidase commercial enzyme (Fig. 16) clearly indicates the presence of one major peak of sucrose (peak 7) and minor ones of fructose and glucose, peaks 4 and 6, respectively; hence, the presence of these sugars could influence the effect of enzymatic hydrolysis on the liberation of sugars from the glycosylated phenolic compounds and flavonoids from maple products. Although sucrose and inverted sugar were described as the major components in maple sap (Koelling and Heiligmann, 1996c; Stuckel and Low, 1996), as the author is aware there is little information on the nature of sugars associated to phenolic compounds and flavonoids in maple sap.

Table 18. HPLC analysis of the sugar components recovered from the glycosylated fractions of maple sap obtained at different periods of the maple sap season, after hydrolysis with the hesperinidase enzyme preparation, using LLSD.

Peak No	Retention Time (min)[a]	Tentative Identification[b]	Relative Change in Peak Area (%)[c,d,e] Maple Sap Season Periods (%)[f] 0	25	50	75	100
2	7.05	Rhamnose	+281.18	+262.99	+314.94	+214.07	+605.75
3	8.25	Undetermined	-51.25	-59.32	-96.46	-87.82	-100.00
4	10.37	Arabinose & fructose	-57.92	-44.39	-57.21	-97.29	-96.01
6	13.19	Glucose & mannose	-52.17	-55.44	-64.33	-98.11	-98.06
7	19.28	Sucrose	+24.35	-4.72	-20.65	+54.50	-14.03

[a]Average retention time, calculated from triplicates, with a relative standard deviation of less than 5%.

[b]Tentative identification performed by comparing the retention time of the compounds with those of the standard compounds.

[c]The (–) sign indicates a decrease in the peak area and the (+) sign is an increase in peak area.

[d]Relative changes in the peak areas, calculated by using this formula: [(Peak area of hydrolyzed maple extract - Peak area of maple extract)/ Peak area of maple extract]*100.

[e]Average peak area, calculated from triplicates, with a relative standard deviation of less than 5%.

[e]Maple sap samples collected at different periods during the maple sap season (0, 25, 50, 75 and 100%).

Table 19. HPLC analysis of the sugar components recovered from the glycosylated fractions of maple syrup obtained at different periods of the maple sap season, after hydrolysis with the hesperinidase enzyme preparation, using LLSD.

Peak No	Retention Time (min)[a]	Tentative Identification[b]	Relative Change in Peak Area (%)[c,d,e] Maple Sap Season Periods (%)[f] 0	25	50	75	100
2	7.05	Rhamnose	+158.37	+216.54	-0.32	+18.87	-16.86
3	8.25	Undetermined	-100.00	-100.00	-80.65	-95.01	-88.75
4	10.37	Arabinose & fructose	-100.00	-96.98	-94.93	-98.27	-96.02
6	13.19	Glucose & mannose	-98.10	-98.00	-96.68	-98.48	-98.19
7	19.28	Sucrose	+32.37	+64.11	+104.22	+6.37	+20.96

[a]Average retention time, calculated from triplicates, with a relative standard deviation of less than 5%.

[b]Tentative identification performed by comparing the retention time of the compounds with those of the standard compounds.

[c]The (–) sign indicates a decrease in the peak area and the (+) sign is an increase in peak area.

[d]Relative changes in the peak areas, calculated by using this formula: [(Peak area of hydrolyzed maple extract - Peak area of maple extract)/ Peak area of maple extract]*100.

[e]Average peak area, calculated from triplicates, with a relative standard deviation of less than 5%.

[e]Maple syrup samples collected at different periods during the maple sap season (0, 25, 50, 75 and 100%).

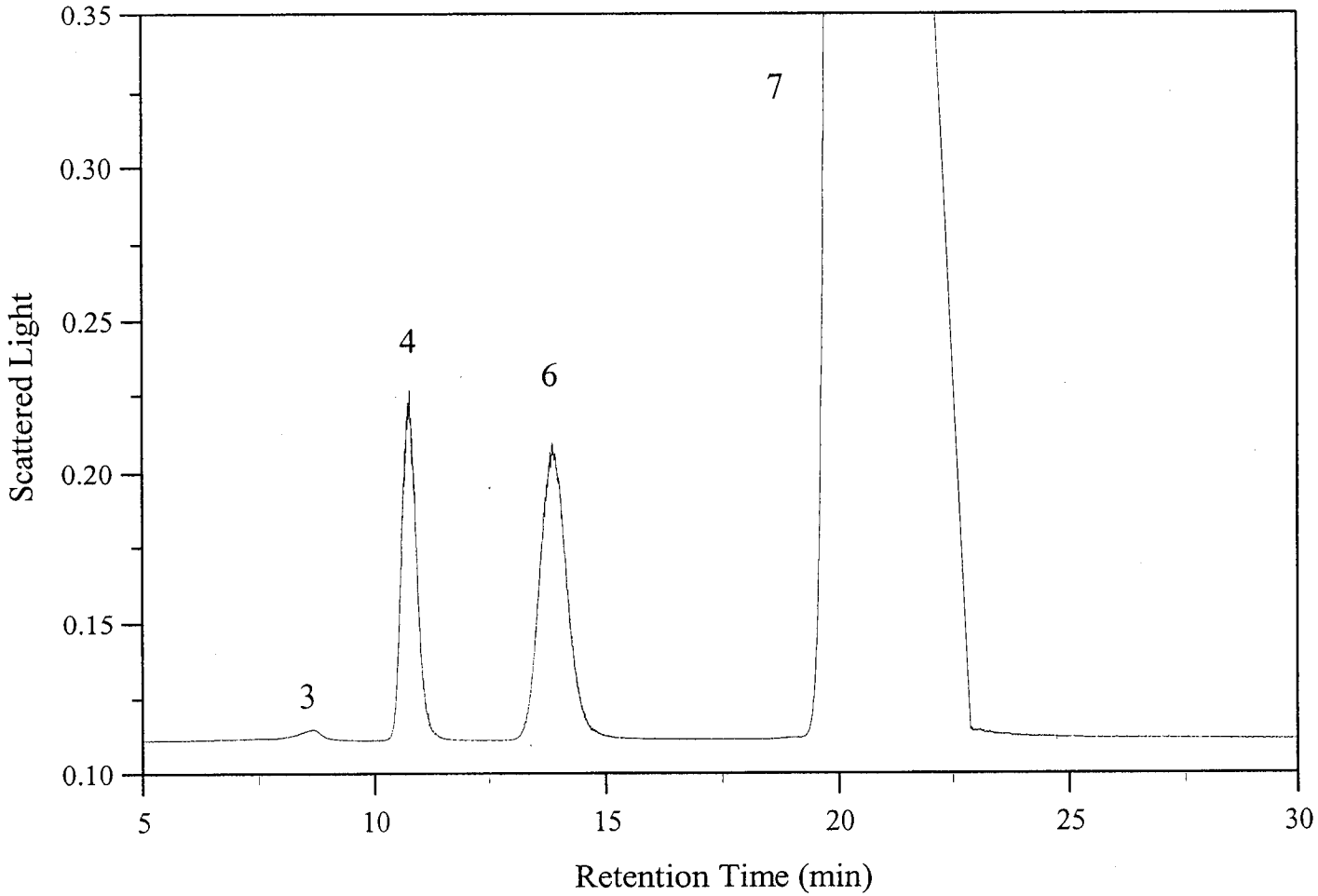


Figure 16. HPLC chromatogram of the sugar components, fructose (peak 4), glucose (peak 6) and sucrose (peak 7), obtained from the commercial hesperinidase enzyme extract, using LLSD.

4.4. Changes in Phenolic Compound and Flavonoid Profile During the Maple Sap Season

The average retention time and relative peak area for each peak during the different periods of the maple sap season were calculated and statistical analysis was performed from data obtained from HPLC analysis of triplicate maple samples. Precision, which represents the relative standard deviation, was less than 5.0% (Tables 6, 7, 8, 9, 10 and 11).

4.4.1. Changes in Phenolic Compound and Flavonoid Profile in Maple Sap

4.4.1.1. Maple Sap

The analysis of the seasonal evolution of the phenolic compounds and flavonoids in maple sap (Table 6) indicates that peaks 27 and 29 were the only two compounds highly present during the different periods of the maple sap season, with values ranging from 16.7 to 12.6% for peak 27 and from 12.5 to 10.9% for peak 29 obtained at 0 and 100% of the sap season, respectively.

Moreover, the results (Table 6) show that the relative predominance of peaks 11, 13, 19, 21, 27, 29, 35 and 37 tended to decrease as the maple sap season evolved. Peak 15 appears to be predominant only at 25 and 75% of the maple sap season, whereas peak 36 was not detected at 0% of the maple sap season, but appeared at 50% of the sap season. The relative area of peak 24 was constant at approximately 3.5% throughout the sap season and increased to 16.0% at 100% of the sap season. A similar trend was observed for peak 44; however, its greatest predominance was seen at 75% of the sap season. The results show that peak 36 occurred during the middle of the sap season and its relative concentration remained constant for the rest of the maple sap season. The relative predominance of peak 48 increased during the sap season, but returned to its initial value at the end of the sap season. The overall results are in agreement with those reported by Deslauriers (2000), with the exception being peaks 15 and 21, where the opposite seasonal evolution trend was observed.

4.4.1.2. Glycosylated Fractions of Maple Sap

The analysis of the seasonal evolution of the phenolic compounds and flavonoids obtained from the glycosylated fractions of maple sap (Table 7) indicates a similar trend to that observed for maple sap (Table 6). Peaks 27 and 29 were the only two compounds highly present during the different periods of the maple sap season, with values ranging from 19.1 to 8.7% for peak 27 and from 14.2 to 10.2% for peak 29 obtained at 0 and 100% of the sap season, respectively.

The results (Table 7) show that the predominant peaks 12, 19, 21, 27, 29 and 35 in the glycosylated fractions of maple sap tended to decrease during the maple sap season, whereas that of peaks 28 and 38 remained constant. However, the presence of peaks 24 and 39 increased as the maple sap season evolved. As noticed in maple sap, peak 36 and 45 was not detected at 0% of the maple sap season but appeared at 25% of the maple sap season.

4.4.1.3. Aglycon Fractions of Maple Sap

The analysis of the seasonal evolution of the phenolic compounds and flavonoids obtained from the aglycon fractions of maple sap (Table 8) indicates a similar trend to that observed in for maple sap (Table 6). Peaks 44, 48 and 49, the predominant compounds in the aglycon fractions, were present during the different periods of the maple sap season, with values ranging from 12.4 to 12.0% for peak 44, from 27.8 to 12.7% for peak 48 and from 9.6 to 9.3 for peak 49 obtained at 0 and 100% of the sap season, respectively.

The results (Table 8) show that peaks 35 and 37 were present throughout the maple sap season, with relative peak areas of 12.7 and 17.9%, respectively, at 25% of the sap season. The results also indicate a decrease in the predominance of peaks 43 and 50 throughout the sap season. Peaks 41 and 42 were not detected at the beginning of the sap season, but appeared at 25 and 50% of the sap season, respectively; the relative area of peak 41 continued to increase during the season, whereas peak 42 remained the same.

4.4.2. Changes in Phenolic Compound and Flavonoid Profile in Maple Syrup

4.4.2.1. Maple Syrup

The analysis of the sap seasonal evolution of the phenolic compounds and flavonoids in maple syrup (Table 9) indicates that peaks 19, 20, 21, 27 and 29 were highly present compounds during the different periods of the maple sap season, with values ranging from 31.6 to 1.03% for peak 19, from 10.1 to 5.8% for peak 20, from 17.4 to 3.3% for peak 21, from 10.3 to 4.9% for peak 27 and from 9.5 to 3.3% for peak 29 obtained at 0 and 100% of the sap season, respectively.

The results (Table 9) show that the relative predominance of peaks 19, 20, 21, 27, 29 and 37 tended to decrease as the maple sap season evolved. Peak 2 was not detected throughout the sap season, but appeared at the end of the sap season as a predominant compound. Generally, the relative areas of peaks 30, 33, 33', 34, 36, 44 and 48 remained constant during the maple sap season, but decreased at the end. For most parts, these results are in agreement with those reported by Deslauriers (2000), with the exception of peaks 9 and 20, where the opposite seasonal trend was found.

4.4.2.2. Glycosylated Fractions of Maple Syrup

The analysis of the seasonal evolution of the phenolic compounds and flavonoids obtained from the glycosylated fractions of maple syrup (Table 10) indicates a similar trend to that observed in maple syrup (Table 9). Peaks 19, 20, 21, 27 and 29 were highly present compounds during the different periods of the maple sap season, with values ranging from 28.3 to 1.21% for peak 19, from 3.6 to 6.2% for peak 20, from 8.1 to 2.0% for peak 21, from 12.5 to 5.7% for peak 27 and from 11.3 to 4.5% for peak 29 obtained at 0 and 100% of the sap season, respectively.

The results (Table 10) show that the relative predominance of peaks 19, 21, 27, 29 and 37 tended to decrease as the maple sap season evolved. Peak 2 was not detected throughout the sap season, but appeared at the end of the sap season as a predominant compound. The relative areas of peaks 9, 13, 28, 30, 33, 33', 34, 36 and 38 remained constant during the maple sap season, but those of peaks 28, 30, 33, 33', 34, 36 and 38 tended to decrease at the end. Overall, these results are in agreement with those observed

in maple syrup, with the exception being peak 20 that tends to become predominant as the maple sap season evolves.

4.4.2.3. Aglycon Fractions of Maple Syrup

Analysis of the sap seasonal evolution of the phenolic compounds and flavonoids obtained from the aglycon fractions of maple syrup (Table 11) indicates a similar trend to that observed in maple syrup (Table 9). Peaks 42, 44, 48 and 49 were predominant during the different periods of the maple sap season, with values ranging from 7.5 to 8.5% for peak 42, from 7.5 to 12.1% for peak 44, from 14.5 to 6.4% for peak 48 and from 7.6 to 4.3 for peak 49 obtained at 0 and 100% of the sap season, respectively.

The results (Table 11) show that the relative predominance of peaks 39, 40, 48, 49, 50, 51 and 52 tended to decrease as the maple sap season evolved. Peak 6 was detected at the beginning of the maple sap season, but disappeared at the end of the season. The results show that the relative areas of peaks 41 and 43 gradually increased until 50% of the maple sap season and then stared to decrease. Generally, the presence of peaks 36, 37 and 42 remained constant throughout the maple sap season.

5. CONCLUSION

Using a wide range of supports, including C18 Extra-Clean, DSC-18, DPA-6S, Oasis HLB and Amberlite XAD-2, for the separation of the glycosylated phenolic compounds and flavonoids from aglycons, Amberlite XAD-2 resin was selected as the most efficient. Commercial hesperinidase enzyme was also selected, among a series of commercial enzyme preparations, for the hydrolysis of the glycosylated fractions of maple products.

A series of selected phenolic compounds, including coniferol, syringaldehyde, and HBA and HCA derivatives were tentatively identified in maple products during various periods of the maple sap season. The presence of (+)-catechin, (-)-epicatechin, flavanols, dihydroflavonols and flavone related compounds was also demonstrated in maple products. The experimental results also indicated that the major phenolic compounds and flavonoids present in the glycosylated fractions of maple products, were present as aglycons, including HCA, HBA, flavanol and flavone derivatives; the respective glycosylated compounds were present at a lower concentrations. The experimental findings also suggested the presence of rhamnose, fructose, glucose and sucrose in the glycosylated fractions of maple products. In addition, as the maple sap season evolved, the relative predominance of sucrose decreased, while the other sugar components increased. HPLC analysis of the sugar components of the hydrolyzed glycosylated fractions of maple products suggested that rhamnose bound to the phenolic compounds and flavonoids were liberated by the enzymatic hydrolysis of these glycosylated fractions.

The glycosylated phenolic compounds and flavonoids obtained from maple sap and subsequent maple syrup products during the 0, 25, 50, 75 and 100% of the maple sap season were recovered, separated and characterized. The overall results indicated that maple sap obtained at the later stages of the maple sap season contained higher levels of glycosylated phenolic compounds and flavonoids compared to those found in maple sap at the earlier stages of the season. The opposite trend was observed for the glycosylated phenolic compounds and flavonoids in maple syrup.

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