Analyse des effets du procédé de jus sur le taux de récupération et la conservation des composés phénoliques de la canneberge et sur leurs propriétés

Thèse présentée à l'INRS-Institut Armand-Frappier pour l'obtention du grade de Philosophiae Doctor (*Ph. D.*) en Biologie

Par

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Résumé

La canneberge est une baie attrayante, tant au niveau de sa couleur, sa saveur, que ses bienfaits santé. L'engouement pour ce fruit de l'Amérique du Nord (*Vaccinium macrocarpon*) n'a fait que croître depuis la publication d'études faisant état de ses bienfaits potentiels liés à sa teneur élevée en anthocyanes, flavonols, flavan-3-ols, proanthocyanidines et dérivés d'acides phénoliques.

Plusieurs études ont évalué les propriétés antimicrobienne et antioxydante de la canneberge. L'activité antimicrobienne spécifique à la canneberge congelée, au concentré de jus et à la poudre a été démontrée sur plusieurs bactéries pathogènes, incluant entre autres les bactéries *Helicobacter pylori*, *Salmonelle*, *Staphylococcus aureus*, *Escherichia coli* et *Campylobacter*. Les propriétés antioxydantes de la canneberge sont bien documentées dans la littérature et selon la méthode de mesure des capacités antioxydantes *Oxygen Radical Absorbance Capacity* et d'autres méthodes, le fruit se classe parmi les meilleurs antioxydants.

À cause de sa saveur amère et son goût acidulé, la canneberge est rarement consommée à l'état naturel. Elle est plutôt transformée en jus ou en concentré de jus, et ensuite mélangée à de l'eau et du sucre pour faire une boisson de jus de fruit. Très peu d'études ont tenté d'évaluer les effets des conditions de procédés de transformation sur les composés bioactifs et les propriétés de la canneberge. La congélation-décongélation du fruit avant le broyage semble augmenter la teneur en anthocyanes du jus et l'usage d'enzymes améliore le taux d'extraction de jus. Puisque la pectinase entraîne toutefois une dégradation et un brunissement de la couleur du jus, les effets de différents dosages et de mélanges d'enzymes sur la couleur ont également été testés. Récemment, les chercheurs ont surtout déployé leurs efforts à la caractérisation des composés bioactifs dans différents produits à base de canneberges (fruit frais et congelé, jus, confiture, concentré, poudre et marc). C'est pourquoi nous avons décidé d'analyser les effets du procédé de jus sur le taux de

récupération des phénols, les propriétés antimicrobiennes et les propriétés antioxydantes de la canneberge.

Nous avons confirmé que le broyage et la dépectinisation du fruit augmentait le taux de récupération des phénols totaux. Malgré les traitements de chaleur, hydrolyse enzymatique et d'oxydation, des quantités importantes de phénols ont été mesurées dans le marc (résidu de pressage du jus). Nous avons également constaté que les composés phénoliques hydrophiles et anthocyaniques extraits des échantillons des canneberges les plus transformés étaient les plus efficaces pour inhiber la croissance des bactéries *Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella.* Typhimurium et *Enterococcus faecium* resistant à la vancomycine. Le procédé a toutefois nuit aux propriétés antimicrobiennes des composés phénoliques apolaires de la canneberge contre les bactéries *Staphylococcus aureus, Listeria. monocytogenes, Escherichia coli* O157:H7 *et Enterococcus faecium* résistant à la vancomycine. Dans l'ensemble, le procédé de jus a diminué les capacités antiradicalaire et inhibitrice de peroxydation des lipides des composés phénoliques hydrophiles de la canneberge. La capacité inhibitrice de peroxydation des lipides des composés phénoliques apolaires et des anthocyanes a été réduite lors du broyage, mais à l'inverse cette étape a augmenté leur capacité antiradicalaire. L'évaporation n'a pas nuit au pouvoir antiradicalaire du jus de canneberges.

Jacinthe Côté, PhD

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Abréviations

APCI	Atmospheric pressure chemical ionization
APESI	Atmospheric pressure electrospray ionization
API	Atmospheric pressure ionization
BHI	Brain heart infusion
C-C	Carbon-carbon
CFU	Colony forming unit
CID	Collision-induced dissociation
CJ	Cranberry juice
CMI	Concentration minimale inhibitrice
C-O	Carbon-oxygen
DAD	Diode array detection
DNA	Desoxy ribonucleic acid
DPD	N,N-diethyl-p-phenylenediamine
DPPH	2,2'-diphenyl-1-picrylhydrazyl
DW	Dry weight
E1	Water-soluble phenolic compounds
E2	Apolar phenolic compounds
E3	Anthocyanins
EAG	Équivalent d'acide gallique
EC_{50}	Effective concentration
ERV	*Enterococcus faecium* resistant to vancomycin
ESI	Electrospray ionization
ET	Équivalent Trolox
FRS	Free radical scavenging
GAE	Gallic acid equivalent
HBA	Hydroxybenzoic acid
HCA	Hydroxycinnamic acid
HPLC	High-performance liquid chromatography
IPL	Inhibition de la peroxydation des lipides
LDL	Low-density lipoprotein
LPI	Lipid peroxidation inhibition
MIC	Minimum inhibitory concentration
MS	Mass spectrometer ou Matières sèches
MTC	Maximal tolerated concentration
OD	Optic density
ORAC	Oxygen radical absorbance capacity
PPO	Polyphenol peroxidase
PRL	Piégeage des radicaux libres
PT	Phénols totaux
ROS	Reactive oxygen species
SPE	Solid-phase extraction
TBARS	Thiobarbituric acid reactive substances
TE	Trolox equivalent
TP	Total phenolic compound
UPLC	Ultra performance liquid chromatography
UV/Vis	Ultraviolet/visible

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Introduction

La canneberge fait partie de la famille des Ericaceae, tout comme le bleuet (*Vaccinium angustifolium*) et la myrtille (*Vaccinium myrtillus*). La culture de la canneberge en Amérique du Nord (*Vaccinium macrocarpon*), est une activité agricole d'importance dans les régions du Nord-Est de l'Amérique du Nord (Massachusetts, Michigan, New Jersey, l'Oregon, Québec, Washington et Wisconsin). Selon des données récentes obtenues par l'organisme Nord-Américain le Cranberry Institute, les récoltes de l'Amérique du Nord culture totaliserait environ 850 millions de livres par an. Ce volume de canneberges serait transformé principalement en trois type de produits alimentaires: frais et surgelés (3%); sauce et purée (2%); sucrée et séchée (25%) et en jus et concentré de jus (70%).

La canneberge est une baie attrayante, tant au niveau de sa couleur, sa saveur, que de ses bienfaits santé. L'engouement pour ce fruit n'a fait que croître depuis la publication d'études faisant état de ses bienfaits potentiels liés à sa teneur élevée en anthocyanes, flavonols, flavan-3-ols, proanthocyanidines et dérivés d'acides phénoliques (Zapsalis et Francis, 1965; Füleki et Francis, 1967; Breeze, 1972; Robards et Antolovich, 1997; Kren et Martinkova, 2001). Ces composés phytochimiques ont suscité beaucoup d'attention surtout en raison de leurs propriétés antioxydantes, antibactériennes, anti-inflammatoires et antimutagène (Kaur et Kapoor, 2001; Rice-Evans, 2001; Dixon et al, 2005; Heinonen, 2007; Neto, 2007; Ruel et Couillard, 2007).

L'activité antimicrobienne spécifique à la canneberge congelée, au concentré de jus et à la poudre a été démontrée sur plusieurs bactéries pathogènes, incluant entre autres les bactéries *Helicobacter pylori*, *Salmonelle*, *Staphylococcus aureus*, *Escherichia coli* et *Campylobacter* (Burger *et al.*, 2000; Burger *et al.*, 2002; Leitao *et al.*, 2005; Vattem *et al.*, 2005b; Neto, 2007). Plusieurs

mécanismes d'action impliquant certains composés bioactifs spécifiques à la canneberge ont été proposés pour inhiber l'adhérence des bactéries aux cellules épithéliales. Les tanins polymériques, et en particulier les proanthocyanindines constitués principalement de tétramères et de pentamères d'épicatéchine et avec au moins une liaison de type A, sembleraient être l'élément de protection contre les bactéries pathogènes (Foo *et al.*, 2000b; Heinonen, 2007; Seeram & Heber, 2007).

Les propriétés antioxydantes de la canneberge sont également bien documentées dans la littérature et selon la méthode de mesure des capacités antioxydantes Oxygen Radical Absorbance Capacity (ORAC) et d'autres méthodes, le fruit se classerait parmi les meilleurs antioxydants. Les pigments anthocyanes senblent être les principaux responsables de cette activité, bien que les flavonols, les flavanols, les proanthocyanidines et les dérivés de l'acide benzoïque et cinnamique) ont également démontré un pouvoir antioxydant intéressant. Les composés phénoliques et polyphénoliques de la canneberge ont démontré leur pouvoir antiradicalaire contre les radicaux libres, tels que les anions superoxydes, les peroxydes d'hydrogène et les radicaux hydroxyles, et peuvent également inhiber la peroxydation des lipides, des protéines et ainsi que l'oxydation des lipides dans des liposomes (Wang & Jiao, 2000; Wu *et al.*, 2004; Seeram & Heber, 2007).

À cause de sa saveur amère et son goût acidulé, la canneberge est rarement consommée à l'état naturel. Elle est plutôt transformée en jus ou en concentré de jus, et ensuite mélangée à de l'eau et du sucre pour faire une boisson de jus de fruit. En raison de tous les bienfaits associés à la consommation de produits à base de canneberges, une attention particulière doit être accordée aux changements que peuvent subir ses composés bioactifs lors de la transformation du fruit et qui pourraient affecter leurs propriétés. La large variabilité des composés phénoliques présents dans les fruits et leur sensibilité aux conditions de transformation représentent un défit pour les industriels qui veulent améliorer la fonctionnalité de leurs produits. Avec la demande constante du consommateur

pour la mise sur le marché d'aliments fonctionnels, des stratégies innovatrices doivent être développées dans le but d'enrichir la diète avec des composés phénoliques et des flavonoïdes ayant le maximum de propriétés biologiques. Nous avons donc posé comme hypothèse que le contrôle des différentes étapes du procédé du jus et l'étude de la teneur en poly phénols du sous-produit (marc) permettrait d'uniformiser et d'optimiser le contenu en poly phénols pour le développement d'un jus ayant de meilleures propriétés biologiques.

Le principal objectif de ce projet était d'optimiser le rendement de la récupération et la préservation des propriétés biologiques des composés bioactifs de la canneberge tout au long du procédé de production de jus. Pour ce faire, trois techniques d'extraction ont été développées et standardisées pour récupérer la gamme la plus large possible de poly phénols présents dans le fruit, soit les composés hydrosolubles, les composés apolaires et les anthocyanes, pour ensuite déterminer leurs propres propriétés biologiques. Une fois que cela fait, il a été possible de déterminer comment les différentes étapes du procédé de jus pouvaient influencer les propriétés antibactérienne, antioxydante et antiradicalaire de la canneberge. La capacité des trois extraits phénoliques à inhiber des bactéries responsables de la putréfaction ou des pathogènes d'intérêt en alimentation (*S.* Typhimurium SL1344, *E. coli* O157:H7 et ATCC 25922, *S. aureus* ATCC 29213, *Listeria* 2812 1/2a, *Pseudomonas aeruginosa* ATCC15442) et en santé publique (*Enterococcus* résistant à la vancomycine) a été vérifiée pour le pH physiologique (pH 7). L'inhibition des pathogènes a été évaluée par dénombrement sur milieu gélosé contenant les échantillons à tester des cellules formatrices de colonie. L'absence de colonie a été considérée comme étant la concentration minimale inhibitrice (CMI). La concentration maximale tolérée (CMT) est la concentration maximale n'affectant pas la croissance bactérienne. Le pouvoir antioxydant des trois extraits phénoliques a été mesuré au pH naturel de la canneberge (pH 2.5) et au pH physiologique (pH 7) en adaptant une

technique de peroxydation non enzymatique des microsomes de foie de rat en une microtechnique utilisant une microplaque. La concentration de TBARS (Thiobarbituric reactive substances) produits lors de la peroxydation de liposomes en présence de fer et d'acide ascorbique a été mesurée par spectrophotométrie, et le pouvoir antioxydant déterminé par rapport à cette absorbance. Les propriétés antiradicalaires ont été évaluées en utilisant une microtechnique développée dans nos laboratoires. Cette méthode consiste à mesurer l'effet de la présence d'agents antioxydants contre les radicaux libres oxygénés ($\cdot O_2^-$, $\cdot OH$ $^1O_2$) et leurs sous-produits (H_2O_2, HOCl) générés par l'électrolyse d'une solution saline. L'activité antiradicalaire de l'échantillon correspondait à sa capacité à inhiber les espèces oxydantes accumulées et à limiter ainsi l'oxydation de la DPPD. La valeur de l'absorbance étant proportionnelle au degré d'oxydation de la DPPD par les radicaux libres, le pouvoir antiradicalaire des trois extraits phénoliques en résultant a pu être ainsi mesuré au pH naturel de la canneberge (pH 2.5) et au pH physiologique (pH 7).

CHAPITRE 1

Publication 1: Bioactive compounds in cranberries and their biological properties

Bioactive compounds in cranberries and their biological properties

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1.1. Contributions des auteurs

J'ai réalisé les recherches et les lectures nécessaires pour la rédaction de cette revue de littérature. Le Dr. Stéphane Caillet, codirecteur du projet et associé de recherche à l'INRS-Institut Armand-Frappier a participé aux discussions scientifiques et a corrigé le manuscrit. Le Dr. Gilles Doyon, chercheur scientifique au Centre de recherche et de développement sur les aliments de Saint-Hyacinthe (Québec), a participé aux discussions scientifiques et a corrigé le manuscrit. M. Jean-François Sylvain, directeur technique de Canneberges Atoka Inc., a approuvé le projet et participé aux discussions scientifiques. Prof. Monique Lacroix était la responsable scientifique et la coordinatrice du projet de recherche. De plus, Prof. Lacroix a supervisé l'élaboration des protocoles et les discussions scientifiques entourant ce projet. Elle a révisé le manuscrit.

1.2. Résumé en français

Composés bioactifs dans les canneberges et de leurs propriétés biologiques

La canneberge est une baie attrayante, tant au niveau de sa couleur, sa saveur, que ses bienfaits santé. C'est un des trois seuls fruits indigènes à l'Amérique. Au cours des dix dernières années, l'engouement pour ce fruit de l'Amérique du Nord (*Vaccinium macrocarpon*) n'a fait que croître depuis la publication d'études faisant état de ses bienfaits potentiels liés à sa teneur élevée en anthocyanes, flavonols, flavan-3-ols, proanthocyanidines et dérivés d'acides phénoliques. La présence de ces composés phytochimiques contribuerait aux propriétés de la canneberge qui permettraient de prévenir de nombreuses maladies et infections, y compris les maladies cardio-vasculaires, divers cancers, et des infections impliquant des voies urinaires, la santé buccale, et les ulcères et cancers de l'estomac causés par la bactérie *Helicobacter pylori*. Au cours des dernières années, des percées importantes au niveau de la recherche ont permis de mieux comprendre les mécanismes par lesquels les composés phytochimiques exercent leurs bienfaits biologiques, mais les fondements scientifiques de ces théories restent à être prouvés. Dans cet article, les caractéristiques de la canneberge, ainsi que ses propriétés antioxydante, antiradicalaire, antibactérienne, antimutagène et anticancer sont expliquées.

1.3. Abstract

Cranberries are healthy fruit that contribute color, flavour, nutritional value and functionality. They are one of only three fruits native to America. Over the past decade, public interest for the North American cranberry (*Vaccinium macrocarpon*) has been rising with reports of their potential health benefits linked to the numerous phytochemicals present in the fruit: the anthocyanins, the flavonols, the flavan-3-ols, the proanthocyanidins, and the phenolic acid derivatives. The presence of these phytochemicals appears to be responsible for the cranberry's property of preventing many diseases and infections, including cardiovascular diseases, various cancers, and infections involving the urinary tract, dental health and *Helicobacter pylori*-induced stomach ulcers and cancers. Recent years have seen important breakthroughs in our understanding of the mechanisms through which these compounds exert their beneficial biological effects, yet these remain to be scientifically substantiated. In this paper these characteristics, as well as the antioxidant, radical scavenging, antibacterial, antimutagen and anticarcinogen properties of cranberry major bioactive compounds are explained.

Keywords: Anthocyanins, flavonoids; proanthocyanidins; phenolic acids; antibacterial, antioxidant, antimutagen.

1.4. Introduction

Cranberries are healthy fruit that contribute color, flavour, nutritional value, and functionality. They are one of the only three native Northen American fruits. The North American cranberry (*Vaccinium macrocarpon*) is recognized by the US Department of Agriculture, USDA, as the standard for fresh cranberries and cranberry juice cocktail. The European variety, grown in parts of central Europe, Finland, and Germany, is known as *Vaccinium oxycoccus*. This is a smaller fruit with anthocyanins and acid profiles slightly different to that of the North American variety (Girard and Sinha, 2006).

Phenolic acids, flavonoids and tannins are phytochemicals ubiquitous in food of plant origin (Lugasi and Hovari, 2000). They constitute one of the most abundant groups of natural metabolites and are now recognized for their important contribution to both human and animal diet and health (Vattem *et al.*, 2005a; Vattem & Shetty, 2005). These phytochemicals occur in virtually every plant and in all parts of the plant. Their quantitative distribution can vary between different organs of the plants and within different populations of the same plant species, and for this reason they are often used for the plant taxonomical classification (Robards & Antolovich, 1997; Merken and Beecher, 2000a). The predominant bioactive compounds found in cranberries are the flavonols, the flavan-3-ols, the anthocyanins, the tannins (ellagitannins and proanthocyanidins) and the phenolic acid derivatives. These phytochemicals are commonly associated with the fruit organoleptic (sensory) qualities and have also shown diverse biological properties and physiological activities in animals (Robards & Antolovich, 1997; Kren and Martinkova, 2001). Numerous clinical trials and epidemiological studies have established an inverse correlation between intake of phenolic-rich fruits and vegetables and the occurrence of diseases such as inflammation, cardiovascular disease, cancer, and aging-related disorders (Willet, 2001).

The objective of this paper is to provide an overview of the main bioactive compounds of cranberry, their characteristics and their biological properties. This paper is organized in two parts; the first part presents the main phytochemical composition of cranberry. In the second part, the antioxidant, radical scavenging, antibacterial, antimutagen and anticarcinogen properties of cranberry are reviewed in detail.

1.5. Cranberry bioactive compounds

Cranberries and cranberry products are known for their elevated concentration in total polyphenols (Vinson *et al.*, 2008). On a fresh weight basis, the order of amount of total polyphenols in cranberry foods is as follows: dried > frozen > sauce > jellied sauce. On a serving size basis for all cranberry poducts, the order is: frozen > 100% juice > dried > cocktail > sauce > jellied sauce (Table 1.1).

More specifically, cranberries are rich in anthocyanins, in tannins (ellagitannins and proanthocyanidins), and have significant concentrations of the flavonoids: flavonols and flavan-3-ols (Neto, 2007; Ruel & Couillard, 2007; USDA, 2004 and 2007). Recently, many authors have also reported the presence of certain phenolic acid derivatives in cranberries: hydroxycinnamic acid, HCA, and hydroxybenzoic acid, HBA (Neto, 2007; Ruel & Couillard, 2007). Although these phenolic acids occur naturally in their free forms, they are generally considered as structural moieties in polyphenol compounds i.e. embedded within a polyphenolic structure (Seeram and Heber, 2007).

Flavonoids are defined as naturally-occurring organic species that possess two six-carbon aromatic centers, called the A and B rings, and a three-carbon bridge, called the C ring which forms a phenol bridge with oxygen (Robards & Antolovich, 1997; Haslam, 1998; Gee and Johnson, 2001). They are further classified according to the degree of unsaturation and oxidation of their three-carbon heterocycle segment (Fig. 1.1). Additional structural complexities are introduced by the common occurrence of glycosylation, where one or more of the hydroxyl groups of the flavonoid aglycone are bound to a sugar or sugars by an acid-labile hemiacetal bond to form what are called *O*-glycosides (Robards & Antolovich, 1997; Merken and Beecher, 2000a; Escarpa and Gonzalez, 2001; Rice-Evans, 2001; Cuyckens and Claeys, 2004). This glycosylation step has a profound effect on the plant's economy, as the glycosylated flavonoids have greater sap solubility and more plant mobility

Table 1.1. Polyphenols in different cranberry products based on serving size (Vinson *et al.*, 2008).

Cranberry Products	**Total polyphenols** (as mg catechin/serving size)
Frozen (97.5 g, ½ cup)	632
Dried (40 g, 1/3 cup)	352
Sauce (57 g, ¼ cup)	170
Jellied sauce (57 g, 1/2" thick, approx 8 slices per can)	122
Cocktail, 27% juice (240 mL)	176
Juice, 100% (240 mL)	540

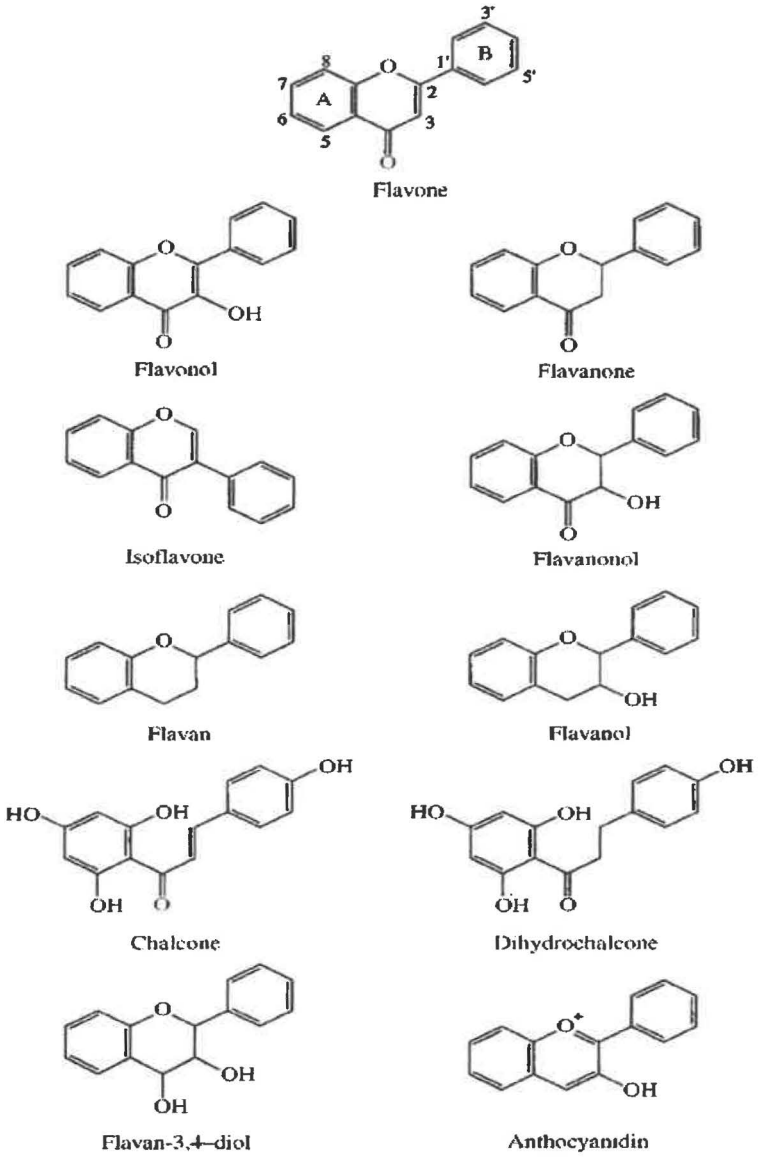


Fig. 1.1 Structure of the various classes of flavonoids. Reproduced with permission from Kevin Robards (Robards & Antolovich, 1997).

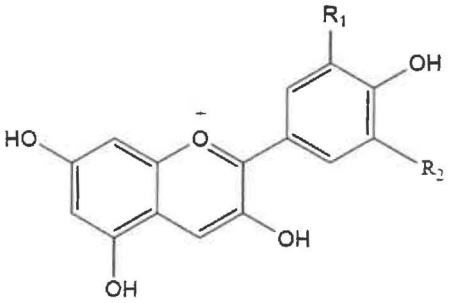
than the parent aglycone. This greater water solubility also permits their storage in the cell vacuole where they are commonly found (Harborne, 1964; Shahidi and Naczk, 1995; Robards & Antolovich, 1997; Cuyckens and Claeys, 2004). Studies have shown that glycosylated flavonoids can be better absorbed than aglycons (Hollman *et al.*, 1997), and tend to form more stable aroma precursors (Mayorga *et al.*, 2001). Glucose is the sugar most commonly encountered glycosylated to flavonoids, although galactose, rhamnose, arabinose and xylose are not unusual. Glycosylation with mannose, fructose, glucoronic and galacturonic acids is exceptionally rare. Disaccharide and more complexe carbohydrates have also been found in association with flavonoids: the more common are rutinose (rhamnosyl-(α1→6)-glucose) and neo-hesperidose (rhamnosyl-(α1→2)-glucose), but occasionally tri- and tetrasaccharides can be found (Shahidi and Naczk, 1995; Robards & Antolovich, 1997; Cuyckens and Claeys, 2004, Rijke *et al.*, 2006). The stereochemistry of the glycosidic linkages is another important characteristic of glycosylated flavonoids. In principle, any of the hydroxyl groups can be glycosylated but certain positions seem favoured. For example, the 3- and 7-hydroxyls in flavonols and flavan-3-ols, and the 3- and 5- hydroxyl in anthocyanidins are common glycosylation sites (Robards & Antolovich, 1997; Cuyckens and Claeys, 2004). The linkage can either be *O*-glycosyl or *C*-glycosyl, but glycosylated flavonoids usually occur as *O*-glycosyl (Bohm, 1998; Rijke *et al.*, 2006). The *C*-glycosylflavonoids are characterized by having one or two sugar units directly linked to the aromatic nucleus through carbon-carbon bonds, whereas the *O*-glycosylation can occur at any of the hydroxyl groups although certain ones, such as 3-*O*-glycosyl and 7-*O*-glycosyl seem favoured (Stafford, 1990; Bohm, 1998).

1.5.1. Anthocyanins

Anthocyanins are generally found in fruit and more specifically in red, purple, and blue berries (Nijveldt *et al.*, 2001; Higdon, 2007). Their concentrations in food tend to increase as fruit ripens in response to climatic factors (light, temperature) (Bohm, 1998). The anthocyanin content of a cranberry averages 95 mg/100 g for a ripe fruit at harvest, with reports of anthocyanin content as high as 124 mg/100 g of fresh fruit weight (USDA, 2007). The concentration can also vary quite significantly among cranberry cultivars: the early black cultivar appear to be significantly higher in anthocyanins compared to other cranberry cultivars (Neto, 2007).

Anthocyanins consist of an anthocyanidin molecule bound to one or more sugar moieties (Robards & Antolovich, 1997). The six most common anthocyanidins are cyanidin, delphinidin, malvidin, perlagonidin, petunidin and peonidin and their chemical structures are shown in figure 1.2 (Robards & Antolovich, 1997; Merken and Beecher, 2000a). Glycosylation of anthocyanidins almost always occurs at the C_3 position with glucose, arabinose, and galactose being the most common sugar moieties (Strack and Wray, 1994; Robards & Antolovich, 1997). In fact, the formation of anthocyanidin 3-*O*-glucosides is considered a necessary step in anthocyanins biosynthesis (Robards & Antolovich, 1997). Anthocyanins with sugars at both the C_3 and C_5 positions and 3,7-diglycosides do also occur as they are considered more stable than C_3 *O*-glycosylanthocyanins (Strack and Wray, 1994; Bohm, 1998). Anthocyanins are frequently found acylated with aromatic and aliphatic acids such as *p*-coumaric acid, caffeic acid, ferulic acid, gallic acid, acetyl malonic acid, malic acid, etc. (Bohm, 1998, Iwashina, 2000). In acidic medium, the aglycone can exist in cationic form with numerous mesomeric forms (Robards & Antolovich, 1997).

The major anthocyanins in cranberry are galactosides and arabinosides of cyanidin and peonidin (Macheix *et al.*, 1990; Neto, 2007). However the following anthocyanins were also detected



Anthocyanidin	R_1	R_2
Cyanidin	H	OH
Delphinidin	OH	OH
Malvidin	OMe	OMe
Pelargonidin	H	H
Peonidin	H	OMe
Petunidin	OH	OMe

Figure 1.2. Molecular structure of anthocyanidins. Reproduced with permission from Joanne Holden (USDA, 2007).

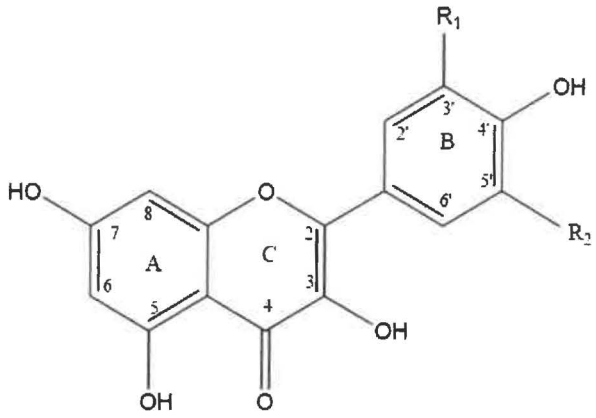
at low concentrations: cyanidin 3-*O*-glucoside, peonidin 3-*O*-glucoside, peonidin 3-*O*-galactoside, peonidin 3-*O*-arabinoside, peonidin 3,5-digalactoside, delphinidin 3-*O*-glucoside, delphinidin 3-*O*-galactoside, delphinidin 3-*O*-arabinoside, pelagonidin 3-*O*-galactoside, pelagonidin 3-*O*-arabinoside, petunidin 3-*O*-galactoside, malvidin 3-*O*-galactoside, malvidin 3-*O*-arabinoside (Macheix *et al.*, 1990; Wu and Prior, 2005; Lin and Harnly, 2007).

1.5.2. Flavonols

Flavonols are found in abundance in Ericaceae fruits such as cranberry, blueberry and bilberry (Robards & Antolovich, 1997; King and Young, 1999; Nijveldt *et al.*, 2001; Heinonen, 2007). They are known to be concentrated mainly in the skin of fruits (Hawker *et al.*, 1972; Wildanger and Herrmann, 1973; Price *et al.*, 1999). On a weight basis, the cranberry is one of the leading fruit sources of flavonols. According to Neto *et al.* (2007) and the Database for the flavonoid content of selected foods (USDA, 2007), the average flavonol content in cranberry is 20-30 mg/100 g fresh fruit weight, although contents as high as 48 mg/100 g were reported.

As shown in figure 1.3, the most predominant type of flavonols includes isorhamnetin, kaempferol, myricetin and quercetin. The flavonols are characterized by the absence of any substituant at the C_3 position. Because of the double bond present in their central aromatic ring, they are renowned for their planar structure (Bohm, 1998; Iwashina, 2000; Nijveldt *et al.*, 2001).

Approximately 75% of the cranberry flavonols consist of quercetin glycosides (quercetin 3-*O*-galactoside), although other flavonols have also been reported in the fruit at lower concentrations: quercetin, quercetin 3-*O*-glucoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinopyranoside, quercetin 3-*O*-arabinofuranoside, quercetin 3-*O*-rhamnoside, myricetin, myricetin 3-*O*-galactoside, myricetin 3-*O*-xylopyranoside, myricetin 3-*O*-arabinopyranoside, myricetin 3-*O*-arabinofuranoside,



Flavonol	R_1	R_2
Isorhamnetin	OMe	H
Kaempferol	H	H
Myricetin	OH	OH
Quercetin	OH	H

Figure 1.3. Molecular structure of flavonols. Reproduced with permission from Joanne Holden (USDA, 2007).

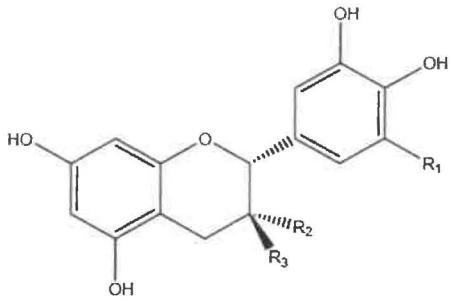
myricetin 3-*O*-rhamnoside, kaempferol 3-glucoside and kaempferol derivative (Macheix *et al.*, 1990; Häkkinen, 1999; Zheng and Wang, 2003; Chen and Zuo, 2007; Lin and Harnly, 2007; Neto, 2007).

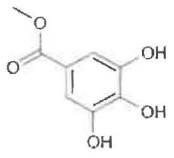
Flavonols are often found in nature as acylated derivatives, involving linkages between aliphatic and aromatic acids (hydroxybenzoic, gallic, *p*-coumaric, caffeic, ferulic, and sinapic acids) and the sugar hydroxyls (Stafford, 1990; Bohm, 1998). The predominant type of glycosylated flavonols in fruits are 3-*O*-monoglycosides with the sugars occurring in the following order: glucose > galactose > rhamnose > glucuronic acid.

1.5.3. Flavan-3-ols and Tannins

Flavan-3-ols are important constituents of fruits, and their presence has been reported in cranberry (Macheix *et al.*, 1990; Lin and Harnly, 2007). They share the same flavonoid molecular structure with flavonols, but as shown in figure 1.4, they lack the C_4 carbonyl group (Iwashina, 2000; Escarpa and Gonzalez, 2001). In contrast to other flavonoids, they are known to occur naturally in plant food as aglycons of catechin and epicatechin as they are soluble enough to be maintained in the vacuole without needing glycosylation. The flavan-3-ol content of a cranberry averages 7 mg/100 g for a ripe fruit at harvest, with content as high as 11 mg/100 g fresh fruit weight being reported (USDA, 2007).

Through reactions catalyzed by light, heat, and oxygen, flavan-3-ols tend to combine with esters of gallic acid and ellagic acid to form compounds refered to as hydrolyzable tannins, such as catechingallate, epigallocatechin, epicatechin gallate, gallocatechin and epigallocatechin gallate, as shown in figure 1.4 (Robards & Antolovich, 1997; Merken and Beecher, 2000a; Escarpa and Gonzalez, 2001; Higdon, 2007; Szajdek and Borowska, 2008).





Gallate

Flavan-3-ol	R_1	R_2	R_3
(+)-Catechin (C)	H	H	OH
(+)-Catechin-3-gallate (CG)	H	H	Gallate
(-)-Epicatechin (EC)	H	OH	H
(-)-Epicatechin-3-gallate (ECG)	H	Gallate	H
(-)-Epigallocatechin (EGC)	OH	OH	H
(-)-Epigallocatechin-3-gallate (EGCG)	OH	Gallate	H
(+)-Gallocatechin (GC)	OH	H	OH
(+)-Gallocatechin-3-gallate (GCG)	OH	H	Gallate

Figure 1.4. Molecular structure of flavan-3-ols. Reproduced with permission from Joanne Holden (USDA, 2007).

Flavan-3-ols can also form oligomeric and copolymeric compounds with a high degree of polymerization through *O*- and *C*-glycosyl linkages with sugars and other *C*- substitutions through acylation and complexation with anthocyanins and noncyanic flavonoids such as phenolic acids or metal ions (Stafford, 1990; Bohm, 1998; Iwashina, 2000; Lazarus *et al.*, 2001; Dixon *et al.*, 2005; Higdon, 2007; Szajdek and Borowska, 2008). Cranberries have a high content of oligomeric and polymeric pigments, also referred to as condensed non-hydrolyzable tannins or proanthocyanidins, which have structures similar to those found in wine (Krueger *et al.*, 2004). According to the USDA Database for the proanthocyanidin content of selected foods (USDA, 2004), the proanthocyanidin content of raw cranberries can average 410 mg/100 g fresh fruit weight, although contents as high as 505 mg/100 g were reported. The proanthocyanidin concentration appears to be significantly higher in cranberry fruit of the early black cultivar, compared to most other cranberry cultivars (Neto, 2007). These tannins are generally located in vacuoles of intact plant cells. The seed coats of many plant species also accumulate proanthocyanidins, which frequently cause poorly-soluble, brown or red-brown pigmentation once the seeds have matured (Leung *et al.*, 1979; Shirley *et al.*, 1992 & 1995; Nozzolillo and Ricciardi, 1992; Marles *et al.*, 2003).

In proanthocyanidins, the successive flavan-3-ol monomeric units are generally linked together by C-C interflavan bonds occuring between the C_4 position of the upper unit and the C_6 or C_8 position of the lower unit (4β→6 or 4β→8) (Hammerstonne *et al.*, 2000; Gu *et al.*, 2004; Dixon et al., 2005). This type of condensed tannin, as shown in figure 1.5, is also called B-type proanthocyanidin oligomer; they differ only in the arrangement of (+)-catechin and (–)-epicatechin starter and extension units. B-type proanthocyanidins containing gallic acid esters have also been reported (Hammerstonne *et al.*, 2000). Their formation appears to be under strict enzymatic control because the different types of dimers are characteristic of specific plant species: proanthocyanidin B_1,

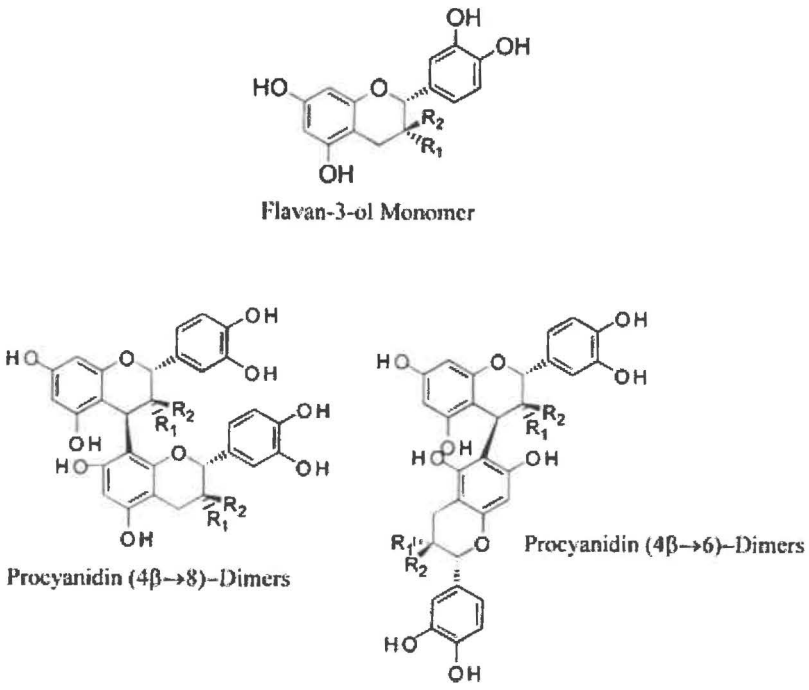


Figure 1.5. Representative structures of flavan-3-ol monomers and their dimers. When R_1 = OH and R_2 = H, the monomer is (-)-epicatechin. When R_1 = H and R_2 = OH, then the monomer is (+)-catechin. Reproduced with permission from John F. Hammerstone (Hammerstone *et al.*, 2000).

for example, is found in cranberry, whereas B_3 is found mainly in strawberry and B_4 in raspberry and blackberry (Fletcher *et al.*, 1977). Another structural variation is known to occur, although less frequently, in condensed tannins where flavan-3-ol units are linked together by a mixture of C-C bonds and interflavonoid bonds by C-O oxidative coupling between both C_2 and C_4 of the upper unit and the oxygen at C_7 and positions C_6 or C_8, respectively, of the lower unit (4β→8 and 2β→O→7). Shown in figure 1.6, these condensed tannins are referred to as A-type proanthocyanidin oligomers (Porter, 1994; Foo *et al.*, 2000a; Gu *et al.*, 2004; Neto, 2007). Due to the complexity of this conversion, A-type proanthocyanidins are not encountered as frequently in nature in comparison to B-type proanthocyanidins (Lazarus *et al.* 2001; Dixon et al., 2005). Cranberry proanthocyanidins tend to occur as tetramers to decamers, but also as polymers of epicatechin units, with the two types of linkages between epicatechin units, the B-type and the less common A-type (USDA, 2004; Howell, 2005; Neto, 2007). Proanthocyanidin characterization in cranberry juice cocktail has shown the presence of a series of polyflavan-3-ol oligomers composed of 4-10 repeating unit structures of epicatechin and epigallocatechin with one or more A-type interflavanyl linkages (Howell *et al.*, 2005). The presence of epicatechin-anthocyanin dimers, trimers and tetramers was also detected in organic cranberry juice and early black cultivar fruit (Neto *et al.*; 2008).

1.5.4. Phenolic Acids

Phenolic acids contribute to the characteristic and unique flavor of berries (Vattem et al., 2005a; Vattem & Shetty, 2005). This family of compounds includes derivatives of hydroxycinnamic acid, HCA, and of hydroxybenzoic acid, HBA. They both have very similar molecular structures composed of a backbone phenol ring, although HCA has an additional ethylenic chain attached to the aromatic ring. Compounds classified in these two families of acid derivatives can otherwise differ by

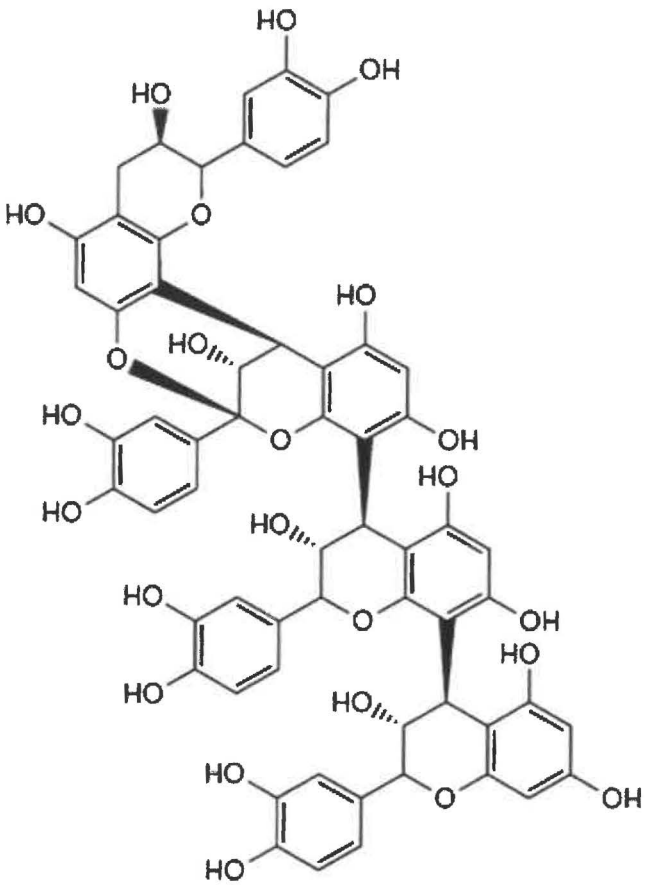


Figure 1.6. Structure of a typical cranberry proanthocyanidin tetramer composed of epicatechin units with one A-type linkage. Reproduced with permission from Catherine C. Neto (Neto, 2007).

the number and position of hydroxyl and methyl groups attached to the phenol ring. The presence of HCA derivatives in plant foods is more frequent than that of the HBA derivatives (Macheix and Fleurinet, 1998; Escarpa and Gonzalez, 2001).

The HCA derivatives are the most widely distributed group of phenolic compounds (Shahidi and Naczk, 1995; Escarpa and Gonzalez, 2001). Liquid chromatography combining mass spectrometer analysis of various cranberry cultivars allowed the detection of hydroxycinnamate esters in the whole fruit in quantities averaging 15-20 mg/100 g fresh fruit (Kondo, 2006). The HCA derivatives reported in cranberry includes compounds such as ferulic acid, *p*-coumaric acid, coumaroylglucose, feruloylglucose, glycosylated sinapic acid, caffeoylglucose and diglucoside of caffeoylglucose, (Macheix *et al.*, 1990; Häkkinen *et al.*, 1999). As shown in figure 1.7, this class of phenolic compounds consists of an aromatic ring onto which a three-carbon side-chain is attached (Harborne, 1998; Sakakibara, 2003). They rarely occur in free form; they are instead associated to other types of compounds. When they do occur as simple phenolic acid, it is generally after they have gone through brutal processes, such as contamination by microorganism or technological processing (freezing, sterilization, fermentation) (Macheix *et al,* 1990; Shahidi and Naczk, 1995). Ferulic, *p*-coumaric, caffeic and sinapic acids are often found combined with sugars by means of glycosidic linkage, or with organic acids such as quinic acid (Harborne, 1964; Escarpa and Gonzalez, 2001). Common glycosylated HCA derivatives include esters *p*-coumaroylglucose and caffeoylglucose (Shahidi and Naczk, 1995).

The concentration of HBA derivatives is generally low in food of plant origin, the exception being for the majority of berries, whose content in protocatechuic, ellagic and gallic acids is very high (Shahidi and Naczk, 1995; Tomas-Barberan and Clifford, 2000). The presence of ellagic and p-

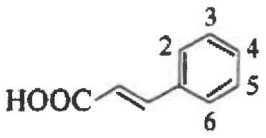


Figure 1.7. Molecular structure of hydroxycinnamic acid. Reproduced with permission from Kazuki Kanazawa (Sakakibara *et al.*, 2003).

hydroxybenzoic acids was reported in cranberries by Häkkinen *et al.* (1999), whereas Zheng and Whang (2003) reported the presence of vanillic acid. HBA derivatives are the simplest structure within the congregation of polyphenolic compounds, with their six-carbon structure shown in figure 1.8 (Macheix and Fleurinet, 1998; Escarpa and Gonzalez, 2001). They are also found glycosylated: glycosides of 4-hydroxybenzoic, protocatechuic, vanillic and syringic acids were reported in plant food products (Shahidi and Naczk, 1995; Tomas-Barberan and Clifford, 2000). Due to reactions catalyzed by light, heat, and oxygen, ellagic acid esters readily combine with flavan-3-ols to form hydrolyzable tannins called ellagitannins. Monomeric ellagitannins can further polymerize and form oligomeric and polymeric ellagitannins. These complex tannins can form the structural components of the plant cell wall (lignin) and cell membrane (Marwan and Nagel, 1986; Chen *et al.*, 2001). High contents of ellagitannins have been reported in cranberry (Kähkönen *et al.*, 2001; Lei *et al.*, 2001; Vattem and Shetty 2003; Kaponen *et al.*, 2007).

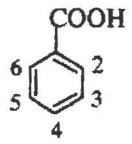


Figure 1.8. Molecular structure of hydroxybenzoic acid derivatives. Reproduced with permission from Kazuki Kanazawa (Sakakibara *et al.*, 2003).

1.6. Biological properties of cranberry bioactive compounds

Cranberries (*Vaccinium macrocarpon*) have been identified with beneficial properties toward bacterial infections involving the urinary tract disorders, dental decay, as well as stomach ulcers and cancers (Heinonen, 2007). Although berry phenolics are potent *in vitro* antioxidants, they exert *in vivo* biological activities beyond antioxidation and can have complementary and overlapping mechanisms of action (Seeram & Heber, 2007). *In vitro* experimental systems showed that berry phenolics possess antioxidant and free radical-scavenging activities, but also metal chelation, antiproliferative, anticarcinogenic, antibacterial, anti-inflammatory, antiallergenic, and antiviral properties (Lee, 2000; Merken and Beecher, 2000a and 2000b; Nijveldt *et al.*, 2001; Higdon, 2007). This would explain why they exhibit such strong physiological activities against infections, cancerous mutations and cancers, as well as against allergies, inflammation, virus, hypertension, arthritis and AIDS (Robards & Antolovich, 1997; Merken and Beecher, 2000a).

1.6.1. Antimicrobial Properties

Juice of the American cranberry (*Vaccinium macrocarpon*) has long been consumed for the prevention of urinary tract infections. Its ability to protect the urinary tract from adherence of uropathogenic bacteria such as *Escherichia coli* and other pathogens, has led doctors to recommend drinking cranberry juice as a treatment for various urinary tract infections and prostatitis. For more than a decade, the tannic components of cranberry have been proposed to inhibit bacterial adherence to the epithelial cells by competing for the bindings of both these fimbriae. In a 2008 Cochrane clinical study review, the authors Jepson and Craig concluded that there was some evidence that cranberry juice may decrease the number of symptomatic urinary tract infections over a 12 month period, particularly for women with recurrent urinary tract infections. All this knowledge has led to

the first ever health claim on berry phenolics issued by the French Food Safety Authority in April 2004: "cranberry proanthocyanidins, in a daily dose of 36 mg, help reducing the adhesion of certain *E. coli* bacteria to the urinary tract" (Heinonen, 2007).

In the past decade, cranberry antimicrobial activity was demonstrated toward other groups of illness-causing pathogenic bacteria, including *Helicobacter pylori*, *Salmonella*, *Staphylococcus aureus*, *Escherichia coli*, and *Campylobacter*, to name a few. Recent *in vitro* studies indicated that the presence of high molecular weight components in cranberry could also inhibit the siallylactose-specific (S fimbriae) adhesion of *Helicobacter pylori* strains to immobilized human mucus, erythrocytes and cultured gastric epithelial cells (Burger *et al.*, 2000; Burger *et al.*, 2002; Vattem *et al.*, 2005b; Neto, 2007). This action against the *Helicobacter pylori* is thought to have a potential inhibitory effect on the development of peptic ulcers and gastric cancer (Neto, 2007). Puupponen-Pimia *et al.* (2005) have also reported a significant inhibitory effect of various concentrations of cranberry extracts (*Vaccinium oxycoccus*) against *Salmonella enteric* sv. Typhimurium and *Staphylococcus aureus*. When freeze-dried berry powder of 2 mg/mL was used, both pathogenic strains of bacteria were clearly affected, *Staph. Aureus* stronger than *Salm.* Sv. Typhimurium. The effect of anthocyanin-and proanthocyanin-rich fractions isolated from cranberry juice were studied for their antibacterial activity by Leitao *et al.* (2005). *Staphylococcus aureus* was the only strain to exhibit some susceptibility to four out of the 10 anthocyanin-rich fractions tested, whereas a variable susceptibility of *S. aureus*, *Enterococcus feacalis* and *Micrococcus luteus* to procyanidin-rich fraction was observed.

Cranberry has also showed potential inhibitory effect against bacteria involved in dental caries and periodontal diseases (Bodet *et al.*, 2008). Dental caries are caused principally by *Streptococcus mutans* infection but also by *Actinomyces spp.* and *Lactobacillus spp.* (Beighton,

2005). During the formation of dental caries by *Steptococcus mutans*, the microorganism synthesizes extracellular glucans from sucrose using glucosyltransferases. The glucans can then promote the accumulation of the streptococci and other microorganism on the tooth surface and help in forming a biofilm to eventually carry out glycolysis of multiple carbohydrates efficiently. Duarte and his group (2006) have investigated the influence of cranberry components on the activities of glucosyltransferase B adsorbed onto a saliva-coated hydroxyapatite surface. Their results indicated that compared to the fractions (flavonols, proanthocyanidins, anthocyanins), the crude cranberry extract showed the highest percentage of inhibition of glucosyltransferase activity. Cranberry phenolic compounds may also play an important role against periodontal diseases, a group of inflammatory disorders initiated by specific gram-negative bacteria, and that lead to connective tissue destruction. The gingivite (gum inflammation) and parodontite (progressive destruction of tooth support – parodontal ligament and alveolar bone) are mainly caused by infections by *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia* (Sorsa, 1990). Proteolytic enzymes, including matrix metalloproteinases and elastase, produced by resident and inflammatory cells in response to periodontopathogens and their products, play a major role in gingival tissue destruction (Palcanis *et al.*, 1992; Sorsa *et al.*, 2004). Recent *in vitro* studies have demonstrated the efficient inhibitory effect of cranberry fractions on the lipopolysaccharide-induced metalloproteinases and elastase (Bodet *et al.*, 2006, 2007, and 2008). Certain extracts obtained from cranberry juice were also shown to be effective in decreasing the congregation and salivary concentration of *Streptococcus mutans* which causes tooth decay (Weiss *et al.*, 1998 and 2002).

Adherence of pathogens to the host tissue is one of the most important prerequisite steps for bacteria colonization and subsequent infection. Inability of the pathogen to bind to the cell surface causes the microorganism to be washed away without causing any infection. Investigations into the

mechanism of adherence to host tissue has led to an understanding that these are mediated by specific glycoprotein receptors called fimbriae or lectins on the bacterial cell surface which can specifically bind to sugars present on the mucosal or intestinal cell surfaces of the host tissue (Zafriri *et al.*, 1989; Sharon & Ofek, 2002). This type of binding, however, occurs only via specific types of fimbriae-mediated adhesion: type 1 fimbriae (mannose sensitive) and type P fimbriae [α-Gal(1→4) β-Gal], which is mannose resistant (Zafriri *et al.*, 1989; Sharon & Ofek, 2002).

Over the last decade, specific bioactive components of cranberry were proposed to inhibit bacterial adherence to the epithelial cells. The polymeric tannins and in particular, the proanthocyanidins consisting primarily of epicatechin tetramers and pentamers with at least one A-type linkage, seem to be the protecting element against pathogenic bacteria (Foo *et al.*, 2000b; Heinonen, 2007; Seeram & Heber, 2007).

Several mechanisms could explain the effect of the A-type proanthocyanidin in the growth inhibition of bacteria: the destabilization of cytoplasmic membrane, the permeabilization of cell membrane, the inhibition of extracellular microbial enzymes, the direct actions on microbial metabolism and the deprivation of the substrates required for microbial growth (Heinonen, 2007). Proanthocyanidins are known to bind metals and form complexes involving their *o*-diphenol groups, a property often viewed as imparting negative traits with regards to bioavailability of essential mineral micronutrients, especially iron and zinc (House, 1999). Iron depletion can severely limit bacterial growth, and it has been suggested that the ability to bind iron represents another mechanism through which proanthocyanidins inhibit bacterial activity (Dixon et al., 2005).

1.6.2. Antioxidant and radical-scavenging properties

Oxidative stress is a widely used and ill-defined term that refers to the imbalance in the rate at which the intracellular content of reactive oxygen species (ROS) increases relative to the capacity of the cell to dispose of these free radicals, most likely due to insufficient antioxidant reserves or intake. Free radicals or ROS are produced continuously in humans through a number of normally occurring cellular events, such as energy production and detoxification of the body, or are generated by exhaustive exercise. They are also found in atmospheric pollution and tobacco smoke. Free radicals have the capacity to alter the integrity of large biomolecules such as lipids, proteins, and DNA, resulting in an increased risk of inflammatory diseases, cardiovascular disease, some cancers, diabetes, Alzheimer's disease, cataracts, and age-related functional decline (Ruel & Couillard, 2007; Seeram & Heber, 2007).

In biology, compounds that can retard or prevent the effects of oxidation have been broadly considered as antioxidants, including compounds that either inhibit specific oxidising enzymes or react with oxidants before they damage critical biological molecules (Ruel & Couillard, 2007). Effective antioxidants are radical scavengers that break down radical chain reactions caused by reactive oxygen/nitrogen species or that can inhibit the reactive oxidants from being formed in the first place (Huang *et al.*, 2005). These reactions are based on a compound's ability to donate hydrogen as well as to stabilise the resulting antioxidant radical by electron delocalisation (resonance) and/or intramolecular hydrogen bonding or by further oxidation. The loss of hydrogen may take place by donation of an electron followed by deprotonation (Frankel & Meyer, 2000; Higdon, 2007).

Considerable *in vitro* evidence exists, showing that cranberry phenolics are powerful antioxidants. However, the great diversity of methods applied to studying both the radical-scavenging

activity and the antioxidant activity has resulted in great differences in the outcome (Heinonen, 2007). Still, cranberry phenolics appear to have free radical-scavenging properties against superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$), and can also inhibit lipid peroxidation, as well as protein and lipid oxidation in liposomes (Wang & Jiao, 2000; Wu *et al.*, 2004; Seeram & Heber, 2007). This could explain why they prevent the oxidation of bulk lipids. The formation of methyl linoleate hydroperoxides for example, was inhibited over 90% by cranberry phenolics at concentrations as low as 500 μg/mL (Heinonen, 2007).

Antioxidant activity will differ depending on the phenolic molecular structure (Bravo, 1998; Ruel & Couillard, 2007). Flavonoids with adjacent dihydroxy substituents on the B ring, for example, have been shown to be effective in the scavenging of radicals. In the group of flavonoids, this catechol unit is found in the flavonol quercetin, the anthocyanidin cyanidin, the flavan-3-ol catechins, and the proanthocyanidin procyanidin (Zheng and Wang, 2003). Despite their apparent lack of effect in bulk lipids, anthocyanins exhibit good free radical-scavenging activities and are powerful antioxidants in lipid-containing hydrophilic environments such as emulsified lipids, liposomes, and LDL (Heinonen, 2007). Indeed, anthocyanin isolates and anthocyanin-rich mixtures of bioflavonoids were shown to provide protection from lipid peroxidation (Ramirez-Tortosa *et al.*, 2001; Acquaviva *et al.*, 2003; Lazze *et al.*, 2003). However, the antioxidant potential anthocyanins can vary significantly across species and cultivars of cranberries. Dimeric and oligomeric B-type procyanidins possess greater scavenging activity toward peroxyl radicals than catechins, which is probably due to the presence of their interflavonoid linkage that increases the electron delocalization capacity of the phenyl radical (Ursini *et al.*, 2001). Polymerized chains of catechin monomers that form procyanidin trimers usually have decreased ability to prevent radical damage in lipid system, but their antioxidant activity is increased in aqueous phase (Plumb *et al.*, 1998).

In cranberry, anthocyanins are among the principal antioxidant constituents, although hydroxycinnamates such as chlorogenic acid, flavonols, and proanthocyanidins are also effective antioxidants (Heinonen, 2007). Indeed, Zheng and Wang (2003) have reported that the anthocyanins contributed 54.2% of the antioxidant activity of cranberries, whereas the flavonols contributed 34.6% of the observed antioxidant activity. When Porter *et al.* (2001) compared the antioxidant activity of six fractions of cranberry phenolic compounds, cinnamic acid derivatives, anthocyanins, flavonols and proanthocyanidins, they found that the fractions containing proanthocyanidin trimers through heptamers and pentamers through nonamers, more efficiently inhibited the oxidation of low-density lipoproteins. Eighty-seven percent of the formation of methyl linoleate hydroperoxide was inhibited by a 100 ppm catechin-proanthocyanidin-rich fraction obtained from cranberry (Määttä-Riihinen *et al.*, 2005). According to Yan *et al.* (2002), the cranberry extract composed primarily of flavonol glycosides showed the best inhibition of oxidative processes measured by DPPH assay (EC_{50} at 30-40 μg/mL) compared to anthocyanin-rich cranberry extract or to crude phenolic cranberry extract. From the compounds isolated from cranberry extract by He and Liu (2006), quercetin, 3,5,7,3',4'-pentahydroxyflavonol-3-O-β-D-glucopyranoside, 3,5,7,3',4'-pentahydroxyflavonol-3-O-β-D-galactopyranoside, and 3,5,7,3',4'-pentahydroxyflavonol-3-O-α-L-arabinofuranoside, showed potent antioxidant activities, with lower median effective concentration, EC_{50}, values of approximately 10 μM.

According to a review of 93 intervention studies concerning the relevance of phenolics antioxidant protection in humans, the ingestion of cranberry juice was reported to increase plasma antioxidant activity and decrease formation of lipid-oxidation products (Heinonen, 2007). Despite these positive results, studies indicate that even with very high flavonoid intakes, plasma and intracellular flavonoid concentrations in humans tend to be 100 to 1000 times lower than

concentrations of other antioxidants, such as ascorbate or glutathione. Moreover, most circulating flavonoids are actually flavonoid metabolites, some of which have lower antioxidant activity than the parent flavonoids. For these reasons, the relative contribution of cranberry phenolics to plasma and tissue antioxidant function *in vivo* is likely to be relatively minor (Higdon, 2007). Although it was initially hypothesized that the biological effects of phenolic compounds would be related to their antioxidant activity, more and more studies suggest that their health properties may involve another variety of mechanisms (Neto, 2007).

1.6.3. Antimutagen and anticarcinogen properties

Current evidence available from cell culture experiments suggests that many of the biological effects of cranberry phenolic compounds are related to their ability to modulate cell signalling pathways. Cells are capable of responding to a variety of different stresses or signals by increasing or decreasing the availability of specific proteins. The complex chain of events that leads to changes in the expression of specific genes is known as cell signalling pathways or signal transduction pathways. These pathways regulate numerous cell processes, including growth, proliferation, and death (apoptosis). Effective signal transduction requires proteins known as kinases that catalyze the phosphorylation of target proteins at specific sites. Cascades involving specific phosphorylations or dephosphorylations of signal transduction proteins ultimately affect the activity of transcription factors – proteins that bind to specific response elements on DNA and promote or inhibit the transcription of various genes (Higdon, 2007).

It is believed that, through this mechanism, phenolics could interfere in several steps leading to the development of malignant tumors, including the inactivation of carcinogens and the inhibition of mutant gene expression. Many studies have also shown that they can activate detoxification

enzymatic systems (Phase II) and prevent oxidative damage to the DNA, an important factor in the age-related development of some cancers (Mitscher *et al.*, 1996; Bravo, 1998; Halliwell, 1999; Vattem *et al.*, 2005a; Vattem & Shetty, 2005). Apoptosis or programmed cell death is another major mechanism through which phenolics could suppress cancer (Ramos *et al.*, 2005). Numerous studies with cell cultures have shown that phenolics, through their effect on cell signalling pathways and by selectively inhibiting kinases, may alter growth factor signalling by inhibiting receptor phosphorylation or blocking receptor binding by growth factors (Higdon, 2007). Reports on quercetin in particular have demonstrated its ability to inhibit cancer cell line proliferation *in vitro*, including breast, colon, pancreas, and leukemia. When looking at the effect of quercetin on cancer cell proliferation, studies observed apoptosis in HepG2 hepatoma and colorectal cells, with arrest of the HepG2 cell cycle in G1 phase; inhibition of epidermal growth factor receptor expression and associated tyrosine kinase activity; reduced expression of Ras protein in colon cancer cells and primary colorectal tumors; increased expression of endogenous inhibitors of matrix metalloproteinases; and phytoestrogenic activity involving interaction with the estrogen α- and β-receptors of human mammary MCF-7 cells (Neto, 2007). Anthocyanins have been shown to be a major contributor toward the induction of apoptosis (Seeram & Heber, 2007). Anthocyanin isolates and anthocyanin-rich mixtures of bioflavonoids may also provide protection from DNA cleavage and enzyme inhibition (Ramirez-Tortosa *et al.*, 2001; Acquaviva *et al.*, 2003; Lazze *et al.*, 2003). A refined bioassay designed to assess the potential antitumor activity of compounds showed how a complex mixture containing (–)-epicatechin, (+)-catechin, dimers of gallocatechin and epigallocatechin, and a series of proanthocyanidin oligomers could inhibit phorbol ester-mediated ornithine decarboxylase (Dixon et al., 2005). Ellagic acid has also been shown to be a potent anticarcinogen agent. One of the main mechanisms by which ellagic acid is proposed to have

anticancer benefits is by modulating the metabolism of environmental toxins, thereby preventing initiation of carcinogenesis induced by these chemicals. Ellagic acid antimutagenic activity was proposed as the mechanism through which it inhibits the direct binding of these carcinogens to the DNA (Teel *et al.*, 1986; Zhang *et al.*, 1993; Vattem & Shetty, 2005). Studies have shown that ellagic acid is a potent inhibitor of DNA topoisomerases, which are other types of enzymes involved in carcinogenesis (Vattem & Shetty, 2005).

Studies reporting *in vitro* and *in vivo* antitumor and antiproliferative activity of cranberry have implicated flavonoid-rich extracts as contributing to these activities (Neto, 2007 and 2008). Seeram *et al.* (2004) reported that total cranberry extract (200 μg/mL) could inhibit the proliferation of human oral, colon and prostate tumor cells *in vitro*. Similar effects were also observed with some purified cranberry fractions, including the total phenolic fraction (200 μg/mL), the anthocyanin fraction (7.1 μg/mL), and the proanthocyanidin fraction (6.5 μg/mL). Quercetin (40.90 ± 1.12 μM) and quercertin glucoside 3,5,7,3',4'- pentahydroxyflavonol-3-O-β-D-glucopyranoside (49.22 ± 4.90 μM) isolated from cranberry extract were also reported to inhibit the proliferation of HepG2 human liver cancer cells. These same compounds, quercetin (137.46 ± 2.5 μM) and 3,5,7,3',4'- pentahydroxyflavonol-3-O-β-D-glucopyranoside (23.90 ± 3.86 μM) also inhibited the proliferation of MCF-7 human breast cancer cells (He and Liu, 2006). Cranberry anthocyanins could play a role in the fight against cancer through their displayed anti-angiogenic properties (Roy *et al.*, 2002; Bagchi *et al.*, 2004). An anthocyanin-rich extract (55.96 ± 5.66 pg/mL) obtained from mixture of berries, including cranberries, inhibited the tumor necrosis factor alpha induced expression of vascular endothelial growth factor, and decreased hemangioma formation and tumor growth (Bagchi *et al.*, 2004). Proanthocyanidin oligomers from whole cranberry fruit, in size up to 12 degrees of polymerization and with as many as four A-type linkages, also showed tumor antiproliferative activity (Neto, 2007).

A proanthocyanidin fraction from whole cranberry fruit was observed to selectively inhibit the growth of H460 human large cell lung carcinoma, HT-29 colon adenocarcinoma, ME-180 cervical carcinoma cells and K562 chronic myelogenous leukemia cells with EC_{50} in the range of 20-110 μg/L, while a proanthocyanidin subfraction containing oligomers composed primarily of 4–7 epicatechin units with at least 1 or 2 A-type linkages could inhibit the growth of H460 lung tumors, HT-29 colon and K562 leukemia cells at GI_{50} values ranging from 20 to 80 μg/ml (Neto *et al.*, 2006).

Other *in vitro* evidence suggests that cranberry phenolics could prevent cancer by decreasing cell invasion and metastasis by inhibiting matrix metalloproteinase activity, and inhibiting the inflammatory processes including cyclooxygenase activity (Neto, 2007). It seems that the presence of A-type linkages can influence the tumor inhibitory and selectivity properties of proanthocyanidins. Indeed, when screening a variety of smaller polyflavan-3-ols from different plants against GLC4 lung and COLO 320 colon carcinomas, Kolodziej *et al.* (1995) found that a trimer with an A-type linkage was more cytotoxic than dimers with A-type linkages and trimers with only B-type linkages. Overall, the tumor inhibition by cranberry is likely to involve synergistic activities between the cranberry major phytochemicals: the anthocyanins, the flavonols and the proanthocyanidins.

1.7. Conclusions and future prospects

The data available strongly suggest that cranberry consumption could help protect against cardiovascular diseases, various cancers, and infections involving the urinary tract disorders, dental health and Helicobacter pylori-induced stomach ulcers and cancers. Through the study of the biological properties of cranberry phytochemicals, considerable progress has been made in the understanding of these potential health benefits. The cranberry phytochemical components, the flavonoids, flavonols and flavan-3-ols, the anthocyanins, the tannins (mainly proanthocyanidins) and phenolic acid derivatives, working in synergy, appear to be responsible for the antioxidant, antibacterial and antimutagen properties observed mainly *in vitro*, but also in animal experimental studies and clinical studies. Confirmation of these hypotheses, through mechanistic and clinical studies, may lead to further development of cranberry-based nutraceutical and pharmaceutical preventive and therapeutic treatments.

CHAPITRE 2

Publication 2: Analyzing cranberry bioactive compounds

Analyzing cranberry bioactive compounds

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2.1. Contributions des auteurs

J'ai réalisé les recherches et les lectures nécessaires pour la rédaction de cette revue de littérature. Le Dr. Stéphane Caillet, codirecteur du projet et associé de recherche à l'INRS-Institut Armand-Frappier a participé aux discussions scientifiques et a corrigé le manuscrit. Le Dr. Gilles Doyon, chercheur scientifique au Centre de recherche et de développement sur les aliments de Saint-Hyacinthe (Québec), a participé aux discussions scientifiques et a corrigé le manuscrit. M. Jean-François Sylvain, directeur technique de Canneberges Atoka Inc., a approuvé le projet et participé aux discussions scientifiques. Prof. Monique Lacroix était la responsable scientifique et la coordinatrice du projet de recherche. De plus, Prof. Lacroix a supervisé l'élaboration des protocoles et les discussions scientifiques entourant ce projet. Elle a révisé le manuscrit.

2.2. Résumé en français

Analyse des composés bioactifs de la canneberge

Il y a un engouement croissant du public pour la canneberge de l'Amérique du Nord (*Vaccinium macrocarpon*) en tant qu'aliment fonctionnel en raison de ses bienfaits potentiels liés à sa teneur en composés phytochimiques, incluant les pigments anthocyanes, responsables de sa couleur rouge brillant, et autres métabolites phytochimiques secondaires (flavonols, flavan-3-ols, proanthocyanidines et dérivés d'acides phénoliques). Afin de pouvoir étudier ces composés phénoliques et flavonoïdes, il est primordial de les isoler convenablement de la matrice de l'échantillon. De loin, la chromatographie en phase liquide combinée à un détecteur ultraviolet-visible et à un spectromètre de masse est la technique la plus largement utilisée pour l'analyse et la caractérisation de ces composés. Cette revue porte sur les principaux composés bioactifs de la canneberge, les méthodes d'extraction et de purification, et les conditions d'analyse HPLC employées pour les caractériser. Les différentes stratégies à envisager pour l'extraction, la séparation chromatographique, la détection, ainsi que l'identification des composés et les applications en chromatographie en phase liquide sont discutées, et les informations concernant les méthodes spécifiques à l'analyse des composés bioactifs de la canneberge sont tabulées afin d'offrir au lecteur un moyen rapide d'accéder aux informations pertinentes.

2.3. Abstract

There is a growing public interest for the North American cranberry (*Vaccinium macrocarpon*) as a functional food because of the potential health benefits linked to phytochemical compounds present in the fruit: the anthocyanin pigments, responsible for its brilliant red color, and other secondary plant metabolites (flavonols, flavan-3-ols, proanthocyanidins, and phenolic acid derivatives). Isolation of these phenolic compounds and flavonoids from a sample matrix is a prerequisite to any comprehensive analysis scheme. By far the most widely employed analytical technique for the characterization of these compounds has been high-performance liquid chromatography, HPLC, coupled with ultraviolet-visible and mass spectrometer detection. This review covers the cranberry major bioactive compounds, the extraction and purification methods, and the analytical conditions for HPLC used to characterize them. Extraction, chromatographic separation and detection strategies, analyte determinations, and applications in HPLC are discussed and the information regarding methods of specific cranberry analyte analyses has been summarized in tabular form to provide a means of rapid access to information pertinent to the reader.

Keywords: Anthocyanins, flavonoids; proanthocyanidins; phenolic acids; high-performance liquid chromatography, mass spectrometer detection, photodiode array detection.

2.4. Introduction

Cranberries are healthy fruit that contribute color, flavour, nutritional value, and functionality. They are one of the only three Native American fruits. The North American cranberry (*Vaccinium macrocarpon*) is recognized by the US Department of Agriculture, USDA, as the standard for fresh cranberries and cranberry juice cocktail. The European variety, grown in parts of central Europe, Finland, and Germany, is known as *Vaccinium oxycoccus*. This is a smaller fruit with anthocyanin and acid profiles slightly different to that of the North American variety (Girard and Sinha, 2006).

Phenolic acids, flavonoids and tannins are phytochemicals ubiquitous in foods of plant origin (Lugasi and Hovari, 2000). Their quantitative distribution can vary between different organs of the plants and within different populations of the same plant species and for this reason they are often used for the plant taxonomical classification (Robards & Antolovich, 1997; Merken and Beecher, 2000a). The predominant bioactive compounds found in cranberries are the flavonols, the flavan-3-ols, the anthocyanins, the tannins (ellagitannins and proanthocyanidins) and the phenolic acid derivatives. These phytochemicals are commonly associated with the fruit's organoleptic (sensory) qualities and have also shown diverse biological properties and physiological activities in animals (Robards & Antolovich, 1997; Kren and Martinkova, 2001). Numerous clinical trials and epidemiological studies have established an inverse correlation between intake of phenolic-rich fruits and vegetables and the occurrence of diseases such as inflammation, cardiovascular disease, cancer, and aging-related disorders (Willet, 2001).

The objective of this paper is to provide an overview of the main bioactive compounds of cranberry and techniques used for their extraction, purification, analysis and characterization. This paper is organized in two main parts; the first part briefly presents the phytochemical composition of

cranberry. In the second part, various techniques used for the analysis of cranberry bioactive compounds, including sample extraction and purification, chromatographic separation, and detection strategies are reviewed in detail. These methods are presented in this review as a reference guide for researchers pursuing work in this field.

2.5. Cranberry bioactive compounds

Cranberries are known for their elevated concentration of anthocyanins, as well as their significant content of flavonols, flavan-3-ols, tannins (ellagitannins and proanthocyanidins) and phenolic acid derivatives. The major anthocyanins in cranberry are galactosides and arabinosides of cyanidin and peonidin (Macheix *et al.*, 1990; Neto, 2007). However, the following anthocyanins were also detected at low concentrations: cyanidin 3-*O*-glucoside, peonidin 3-*O*-glucoside, delphinidin 3-*O*-glucoside, delphinidin 3-*O*-galactoside, delphinidin 3-*O*-arabinoside (Macheix *et al.*, 1990; Lin and Harnly, 2007). Approximately 75% of the cranberry flavonols consist of quercetin glycosides (quercetin 3-*O*-galactoside), although myricetin and kaempferol monoglycosides have also been reported in the fruit (Macheix *et al.*, 1990; Häkkinen, 1999; Neto, 2007). The following flavonols were also detected in cranberry, but at much lower concentrations: quercetin, quercetin 3-*O*-glucoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinopyranoside, quercetin 3-*O*-arabinofuranoside and quercetin 3-*O*-rhamnoside, myricetin, myricetin 3-*O*-galactoside, myricetin 3-*O*-xylopyranoside, myricetin 3-*O*-arabinopyranoside, myricetin 3-*O*-arabinofuranoside, myricetin 3-*O*-rhamnoside, (Macheix *et al.*, 1990; Chen and Zuo, 2007; Lin and Harnly, 2007).

The flavan-3-ols in cranberries are mostly aglycones of catechin and epicatechin. Oligomeric polymerized tannins composed of successive flavan-3-ol monomeric units, are also known to occur in cranberries, primarily consisting of dimers, trimers, but also larger oligomers of epicatechin (Howell, 2005; Neto, 2007). The flavan-3-ol monomeric units can be linked together by C-C interflavan bonds occuring between the C_4-position of the 'upper' unit and the C_6 or C_8-position of the 'lower' unit (4β→6 or 4β→8). They form what are called B-type proanthocyanidin oligomers that differ only in the arrangement of (+)-catechin and (–)-epicatechin starter and extension units (B_1–B_4). Proanthocyanidin B_1 is found in cranberries (Fletcher *et al.*, 1977; Hammerstonne *et al.*, 2000; Gu *et*

al., 2004; Dixon et al., 2005). A less frequent structural variation may occur in cranberry proanthocyanidins with the formation of mixed double linkages, including C-C bonds and interflavonoid bonds by C-O oxidative coupling between both C_2 and C_4 of the upper unit and the oxygen at C_7 and positions C_6 or C_8, respectively, of the lower unit (4β→8 and 2β→O→7). This forms what are called A-type proanthocyanidin oligomers (Foo *et al.*, 2000a; Gu *et al.*, 2004; Neto, 2007). Cranberry proanthocyanidins tend to occur as tetramers to decamers, but also as polymers of epicatechin units, with the two types of linkages between epicatechin units, the B-type and the less common A-type (USDA, 2004; Howell, 2005; Neto, 2007). The presence of epicatechin-anthocyanin dimmers, trimers and tetramers was also reported (Neto *et al.*; 2008).

Recently, many authors have also reported the presence of certain phenolic acid derivatives in cranberries, mainly hydroxycinnamic acid, HCA, and hydroxybenzoic acid, HBA (Neto, 2007; Ruel & Couillard, 2007). Although the phenolic acids occur naturally in their free forms, they are generally considered structural moieties in polyphenol compounds, where they are embedded within a polyphenolic structure (Seeram and Heber, 2007). The presence of HCA derivatives has been reported in cranberry and includes compounds such as *p*-coumaric acid, coumaroylglucose, feruloylglucose, glycosylated sinapic acid, caffeoylglucose and diglucoside of caffeoylglucose, (Macheix *et al.*, 1990; Häkkinen *et al.*, 1999). The presence of ellagic and p-hydroxybenzoic acids was described by Häkkinen *et al.* (1999) in cranberries. Due to reactions catalyzed by light, heat, and oxygen, the ellagic acid tends to combine with flavan-3-ols, forming complex tannins called ellagitannins. High content of ellagitannins in cranberry were reported by Kähkönen *et al.* (2001), Lei *et al.* (2001), Vattem and Shetty (2003), and Kaponen *et al.* (2007).

2.6. Analyzing bioactive compounds in cranberry

Choice of isolation and analytical techniques used for the characterization and/or quantification of phenolic and polyphenolic compounds from food matrix and plant materials is dictated by their chemical nature, but also by the sample particle size, and the presence of interfering substances. The chemical nature of plant phenolics varies from simple to highly polymerized substances that include varying proportions of phenolic acids, anthocyanins and tannins, among others. They may also exist in a complex state with carbohydrates, proteins, organic acids and other plant components forming high-molecular weight phenolics (tannins) and their complexes that are generally insoluble. Therefore, phenolic extracts obtained from food matrix and plant materials are always a mixture of different classes of phenolics which will depend on the solvent system used for the extraction or further analyses. Different techniques can be used to characterize and quantify bioactive compounds in food products, although by far the most widely recognized technique has been high-performance liquid chromatography, HPLC, combining ultraviolet visible, UV/Vis, and mass spectrometric, MS, detection instruments (Escarpa and Gonzalez, 2000a; Merken and Beecher, 2000b, Rodriguez-Delgado *et al.,* 2000). The measured phenolic content of a sample can be greatly influenced by the method used for analysis. Lee *et al.* (2008) have demonstrated the importance of reporting the standard used to express the values.

2.6.1. Isolating phenolics from their food matrix: sample preparation and purification

Studies employ different methods to extract, purify and characterize the phenolic compounds in fruit. Isolation of phenolic compounds and flavonoids from a sample matrix is generally a prerequisite to any comprehensive analytical scheme. As shown in Figure 2.1, sample preparation encompasses a series of steps. Following the extraction, additional steps may be required for the

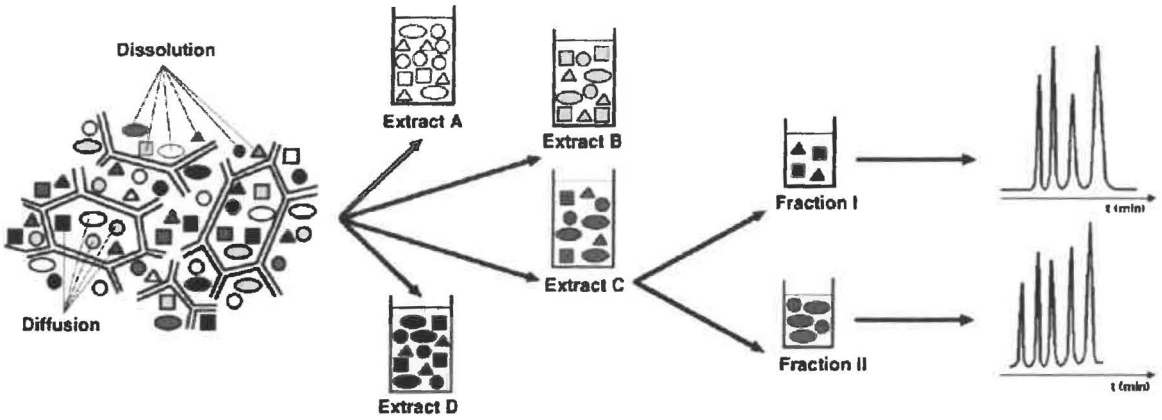

Figure 2.1. Separation methods for the analysis and isolation of plant constituents. Reproduced with permission from Szabolcs Nyiredy (Nyiredy, 2004).

removal of unwanted phenolics and non-phenolic substances such as waxes, fats, terpenes and chlorophylls. Solid phase extraction, SPE, techniques and fractionation based on acidity are commonly used to remove unwanted phenolics and non-phenolic substances (Robbins, 2003; Naczk and Shahidi, 2004). Ultimately, the goal of these procedures is the preparation of a sample extract uniformly enriched in all components of interest and free from interfering matrix components. The procedure must allow quantitative recovery of the compounds of interest whilst avoiding any chemical modifications in the analytes which result in artefacts and unnecessarily complicate subsequent steps (Robards & Antolovich, 1997).

2.6.1.1. Extraction

Extraction methods can vary from exhaustive extraction and pre-concentration procedures to simple liquid-liquid extraction. The choice of a method will depend on both the nature of the analyte (solubility and stability of phenolics in this case), and the physical state of the sample (fruit type, fruit portion – seed/stone, skin, flesh, leaf). Solubility of phenolics is governed by their degree of polymerization (glycosylation, acylation, esterification), as well as by their interactions with other food constituents that can form insoluble complexes (Naczk and Shahidi, 2004). The glycosylation of phenolic compounds, for example, renders them highly polar, whereas those that occur in the free form are much less polar (Bohm, 1998). Accumulation of soluble phenolics is greater in the outer tissues (exocarp and mesocarp layers) of the fruit than in the inner tissues (endocarp and seed). At the subcellular level, soluble phenolic compounds may accumulate in the vacuoles or in the cell walls (Antolovich *et al.*, 2000; Robards & Antolovich, 1997). Phenolic compound extraction from seed coat and pericarp tissues are particularly difficult to assay accurately (Styles and Ceska, 1989; Marles *et al.*, 2003). The fidelity between the phenolic profile of the starting material and that of the isolated extract provides the theoretical basis for judging analytical techniques. The 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). Still, there is no completely satisfactory procedure suitable for extraction of all phenolics or a specific class of phenolic substances in plant materials (Naczk and Shahidi, 2004).

The extraction method and solvent choice are critical elements, as are extraction time and temperature reflecting the conflicting actions of solubilization and analyte degradation by oxidation (Robards, 2003). A variety of extraction techniques can be used to promote physical solvent-sample interactions: sonication, microwave-assisted extraction, high pressure temperature extraction, stirring, and shaking (Robards, 2003; Merken and Beecher, 2000b; Molnar-Perl and Fuzfai, 2005; Rijke *et al.*, 2006). Multiple, repeated extraction (up to six stages) methods are often reported, but only marginally enhance the yield of extraction of additional phenolics. Solvents such as methanol, ethanol, acetone, water, ethyl acetate and, to a lesser extent, propanol, dimethylformamide, and their combinations are frequently used for the extraction of food phenolics (Naczk and Shahidi, 2004). Extraction periods, usually varying from 1 min to 24 h, have been reported; longer extraction times will improve phenolics recovery but also increase the chance of oxidation by air and light. For this reason, extraction procedures frequently incorporate an antioxidant as a stabilizer to the solvent system, such as ascorbic acid or tert-butylhydroquinone. The choice of stabilizer can be influenced by the subsequent procedure as co-elution with plant phenols can occur. The effectiveness of the added stabilizer will inevitably depend on its concentration and activity relative to indigenous phenols, as analyte recovery can be reduced at higher concentrations of stabilizer due to the pro-oxidant action of the latter (Robards, 2003).

Extractions have been performed on freeze-dried extracts of fruit or alternatively, by maceration of fresh, undried fruit with the extracting solvent. In the latter case, the required proportion

of water is lower. Fruit juices will generally require minimal sample preparation beyond filtration and ultracentrifugation. However, the use of solvents of graded polarity can be used with fruit juices for the recovery of specific classes of phenolics which have different solubility (Robards & Antolovich, 1997). A brief summary of various approaches used for the extraction of bioactive compounds from several cranberry samples, including frozen fruit, juice, pomace and powder made from concentrate is presented in Table 2.1.

2.6.1.2. Purification

The Folin-Ciocalteu assay is widely used to determine the total phenolic concentration of samples (Singleton and Rossi, 1965). Since non-phenolic reducing agents (carbohydrates and/or lipoidal material) can react in this assay, a purification step is often necessary to obtain an extract suitable for subsequent characterization and quantification (Robards, 2003; Kalt *et al.*, 2008). This purification or clean-up of extracts is often achieved by selective retention of the compounds on a solid-phase adsorbent (Mateo and Jimenez, 2000, Robards, 2003). Preparatory chromatography has been employed for purification of phenolics extracts, although SPE has been a great alternative to liquid–liquid extraction and is now the most widely used method (Naczk and Shahidi, 2004; Nyiredy, 2004). SPE allows the pre-concentration, as well as the clean-up and the purification of extracts. Non-selective SPE typically on C_{18} alkyl-bonded silica or copolymer sorbents is widely used for analyte extraction and enrichment from aqueous samples and sample extracts.

Sample solution and solvents are usually slightly acidified to prevent compound ionization, which would reduce their retention (Rijke *et al.*, 2006). Pre-packed SPE cartridges may be used in one of two stages, such that the interfering plant constituents of a sample are retained on the cartridge while the components of interest are eluted or vice versa. The first fraction obtained from an SPE cartridge, particularly if aqueous alcohol has been used as extracting solvent, generally contains

Table 2.1. Extraction methods used for the recovery of bioactive compounds from cranberry samples.

Sample	Analyte	Sample Preparation and Extraction Conditions	Ref.
Frozen Cranberries	Flavonol aglycones and glycosides	100g frozen sample thawed in microwave oven and crushed in a food processor. Flavonoid glycosides extraction: in a 100-mL bottle, 5g of homogenized berries diluted in purified water to 15 mL. Then 25 mL of methanol with 2 g/L tert.-butylhydroxyquinone (TBHQ) were added. Hydrolysis induced with 10 ml of 1.2 M HCl. Mixture refluxed 2 h at 85°C, cooled and filtered. A 20-mL filtrate portion was evaporated to dryness by rotary evaporation in 35°C water bath. Residues dissolved in 1.5 ml methanol and filtered on 0.45-mm filter. Flavonoid aglycones extraction: in a 100 mL bottle, 5 g of homogenized berries was diluted in purified water to 25 mL. Then 25 mL of methanol were added. The mixture was flushed with nitrogen, shaken at room temperature for 2 h and the extract centrifuged at 3000 g 10 min at 148°C. A 20-mL filtrate portion was evaporated to dryness by rotary evaporation in 35°C water bath. Extract dissolved in 1.5 ml methanol and filtered on 0.45-mm filter.	Häkkinen & Auriola (1998)
Fresh Cranberries	Gallic acid, catechins, hydroxycinnamic acids, flavonols	100g frozen sample crushed in food processor and concentrated by lyophilization. Freeze-dried sample ground to pass a 0.1-mm sieve and stored at -18°C. In a 100-ml bottle, 0.5 g of freeze-dried sample dissolved in 15 ml of purified water with 80 mg of ascorbic acid. Then 25 ml of methanol was added. Hydrolysis induced with 10 ml of 1.2 M HCl. Solution sonicated 2 min and flushed with N_2. The 50 mL mixture shaken 16 h at 35°C, in the dark, cooled and then filtered. A 15-ml portion was evaporated to dryness by rotary evaporation in 35°C water bath. Extract dissolved in 1.5 ml methanol and filtered on 0.45-mm filter.	Häkkinen *et al.* (1999)
Frozen Cranberries	Anthocyanin, flavonoid glycosides, proanthocyanidin	10 kg washed cranberry fruit homogenized with acetone in a blender 10 min at 4°C. Mixture filtered and pulp discarded. Extract concentrated under reduced pressure to remove acetone. Extract diluted with water and defatted by 3 extractions with an equal volume of petroleum ether. Aqueous phase reduced to a small volume and extracted 4 times with an equal volume of ethyl acetate. Solvent extracts combined, concentrated and freeze-dried to yield 47g of red-colored syrup.	Foo *et al.* (2000a)
Cranberries	Catechins, flavanones, flavones, flavonols	Maceration with 1.2 M HCl in 50% MeOH 2 h at 90° C. Filtration.	Mattila, Astola, and Kumpulainen (2000)
Cranberry juice	Procyanidins	50 mL of juice loaded onto gravity-fed 20 mL column packed with 5 g of Sephadex LH-20 in water. Column rinsed with 10 ml of 20% aqueous methanol (v/v). Procyanidins eluted with 75mL acetone/water/acetic acid (70:29.5/0.5 v/v). Eluate concentrated by rotary evaporation to 2 ml. Solution was brought to 5-mL volume with the extraction solvent and filtered through a 0.45-mm nylon filter.	Hammerstone *et al.* (2000)
Cranberry powder 90X	Proanthocyanidins	10 g cranberry powder dissolved in 25 mL water and loaded on 620 mm column packed with 70 g Sephadex LH-20. Non-phenolic compounds eluted with 500 mL of water, at 1.5 ml/min. Proanthocyanidin eluted with 2.5 L of 80% aqueous acetone (v/v). Eluate concentrated by rotary evaporation under reduced pressure at 35°C.	Porter *et al.* (2001)
Cranberry juice and cranberry juice cocktail	Flavonoids and phenolic compounds	0.5 mL of 10 g/L ascorbic acid added to 3 mL of canned cranberry juice or to 0.8 mL of freshly squeezed cranberry juice. Solution pH adjusted to 7.0 with NaOH solutions. Sample loaded onto C18 Sep-Pak cartridge preconditioned with 8 mL of methanol and 4mL of distilled–deionized water (DDW) at pH 7.0. For acidic compounds, preconditioning with 4 ml of 0.01 M HCl instead of DDW. Neutral sample loaded on C18 Sep-Pak cartridge and washed with 10 mL DDW at pH 7. Acqueous eluate pH adjusted to 2.0 with 2.0 M HCl and loaded onto preconditioned acidic column washed with 5 mL of 0.01 M HCl. Both adsorbed fractions eluted with 12 mL methanol and evaporated using a rotary evaporator until dryness at 35°C. Extracts redissolved in 3.0 mL of methanol and filtered.	Chen et al. (2001)
Frozen Cranberries	Flavan-3-ols and proanthocyanidins	In a 15-mL screw-cap tube, 1g of freeze dried sample combind with 10 mL of acetone/water/acetic acid (70:29.5:0.5 v/v). Sample vortexed 30 s, sonicated at 37 °C for 10 min and then maintained at room temp. 50 min (tube vortexed 30 s after 25 min). Tube centrifuged at 3500 rpm 15 min. Supernatant evaporated at 25 °C under partial vacuum (1.5 torr). Extract dissolved in 6 mL of 30% aqueous methanol (v/v).	Gu *et al.* (2002); Gu *et al.* (2003)

Sample	Analyte	Sample Preparation and Extraction Conditions	Ref.
Cranberry powder 90X	Sugars, organic acids, total phenols, anthocyanins, proanthocyanidins	60g sample dissolved in water, filtered, and loaded onto 4 RP-Flash 40M C_{18} cartridges. Each cartridge eluted with 500 mL of water; water/methanol (85:15 v/v), and methanol/acetic acid (99:1 v/v) to yield 3 fractions: sugars-enriched, organic acids-enriched and polyphenols-enriched fractions that were evaporated in vacuum, reconstituted in water, and freeze-dried.	Seeram *et al.* (2004)
Frozen Cranberries	Catechins and procyanidins	600 g frozen berries were crushed and mixed with 150 mL of ascorbic acid (1 g/100 mL) in water. Phenolic compounds extracted with 600 mL ethyl acetate by shaking 2 h at room temp. Ethyl acetate extract rinsed twice with 200 mL of 0.1 M sodium acetate buffer (pH 7.0) and then with 200 mL of water. Eluate evaporated to dryness under vacuum at 35 °C.	Määttä-Riihinen *et al.* (2005)
Freeze dried cranberry powder	Anthocyanins	In a 15 mL screw-cap tube, 1g of freeze-dried sample combined with 15 mL of methanol/water/acetic acid (85:15:0.5 v/v). Sample vortexed 30 s, sonicated 5 min and maintained at room temp. 10 min (vortexed for 30 s after 5 min). Tube centrifuged at 4550g 10 min and the supernatant removed. Sample extracted once more with 10 mL of methanol/water/acetic acid (85:15:0.5 v/v) using the same procedure, and the supernatants were combined. Supernatant was transferred to a 25 mL volumetric flask, volume completed with extraction solvent.	Wu and Prior (2005); Wu *et al.* (2006)
Cranberry powder 90X	Phenolic acids, anthocyanins, flavonols, proanthocyanidins	4.5 g sample dissolved in 8 mL water and loaded on a 270 mm column packed with 35 g Sephadex LH-20. Non-phenolic compounds eluted with 250 mL water at 1.5 ml/min. Phenolic compounds eluted with 2.5 L 80% aqueous acetone (v/v). Phenolic eluate concentrated by rotary evaporation under reduced pressure at 35°C and freeze-dried.	Lee *et al.* (2006)
Cranberry powder 90X	Total phenols, quercetin	50 g powder blended 1 min with 500 mL of chloroform/methanol (1:1 v/v). Mixture stirred 2 h at room temp. 100 mL of 0.5% NaCl added and the mixture transferred into a separatory funnel. Chloroform layer recovered, mixed with 20 mL of water, and evaporated by rotavap under vacuum at 35 °C until concentrated to less than 20 mL. Acqueous extract freeze-dried and stored at -80 °C.	Raghavan and Richards (2006)
Cranberry pomace	Quercetin	50g sample blended with 500 mL acetone/water (70:30 v/v) 1 min. Mixture was stirred for 2 h at room temp. and filtered. Supernatant concentrated to 150 mL by rotory evaporation under vacuum, and mixed with 3 volumes of ethyl acetate, transferred into a separatory funnel and left to separate overnight. Recovered ethyl acetate phase evaporated under vacuum. Extract freeze-dried and stored at -80 °C.	Raghavan and Richards (2006)
Raw cranberries	Flavonoids	0.2-0.5 g freeze-dried sample homogenized 3 min with 4 mL of 90% aqueous methanol with 0.4 g/L TBHQ. Sample centrifuged. For times, retentat combined with 4 mL of 90% aqueous methanol with 0.4 g/L TBHQ, homogenized and centrifuged. Collected supernate combined, evaporated by N_2 flushing to less than 1 mL and filtered.	Harnly et al. (2006)
Cranberries	Total polyphenols, flavonols, anthocyanins, proanthocyanidins	825 g sample homogenized with 500 mL acetone/methanol/water/formic acid (40:40:19:1 v/v) and macerated 1 h. Mixture filtered and retentate reextracted twice with 250mL solvent mixture. Solution concentrated by rotary evaporation and loaded on a Diaion HP-20 column (25×200mm). Sugars eluted with 300mL DDW and phenolics eluted with methanol. Phenolic solution concentrated by rotary evaporation and extract freeze-dried.	Neto et al. (2006)
Frozen cranberries	Flavonoids glycosides and aglycones	66.5 g sample homogenized with 100 mL 70% aqueous methanol. Mixture filtered and retentate reextracted twice with 100 mL 70% aqueous methanol and filtered as before. Solution concentrated by rotary evaporator. Aqueous extract combined twice with 60 mL petroleum ether. Remaining aqueous solution extracted 4 times with 60.0 mL ethyl ether. Ethyl ether layers combined, filtered and concentrated by rotary evaporator.	Chen & Zuo (2007)

numerous non-flavonoid substances which can interfere in later stages of the analysis: surface waxes, simple and complex carbohydrates, organic acids, including vitamin C, and protein). By washing the adsorbent with aqueous organic solvent following the adsorption step, polar glycosylated constituents can then be eluted while the less polar aglycons are retained. Flavonoids are generally recovered by elution with methanol from a Sep-Pak C_{18} cartridge following the elution of sugars and phenolic compounds with aqueous methanol (Robards & Antolovich, 1997). Table 2.2 summarizes the various approaches that have been used for the purification of bioactive compounds from different types of cranberry extracts or samples.

2.6.2. Chromatographic separation and detection of phenols in cranberry

Once the desired extract is obtained, different techniques can be used to characterize and quantify bioactive compounds in food products. Although modern separation systems such as gas chromatography, capillary zone electrophoresis and micellar electrokinetic capillary chromatography have shown good results, by far the most widely employed technique has been HPLC combining UV/Vis and MS detection (Escarpa and Gonzalez, 2000a; Merken and Beecher, 2000b, Rodriguez-Delgado *et al.*, 2000).

2.6.2.1. Chromatographic separation

Normal-phase chromatography has been used for the separation of flavonoids in food, but with this system there are concerns that highly polar materials may be irreversibly retained on the column, resulting in a gradual alteration of the separation characteristics. Thus, reversed-phase, RP, chromatography has invariably been the method of choice for the separation of the phenolic compounds and flavonoids (Robards & Antolovich, 1997; Lee, 2000; Merken and Beecher, 2000a; Escarpa and Gonzalez, 2001). The separation of these compounds is usually done on a silica-based C_8

Table 2.2. Purification and fractionation methods used for the recovery of bioactive compounds from cranbery extracts.

Sample	Analyte	Stationary Phase	Mobile Phase	Ref.
Frozen Cranberries	Anthocyanin, flavonoid glycosides and proanthocyanidin	600x50 mm column packed with Sephadex LH-20	45g of extract dissolved in 40 mL ethanol and loaded on column. Elution with 100% ethanol and 18 mL portions collected to give predominantly anthocyanin, flavonoid glycosides, and three main (A-C) proanthocyanidin fractions.	Foo *et al.* (2000a)
Cranberry	Proanthocyanidins	600x50 mm column packed with Sephadex LH-20	45g of extract dissolved in ethanol and loaded on column. Sugars, phenolic acids and anthocyanins eluted with 100% ethanol until the eluent clear. Proanthocyanidins eluted with 60% aqueous acetone and sample concentrated by rotary evaporator at 40°C. Extract freeze-dried.	Foo *et al.* (2000b)
Cranberry powder 90X	Proanthocyanidins	215x150 mm column packed with 23 g Sephadex LH-20 equipped with HPLC binary pump and DAD	5 mL extract was applied onto the column. Each fraction monitored at 280, 320, 360 and 520 nm and obtained by elution with solvent applied at 1.0 ml/min: - 1: 100% water; - 2: 50% aqueous ethanol (v/v); - 3: 100% ethanol; - 4: ethanol:methanol (1:1,v/v); - 5: 100% methanol - 6: 80% aqueous acetone (v/v). Each fraction concentrated under vacuum by rotary evaporation at 35°C and stored in 50% aqueous ethanol (v/v) at -80°C.	Porter *et al.* (2001)
Cranberries, cranberry juice and spray-dried cranberry extract	Procyanidins	20 mL columns packed with 5 g Sephadex LH-20, equilibrated 2h in 25 mL water	5 mL extract or 80 mL of juice loaded onto the column. Sugars and phenolic acids were eluted with 25 mL of 20% acqueous methanol. Flavonols and anthocyanins eluted with 40 mL of 60% acqueous methanol and procyanidins eluted with 90 mL methanol. Collected fractions concentrated by rotary evaporation. Procyanidins extract diluted to a final volume of 15 mL methanol. Other fractions were freeze-dried.	Prior *et al.* (2001)
Frozen cranberries	Polymeric procyanidins	60x15 mm column packed with 3 g Sephadex LH-20 equilibrated 4 h with water	6 mL extract loaded onto the column. Sugars and phenolic acids were eluted with 40 mL of 30% acqueous methanol. Procyanidins eluted with 80 mL of 70% aqueous acetone. Fractions evaporated to dryness under vacuum in a SpeedVac at 43°C. Recovered extract dissolved in the extraction solvent, brought to a final 5 mL volume and filtered.	Gu *et al.* (2002)
Cranberry	Flavan-3-ols and proanthocyanidins	60x15 mm column packed with 3 g of Sephadex LH-20 equilibrated 4 h with 30% aqueous methanol	6 mL extract loaded onto the column. Sugars and phenolic acids were eluted with 40 mL of 30% acqueous methanol. Proanthocyanidins eluted with 70 mL of 70% aqueous acetone. Fractions evaporated to dryness under vacuum in a SpeedVac at 25 °C. Recovered extract dissolved in the extraction solvent, brought to a final 5 mL volume solution and centrifuged at 14 000 rpm for 10 min.	Gu *et al.* (2003)
Cranberry powder 90X	Anthocyanins and proanthocyanidins	20 mL columns packed with 5 g of Sephadex LH-20 equilibrated 2 h with water	2 g extract dissolved in 1 mL of water and loaded onto the column. Elution consecutively with 500 mL water/ethanol/acetic acid (1:1:0.1 v/v), 500 mL acetone/water (8:2 v/v). Fractions evaporated in vacuum, reconstituted in water, and freeze-dried.	Seeram *et al.* (2004)
Frozen Cranberries	Catechins and procyanidins	300x20 mm column packed with Sephadex LH-20 equilibrated with 0.1 M sodium acetate buffer (pH 7)	Extract dissolved in 10 mL methanol was loaded onto the column. Phenolic acid residues were eluted with 40 mL of sodium acetate buffer and 50 mL of 30% methanol in water. Catechins eluted with 50 mL of 60% methanol in water. Dimeric and trimeric proanthocyanidins eluted with 50 mL of pure methanol. Fractions were concentrated by vacuum evaporation and this fractionation procedure repeated 1-3 times to collect sufficient amounts of catechins and procyanidins.	Määttä-Riihinen *et al.* (2005)

Sample	Analyte	Stationary Phase	Mobile Phase	Ref.
Cranberry powder 90X	Phenolic Acids, Anthocyanins, Flavonols, Proanthocyanidins	250x 22 mm column packed with 23 g Sephadex LH-20 equipped with HPLC binary pump and DAD	50 mg extract dissolved in 7.5% aqueous ethanol and applied onto the column. Each fraction monitored at 280, 320, 360 and 520 nm and obtained by elution with solvents applied at 2.5 ml/min: - 1A and 1: 100% water; - 2: 50% aqueous ethanol (v/v); - 3: 100% ethanol; - 4: ethanol:methanol (1:1,v/v); - 5: 100% methanol; - 6: 80% aqueous acetone (v/v). Each fraction concentrated under vacuum by rotary evaporation at 35°C, freeze-dried, and stored in 50% aqueous ethanol at -80°C.	Lee *et al.* (2006)
Cranberry	Phenolic acids, Total polyphenols, flavonols, anthocyanins, proanthocyanidins	25×290mm C_{18} column for small phenolics and total polyphenols 25×290mm column packed with Sephadex LH-20 for flavonols, anthocyanins and proanthocyanidins	Extract dissolved in methanol and loaded onto C_{18} column. Smalla phenolics eluted with 100mL water and 100 mL 85% acqueous methanol. Total polyphenols eluted with acidified methanol (5mg/mL formic acid) until clear. Eluates concentrated under vacuum. Total polyphenol extract dissolved in methanol and loaded onto Sephadex column. Flavanols and anthocyanins eluted with methanol/water/formic acid (70:25:5 v/v) until clear. Proanthocyanidin eluted with 200mL of acetone/water (70:30 v/v). Eluates concentrated under vacuum. Proanthocyanidin extract dissolved in methanol, loaded on another Sephadex column. Triterpene hydroxycinnamates eluted with acidified methanol (5mg/mL formic acid). Proanthocyanidins eluted acetone/water (70:30 v/v). Eluated concentrated under vacuum.	Neto *et al.* (2006)
Frozen cranberries	Flavonoids glycosides and aglycones	200x200 mm silica plates	Extract loaded onto silica plates. Elution with ethyl acetate stopped when solvent front was 2 cm from the top edge of plates. Chromatic bands shown under UV lamp and circled with pencil. Chromatic bands scraped out, extracted with 2x2 mL methanol and filtered.	Chen & Zuo (2007)

or 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). In general, the Symmetry and Zorbax XSD-C_{18} columns were found to offer the best separations, followed closely by the Symmetry Shield and Luna columns. None of these columns, however, was optimal for all regions of the chromatograms. For the best separation results over the entire range of polarities of the extracted compounds, two or more columns should be used (Robards & Antolovich, 1997, Lee, 2000). Columns are generally protected with guard columns containing similar packing. Dimensions of the column varies between 100 and 300 mm in length and the internal diameter is usually 4.6 mm (Merken and Beecher, 2000a and 2000b; Escarpa and Gonzalez, 2001).

The employed elution modality, whether isocratic or gradient, depends on the phenol composition of the sample analysed. Isocratic elution has been used for the study of samples whose phenol composition is constituted by the same group or structure family, but the elution patterns most invariably rely on gradient elution with binary solvent system owing to the diversity of phenols (Antolovich *et al.*, 2000). In RP chromatography, the mobile phase usually consists of a mixture of water (the diluent) and an organic modifier. Aqueous acetonitrile, methanol, or a mixture of the two with either formic, acetic or trifluoroacetic acid, ammonium acetate (10 mM), and formate (10 mM) are the mobile phases most frequently reported in the literature (Robards & Antolovich, 1997, Lee, 2000, Robards, 2003; Merken and Beecher, 2000; Naczk and Shahidi, 2004; Molnar-Perl and Fuzfai, 2005; Rijke *et al.*, 2006). Compared to acetonitrile, a 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, as 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). Most flavonoids are ionisable and this tends to reduce their adsorption onto the stationary phase. To circumvent this problem and improve the resolution and reproducibility of successive analysis, 0.1 – 5% of acids (acetic, phosphoric, formic or trifluoroacetic acids) is usually added to the mobile phase to control the pH and suppress ionization (Dalluge et al., 1998; Merken and Beecher, 2000a; Bloor, 2001; Escarpa and Gonzalez, 2001).

HPLC methods developed for phenolic compounds and flavonoids analysis 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; temperatures up to 40°C are sometimes used to reduce the time of analysis and because thermostated columns give more repeatable elution times (Rijke *et al.*, 2006). According to the table presented in a review article by Molnar-Perl and Füzfai (2005) the number of analytes detected simultaneously by most HPLC methods is usually around 10. Such a number of phenolic compounds is usually separated satisfactorily in 45 or 50 min. But when comparing a conventional HPLC system and an ultra performance liquid chromatography system, UPLC, both equipped with columns containing similar stationary phases, a faster separation of standards of phenolic compounds can be obtained by UPLC (Spacil *et al.*, 2008).

Under RP separation conditions, more polar compounds generally elute first. The diglycosides precede monoglycosides, which precede aglycones. The elution pattern for flavonoids containing equivalent substitution patterns is: flavanone followed by flavonol and flavones. This elution pattern also holds for both aglycones and glycosides (Robards and Antolovich, 1997; Nollet, 2000). For anthocyanins, molecular structure–retention characteristics on an RP column are governed by the following factors: substitution of the anthocyanidin B-ring, the position, nature and number of sugar moieties attached to the anthocyanidin and the sugar acylation. Thus, substitution of the B-ring gives

the elution order delphinidin < cyanidin < petunidin < pelargonidin < peonidin < malvidin with hydroxyl groups decreasing and methoxyl groups increasing retention. In general, anthocyanidin glycosylation decreases retention in the order 3,7-diglycosides < 3,5-diglycosides < 3-glycosides and 3-galactosides <3-glucosides < 3-rutinosides, subject to modification by the nature of the sugar moiety (Robards and Antolovich, 1997; Nollet, 2000; Bloor, 2001). Investigation of polymeric pigments using HPLC with RP C_{18} column has been limited because these compounds typically elute together as a broad peak (called an envelope) that can be difficulte to evaluate (Versari *et al.*, 2008). The recent availability of the polystyrene-divinylbenzene RP column provides a great opportunity to improve the analysis of polymeric pigments (Peng *et al.*, 2002). A brief summary of various elution patterns used for the determination of bioactive phenolics from several cranberry samples, including frozen fruit, juice, and powder made from concentrate are presented in Table 2.3.

2.6.2.2. UV-Vis detection

Since all phenolics contain at least one aromatic ring, they can consequently absorb UV light effectively and each class of phenolics has a distinctive UV or UV-Vis spectra where minor differences in structure are often seen as significant differences in their UV spectra. The diode-array detection, DAD, set at specific wavelength with scanning for an entire spectrum (190 to 600 nm) in approximately one second, allows the collection of data that provides the unique scan for specific compounds and their maximum wavelength (Siouffi, 2000). Combined with chromatographic retention properties, these spectral properties enable compound characterization and rapid diagnosis of certain structural features of each eluted band (Robards and Antolovich, 1997; Escarpa and Gonzalez, 2000b; Bloor, 2001; Escarpa and Gonzalez, 2001).

The typical UV/Vis absorption spectra of HCA derivatives has a peak between 305 and 330 nm (band I) and a shoulder between 290 and 300 nm (band II). Commonly occurring HCA

Table 2.3. HPLC characterisation of bioactive compounds from cranberry extracts.

Sample	Analyte	Stationary Phase	Mobile Phase	Detection	Ref.
Cranberry Juice	Anthocyanidins, anthocyanins	Anthocyanidins: ODS (250 x 5 mm) Anthocyanins: PLRP-S (250 x 4.6 mm; 5µm)	Anthocyanidins: (A) 15% acetic acid in water (B) 100% acetonitrile Isocratic: 85% A in B Anthocyanins: (A) 4% phosphoric acid in water (B) 100% acetonitrile Gradient: 6% B in A, 0-10 min; 6-20% B in A, 10-50 min; 20% B in A; 50-65 min	DAD: 260 & 520 nm	Hong & Wrolstad (1990)
Frozen Cranberries	Flavonol aglycones and glycosides	RP-C_{18} column (125x3 mm i.d., 5 µm) protected with a guard column of same material (4x 4 mm)	(A) 1% formic acid in water (B) 100% acetonitrile Gradient: 10-13% B in A, 0-10 min; 13-70% B in A,10-25 min; 70% B, 25-29 min; 70-10% B in A, 29-30 min; 10% B in A, 30-35 min. Flow rate 0.5 mL/min. Injection volume:20 µL for LC-MS, 5 µL for LC-DAD.	DAD: 280 nm + scan 220–450 nm. MSD positive ionization with capillary temp. at 225°C and voltage at 4.5 kV. Mass scan from m/z 200-1000 at 500 ms per cycle.	Häkkinen & Auriola (1998)
Fresh Cranberries	Gallic acid, catechins, hydroxycinnamic acids, flavonols	ODS column (3.5 µm, 100x4 mm) protected with guard column RP-18 (5 µm, 10x4 mm)	(A) 50 mM ammonium dihydrogen phosphate, pH 2.6 (B) 0.2 mM ortho-phosphoric acid, pH 1.5 (C) 20% of solvent A in 80% acetonitrile Gradient elution and flow rate as per Häkkinen & Auriola (1998). Injection volume: 20µL.	DAD: 260, 280, 320 and 360 nm.	Häkkinen *et al.* (1999)
Cranberry juice	Procyanidins	Phenomenex packed column (250 x 4.6 mm) and heated at 37°C	(A) dichloromethane, (B) methanol (C) acetic acid and water (1:1 v/v). Linear gradients of B in A with a constant 4% C Gradient: 14-28.4% B in A, 0-30 min; 28.4-39.2% B in A, 30-45 min; 39.2-86% B in A, 45-50 min. Flow rate 1 mL/min. Injection volume: 5µL.	DAD: 280 nm	Hammerstone, *et al.* (2000)
Cranberries, cranberry juice and spray-dried cranberry extract	Procyanidins	Phenomenex packed column (250 x 4.6 mm) and heated at 37°C	(A) dichloromethane (B) methanol (C) acetic acid and water (1:1 v/v). Linear gradients of B in A with a constant 4% C Gradient: 14-28.4% B in A, 0-30 min; 28.4-39.2% B in A, 30-45 min; 39.2-86% B in A, 45-50 min. Flow rate 1 mL/min. Injection volume: 5µL.	DAD: 280 nm MSD: Post column addition with 10 mM ammonium acetate in methanol at 0.1 mL/min. API-ES negative ionization with capillary voltage at 3.5 kV, nebulizing pressure at 25 psi, drying gas temp. at 350°C and fragmentor at 85 V. Mass scan from m/z 220-2200 at 2.12 s per cycle.	Prior *et al.* (2001)
Cranberry powder 90X	Proanthocyanidin	C18 column (8µm, 250x 4.5 mm)	(A) 0.1% TFA in water (B) 100% methanol Linear elution gradient 90-72% A, 0-10 min; 72-45% A, 10-20 min; 100% B, 20-25 min; Flow rate 2 mL/min. Injection volume: 100 µL.	MALDI-TOF	Porter et al. (2001)

Sample	Analyte	Stationary Phase	Mobile Phase	Detection	Ref.
Cranberry juice and cranberry juice cocktail	Flavonoids and phenolic compounds	XDR-C RP column (15 cm x 4.6 mm, 5 µm)	(A) water–acetic acid (97:3,(v/v) (B) methanol Gradient elution: 100% A, 0-10 min; 90% A, 10-40 min; 30% A, 40-44 min; 100% A 44-47 min. Flow rate: 0.9 mL/min, 0-10 min and 44-47 min; 1.0 mL/min 10-44 min. Injection volume: 15 µL.	DAD: 280 and 360 nm.	Chen et al. (2001)
Frozen Cranberries	Polymeric procyanidins	Phenomenex packed column (250 x 4.6 mm) at 37°C	(A) dichloromethane (B) methanol (C) acetic acid and water (1:1 v/v). Linear gradients of B in A with a constant 4% C Gradient: 14-23.6% B in A, 0-20 min; 23.6-35% B in A, 20-50 min; 35-86% B in A, 50-55 min, 86.0% B in A isocratic, 55-60 min; 86.0-14.0% B 60-65 min, followed by 10 min of reequilibration. Flow rate 1 mL/min. Injection volume: 5 µL.	DAD: 280 nm MSD: Post column addition with 10 mM ammonium acetate in methanol at 0.1 mL/min. API-ES negative ionization with capillary voltageat 3.5 kV; nebulizing pressure at 25 psi; drying gas temp. at 350°C, fragmentor at 85 V. Mass scan from m/z 220-2200 at 2.12 s per cycle.	Gu et al. (2002)
Cranberries	Flavan-3-ols and proanthocyanidins	Phenomenex packed column (250 x 4.6 mm) at 37°C	(A) methylene chloride (B) methanol (C) acetic acid and water (1:1 v/v). Linear gradients of B in A with a constant 4% C Gradient: 14-23.6% B in A, 0-20 min; 23.6-35% B in A, 20-50 min; 35-86% B in A, 50-55 min, 86.0% B in A isocratic, 55-65 min; 86.0-14.0% B 65-70 min, followed by 10 min of reequilibration. Flow rate 1 mL/min . Injection volume: 5 µL.	MS-MS	Gu et al. (2003)
Cranberry powder 90X	Total phenols, anthocyanins and proanthocyanidins	Total polyphenols: Novapak C-18 column (150x3.9 mm i.d., 5 µm) Anthocyanins and proanthocyanidins: Symmetery C18 (100x2.1 mm i.d., 3.5 µm) heated at 30°C	(A) 4% aqueous phosphoric acid (B) acetonitrile Linear gradient: 95% A to 75% A over 60 min. Flow rate: 0.75 mL/min. Injection volume: 50 µL. (A) 2% aqueous acetic acid (B) methanol with 2% acetic acid Gradient: initial 99% A, 80% A at 30 min, 60% A at 45 min, 5% A at 60 min. Flow rate: 0.15 mL/min. Injection volume: 20 µL.	DAD: 520 nm (anthocyanins) and 380 nm (proanthocyanidins) + scan 210–600 nm. MSD-ES positive ionization for anthocyanins with cone voltage at 17 eV; mass scan from m/z 200-600at 1 scan/s. MSD-ES negative ionization for proanthocyanidins; mass scan from m/z 200-2000.	Seeram et al. (2004)
Frozen Cranberries	Catechins and procyanidins	RP-C18 column (125x3 mm i.d., 5 µm) protected with a guard column of same material (4x4 mm)	Catechins: (A) 100% acetonitrile (B) 0.1% TFA in water Linear elution gradient from 5-30% A in B, 0-20 min. Flow rate 0.5 mL/min. Procyanidins: (A) 100% acetonitrile (B) 5% formic acid in water Gradient: 5% A in B, 0-5 min; 5-20% A in B, 5-35 min. Flow rate 0.5 mL/min.	DAD: 280 nm + scan 220–450 nm MSD-ES positive ionization with capillary at 225°C and voltage at 4.5 kV. Mass scan from m/z 200-1000 at 500 ms per cycle.	Määttä-Riihinen et al. (2005)

Sample	Analyte	Stationary Phase	Mobile Phase	Detection	Ref.
Freeze dried cranberry powder	Anthocyanins	Zorbax SB-C18 column (250x4.6 mm i.d.)	(A) 5% formic acid in water (B) methanol Gradient: 5% B, 0-2 min; 5-20% B, 2-10 min; 20% B, 10-15 min; 20-30% B, 15-30 min; 30% B, 30-35 min; 30-45% B, 35-50 min; 45% B, 50-55 min; 45-5% B, 55-65 min, 5% B, 65-68 min. Flow rate of 1 mL/min.	DAD: 520 nm MSD-ES positive ionization with capillary voltage at exit 3.5 kV; capillary offset at 0.5 kV; skim 1 at 25.4 V; nebulizer at 45 psi; dry gas temp at 345°C at 11 L/min.	Wu and Prior. (2005); Wu et al. (2006)
Cranberry powder 90X	Phenolic Acids, Anthocyanins, Flavonols, Proanthocyanidins	C18 column (250x4.5 mm, 8µm)	(A) 0.1% TFA in water (B) 100% methanol. Linear gradient 0-100% A in B, 0-40 min. Flow rate 2 ml/min. Injection volume: 50 µL	DAD: 280, 320, 360 and 520 nm.	Lee et al. (2006)
Cranberries	Total polyphenols, flavonols, anthocyanins, proanthocyanidins	XTerra C8 column (4.6×250mm, 5 µm)	(A) 20 g/L aqueous acetic acid (B) 20 g/L acetic acid in methanol Gradient : 100% A, 0-5min, 100-0% A, 5-45 min, 0% A, 45-60 min. Flow rate 0.8 mL/ min.	DAD: 280 nm (proanthocyanidins), 525 nm (anthocyanins), 350 nm (flavonols), and 308 and 312nm (cis and trans hydroxycinnamoyl ursolic acid)	Neto et al. (2006)
Cranberry Juice cocktail	Flavan-3-ols, hydroxycinnamates, flavonols, anthocyanins	Max-RP column (250x 4.6 mm i.d. 4 µm) maintained at 40°C.	(A) 1% formic acid in water (B) 100% acetonitrile Gradient elution for 60 min with 5-40% A in B. Flow rat 1 mL/min.	DAD: 280 nm (flavan-3-ols), 325 nm (hydroxycinnamates), 365 nm (flavonols) and 520 nm (anthocyanins) + scan from 250-700 nm. MSD: Post column split and 0.3 mL/min directed to MSD-ES negative ionization for hydroxycinnamates, flavan-3-ols, flavonols, and flavanones and positive ionization for anthocyanins. Capillary temp. at 350°C and voltage at 4 kV for negative ionization and 1 kV for positive ionization. Mass scan from m/z 150-2000.	Mullen et al. (2007)
Raw cranberries	Flavonoids	Zorbax XDB-C_{18} column (250x4.6 mm, 5 µm) with guard column (12.5x4.6 mm) of same material and heated at 30°C	(A) methanol, (B) acetonitrile (C) trifluoroacetic acid Gradient elution over the 60 min: A/B/C varied linearly from 90:6:4 at 0 min, to 85:9:6 at 5 min, to 71:17.4:11.6 at 30 min, and to 0:85:15 at 60 min. Flow rate 1.0 mL/min. Injection volume: 5 µL	DAD: 210, 260, 278, 370, and 520 nm+ scan 190-650 nm.	Harnly *et al.* (2006)
Cranberries	Flavonoids: glycosides and aglycones; hydroxycinnamates	C_{18} column (250x4.6 mm i.d., 5 µm) with Sentry guard column (20x3.9mm, 5 µm) heated at 25°C	(A) 0.1% formic acid in water (B) 0.1% formic acid in acetonitrile Gradient: 10-26% B, 0-40 min; 26-65% B, 40-70 min; 65-100% B, 70-71 min; 100% B, 71-75 min. Flow rate 1.0 mL/min. Injection volume: 5 µL	DAD: 270, 310, 350, and 520 nm + scan from 190-650 nm. MSD-ES positive and negative ionization, drying gas at 13 L/min and temp. at 350°C, nebulizer pressure at 50 psi, capillary voltages of 4000 V for positive ionization and 3500 V for negative ionization, fragmentor at 100 and 250 V. Mass scan from m/z 100-2000.	Lin and Harnly (2007)
Frozen cranberries	Flavonoids glycosides and aglycones	RP-C_{18} column (15 cmx 4.6 mm, 5 µm)	(A) water–acetic acid (97:3,(v/v) (B) methanol Gradient: 100% A, 0-10 min; 90% A, 10-40 min; 30% A, 40-44 min; 100% A 44-47 min. Flow rate: 0.9 mL/min, 0-10 min and 44-47 min; 1.0 mL/min 10-44 min. Injection volume: 15 µL	DAD at 280 and 360 nm.	Chen & Zuo (2007)

derivatives – ferulic, sinapic, caffeic and p-coumaric acids - have absorbance maxima at 300 nm (Ibrahim and Barron, 1989; Van Sumere 1989; Lee, 2000). Most HBA derivatives display their maxima at 246-262 nm, with a shoulder at 290-315 nm, except for gallic and syringic acids, which have absorbance maxima at 271 and 275 nm, respectively (Escarpa and Gonzales, 2001).

Flavonoids typically exhibit two major absorption bands in the UV region. Band I absorption occurs in the 300-550 nm range and presumably arises from the B-ring and the substitution pattern and conjugation of the C-ring, and band II occurs in the 240-285 nm range, which is believed to arise from the A-ring. Addition of simple substituents to an aromatic ring capable of electron donation, such as methyl, methoxy and non-dissociated hydroxyl groups generally effect only minor changes in the position of the adsorption maxima, usually inducing a 10-15 nm bathochromic shift for the absorption band associated with the ring involved (Lee, 2000; Merken and Beecher, 2000a; Escarpa and Gonzalez, 2001; Rijke *et al.*, 2006). The UV spectra of flavonols has a band II peak around 240-280 nm and a band I peak around 300-380 nm. The flavan-3-ols also have a UV spectra with a band II peak around 240-280 nm, but because they lack the conjugation between the A- and B-rings, they usually have a small band I peak. Anthocyanins have very characteristic spectra with a peak between 240 and 280 nm (band II) and a strong visible peak between 450 and 560 nm. This latter band renders anthocyanins easy to distinguish from the other flavonoids and typical wavelengths for their analysis are 510 and 520 nm (Escarpa and Gonzalez, 2001, and Merken and Beecher, 2000b). Numerous studies have shown that the maximum wavelength shifts slightly dependent upon the anthocyanin chromophores. For example, the maximal absorbance for malvidin-3-glucoside and cyanidin-3-glucoside in acidified (0.01% HCl) methanol are 534 and 523 nm, respectively (Durst & Wrolstad, 2005; Francis, 1982; Hong & Wrolstad, 1990). The UV spectra of proanthocyanidins have a band II peak around 240-280 nm and a band I peak around 300-380 nm (Hammerstone, *et al.*, 2000; Prior *et*

al., 2001; Gu *et al.*, 2002; Seeram *et al.*, 2004; Määttä-Riihinen *et al.*, 2005; Neto *et al.*, 2006). The wavelengths at which various bioactive compounds found in cranberry have their maximal absorbance are presented in Table 4.

Most glycosides and acyl residues are poor chromophores; consequently no further distinguishing can be achieved by DAD (Rijke *et al.*, 2006). That is the reason why single-stage MS is often used in combination with UV-VIS detection to facilitate the confirmation of the identity of phenolics in a sample with the help of standards and reference data (Rijke *et al.*, 2006).

2.6.2.3. Mass spectrometer detection

Mass spectrometer detection, MSD, is generally used to determine molecular mass and to establish the distribution of substituents on the phenolic ring(s) (Robards, 2003; Lin and Harnly, 2007). Nowadays, MS usually consists of an atmospheric pressure-controlled sample introduction chamber that can accomplish nebulization and vaporization of the eluate, an ion source that ionizes the analytes, a capillary for ion transport and fragmentation, a mass analyzer which separates the ions according to their mass-to-charge ratio, m/z, and an electron multiplier for ion detection (He, 2000).

With the development of atmospheric pressure ionization, API, HPLC-MS coupling has become more efficient and easy to use for the analysis of polyphenolic and phenolic compounds (Cuykens and Claeys, 2004). This type of soft ionization is suitable for analysis of polar, non-volatile, thermolabile and high molecular mass molecules such as food and plant phenolics (Robards, 2003). Today, atmospheric pressure electrospray ionization, APESI and atmospheric pressure chemical ionization, APCI are used almost exclusively for the analysis of phenolics by HPLC-MS (Rijke *et al.*, 2006). APESI offers many advantages in term of sensitivity and capacity for the analysis of large, thermally labile and highly polar compounds. The low level of fragmentation induced by electrospray ionization, ESI, allows a direct analysis of samples without preliminary chromatographic separation.

ESI produces for the most part molecular ions, and under these conditions phenols form intense protonated or deprotonated molecules. ESI used in negative ion mode provides more sensitive detection for the majority of phenols and polyphenols with the exception of anthocyanins, the detection of which is more sensitive in positive ion mode (Robards, 1997 and 2003). Thus, for most of phenol and polyphenol compounds, molecular masses can be obtained through $[M-H]^-$ ions; for the anthocyanins, they are obtained through M^+ ions. To improve the ionization process for ESI in positive ion mode, acetic or formic acid is often added to mobile phases; otherwise positive ion ESI mass spectra will show protonated molecules as base peaks and low amounts of fragment ions will be seen (Häkkinen and Auriola, 1998).

When the chosen ionization technique does not produce many molecular fragments, MS conditions can be adjusted to induce analyte fragmentation by collision-induced dissociation, CID (He, 2000). The collision energy applied during CID processes plays an important role in the fragmentations observed (Cuykens and Claeys, 2004). When molecular ions are broken apart, fragment ions, also known as product ions, are formed. They can provide important structural information about molecules and enable, for example, the distinction of structural isomers of phenol or polyphenol glycosides differing in the nature of the disaccharide linkage or sugar moieties (Niessen, 1999). By the same token, when glycosylated phenolic compounds and flavonoids are subjected to CID conditions, cleavage of their sugar moieties can provide information concerning the aglycone (non-glycosylated) portion of the molecule (Constant et al., 1997). The CID of the $[M-H]^-$ (or M^+) precursor ions yields product ions that determine the molecular mass of the aglycone and glycosides (Robards, 2003). The m/z of the molecular ions and/or fragment ions of various phenolic compounds of interest in cranberry are presented in Table 2.4.

Table 2.4. Characteristics of Bioactive Compounds in Cranberries.

Cranberry Bioactive Compounds	UV/Vis Absorbance λ (nm)	Molecular and Fragment Ions Mass-to-charge ratio (*m/z*)	Ref.
Cyanidin 3-galactosides	280, 320sh, 503; 516	449, 286	1, 2, 3, 4, 5, 6, 7, 8, 9
Cyanidin 3-arabinoside	280, 516	419, 286	
Peonidin 3-galactosides	278, 516	463, 300	
Peonidin-arabinoside	278, 516	433, 300	
Cyanidin 3-glucoside	278, 516	449, 286	1, 3, 4, 5, 6, 9
Peonidin 3,5-digalactoside	278, 516	625, 300	
Peonidin 3-glucoside	278, 516	463, 300	
Peonidin 3-arabinoside	278, 516	433, 300	
Delphinidin 3-glucoside	278, 516	465, 302	
Delphinidin 3-galactoside	278, 516	465, 302	
Delphinidin 3-arabinoside	278, 516	435,302	
Perlagonidin 3-galactoside	278, 516	433, 270	
Perlagonidin 3-arabinoside	278, 516	403, 270	
Petunidin 3-galactoside	278, 516	479, 316	
Malvidin 3-galactoside	278, 516	493, 330	
Malvidin 3-arabinoside	278, 516	463, 330	
Quercetin	250, 268sh, 297sh, 370	303	1, 2, 3, 5, , 7, 10, 11, 12
Quercetin 3-glucoside (isoquercitrin)	253, 263sh, 294sh, 351	465, 303	
Quercetin 3-galactoside	256, 266sh, 352	465, 303	
Quercetin 3-xyloside	256, 266sh, 352	435, 303	
Quercetin 3-arabinopyranoside	256, 266sh, 352	435	
Quercetin 3-arabinofuranoside	256, 266sh, 352	435	
Quercetin 3-rhamnoside (quercitrin)	253, 263sh, 344	449	
Myricetin	255, 267sh, 300sh, 373	319	
Myricetin 3-galactoside	264, 358	481	
Myricetin 3-xylopyranoside	264, 358	451	
Myricetin 3-arabinopyranoside		451	
Myricetin 3-arabinofuranoside	264, 358	451	
Myricetin 3-rhamnoside (myricitrin)	250sh, 262, 298sh, 349	465	
Catechin	242sh, 279	291	3, 11
Epicatechin	229sh, 279	291	3, 5, 7, 11
Ferulic acid	239sh, 291sh, 325	194	3
p-Coumaric acid	226sh, 291sh, 310	163	1, 3, 5, 7, 11, 12, 13
Caffeic acid	246sh, 295sh, 323	179	
Sinapic acid	244, 279, 323	224	
p-Coumaroylglucose		325, 308	
Caffeoylglucose	240, 298sh, 326	341, 179	
Feruloylglucose		339	
Diglucoside of caffeoylglucose			
Chlorogenic acid (3-*O*-caffeoylquinic acid)	248sh, 296sh, 326	353	3, 5, 7, 11
p-Hydroxybenzoic acid	241, 260, 277	105	3, 11, 12
Protocatechui acid	257, 291	154	
Gallic acid	269, 333sh	170	
Ellagic acid	251, 300sh, 358	301	
Ellagitannin			14, 15, 16, 17
Antho-flavan-3-ols		735, 749, 765, 779	18
Proanthocyanidins with DP of 2	280	575.1	19, 20
Proanthocyanidins with DP of 3	280	863.1	
Proanthocyanidins with DP of 4	280	1149, 1283, 1299	
Proanthocyanidins with DP of 5	280	1437, 1571, 1587	
Proanthocyanidins with DP of 6	280	1859, 1875	
Proanthocyanidins with DP of 7	280	2145, 2161	
Proanthocyanidins with DP of 8	280	2435, 2451	
Proanthocyanidins with DP of 9	280	2721, 2737	
Proanthocyanidins with DP of 10	280	3010, 3026	
Proanthocyanidins with DP of 11	280	3300, 3314	
Proanthocyanidins with DP of 12	280	3588	
Antho-polyflavan-3-ols with DP 2	280	1023, 1035, 1051, 1065	18
Glucose / Galactose (hexose)		162	13
Rhamnose (deoxyhexose)		146	
Arabinose / Xylose (pentose)		132	
Fructose		180	
CH_3 – CH bridge		28	18
Quinic acid		191	7

1. Macheix *et al.*, 1990
2. Neto, 2007
3. Sakakibara *et al.*, 2003
4. Wu & Prior, 2005
5. Lin and Harnly, 2007
6. Prior *et al.*, 2001
7. Mullen *et al.*, 2007
8. Nakajima *et al.*, 2004
9. Wu *et al.*, 2004
10. Chen and Zuo, 2007
11. Chen, 2001
12. Häkkinen, 1999
13. Cuyckens & Claeys, 2004
14. Kähkönen *et al.*, 2001
15. Lei *et al.*, 2001
16. Vattem and Shetty, 2003
17. Kaponen *et al.*, 2007
18. Reed *et al.*, 2005
19. Gu *et al.*, 2003
20. Neto *et al.*, 2006

2.7. Conclusions

A large variety of bioactive compounds are found in cranberries: the flavonols (quercetin 3-galactoside), the flavan-3-ols (catechin and epicatechin), the anthocyanins (cyanidin 3-galactoside, cyanidin 3-arabinoside, peonidin 3-galactoside, and peonidin 3-arabinoside), the tannins (ellagitannins and proanthocyanidins) and the phenolic acid derivatives (ferulic acid, *p*-coumaric acid, coumaroylglucose, feruloylglucose, caffeoylglucose, chlorogenic acid, ellagic acid, and *p*-hydroxybenzoic acids). These compounds display many promising health properties, such as antioxidant, antimicrobial, anticarcinogen, etc. The proper choice of techniques for the isolation, the analysis, and the characterization and/or quantification of cranberry bioactive compounds is of great importance to further study these properties. Various extraction, chromatographic separation and detection strategies were mentioned and successfully employed for analyte determinations in cranberries. Extractions were performed using various extracting solvents (methanol, ethanol, acetone, water, ethyl acetate) on freeze-dried cranberry or alternatively, by maceration of fresh, undried fruit. The method of choice for the separation and analysis of cranberry bioactive compounds has invariably been RP chromatography combined to DAD at 280, 320, 365 and 520 nm, and MS detection. Detailed information regarding these methods was presented in this review as a reference guide for researchers pursuing work in this field.

2.8. Abbreviations used

HPLC, high-performance liquid chromatography; UV/Vis, ultraviolet/visible; MS, mass spectrometer; C-C, carbon-carbon; C-O, carbon-oxygen; SPE, solid-phase extraction; UPLC, ultra performance liquid chromatography; DAD, diode array detection; HCA, hydroxycinnamic acid; HBA, hydroxybenzoic acid; API, atmospheric pressure ionization; APESI, atmospheric pressure electrospray ionization; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; CID, collision-induced dissociation.

CHAPITRE 3

Publication 3: Antimicrobial effects of cranberry juice and extracts

Antimicrobial effect of cranberry juice and extracts

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Running title: Antimicrobial activity of cranberries

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3.1. Contributions des auteurs

J'ai imaginé, mis au point, réalisé toutes les expériences et rédigé le manuscrit scientifique. Le Dr. Stéphane Caillet, codirecteur du projet et associé de recherche à l'INRS-Institut Armand-Frappier a participé à la supervision des expériences et aux discussions scientifiques et a corrigé le manuscrit. Le Dr. Gilles Doyon, chercheur scientifique au Centre de recherche et de développement sur les aliments de Saint-Hyacinthe (Québec), a participé aux discussions scientifiques et a corrigé le manuscrit. M. Dominique Dusseault, candidat au doctorat en biologie à l'INRS-Institut Armand-Frappier a corrigé le manuscrit. M. Jean-François Sylvain, Directeur technique de Canneberges Atoka Inc., a approuvé le projet et participé aux discussions scientifiques. Prof. Monique Lacroix était la responsable scientifique et la coordinatrice du projet de recherche. De plus, Prof. Lacroix a supervisé l'élaboration des protocoles et les discussions scientifiques entourant ce projet. Elle a révisé le manuscrit.

3.2. Résumé en français

Les effets antimicrobiens du jus et des extraits canneberges

Les effets antimicrobiens du jus de canneberges et de trois extraits de canneberge (composés phénoliques hydrophiles (E1) et apolaires (E2), et anthocyanes (E3)) ont été étudiés sur sept souches bactériennes (*Enterococcus faecium* résistant à la vancomycine (ERV), *Escherichia coli* O157: H7 EDL 933, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* HPB 2812, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella* Typhimurium SL1344 et *Staphylococcus aureus* ATCC 29213). La concentration minimale inhibitrice (CMI) et la concentration maximale tolérée (CMT) ont été déterminées pour chaque échantillon de canneberges ajusté à pH 7.0. Selon les résultats présentés en µg de phénol / mL, toutes les souches bactériennes testées, autant les bactéries Gram-positives que les Gram-négatives, ont été inhibées par les composés phénoliques de la canneberge, mais à différents degrés. L'extrait riche en composés phénoliques hydrophiles est celui qui a démontré le plus grand pouvoir antimicrobien. La croissance de la bactérie ERV, et dans une moindre mesure, celle des bactéries *P. aeruginosa*, *S. aureus* et *E. coli* ATCC 25922, ont été les plus affectées par la propriété antimicrobienne de l'extrait E1. La croissance de *P. aeruginosa* et d'*E. coli* ATCC a également été affectée par l'extrait de canneberges riche en anthocyanes (E3), même si l'effet antibactérien observé n'était pas aussi important qu'avec E1. En général, les bactéries *L. monocytogenes*, *E. coli* O157: H7 et *S.* Typhimurium se sont montrées les plus résistantes à l'activité antibactérienne des extraits de canneberge. En moins de 30 minutes, les bactéries *L. monocytogenes* et ERV ont été complètement inactivées par l'exposition au jus de canneberges pur neutralisé.

3.3. Abstract

The antimicrobial effect of cranberry juice and of three cranberry extracts (water-soluble (E1) and apolar phenolic compounds (E2), and anthocyanins (E3)) was investigated against seven bacterial strains (*Enterococcus faecium* resistant to vancomycin (ERV), *Escherichia coli* O157:H7 EDL 933, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* HPB 2812, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella* Typhimurium SL1344, and *Staphylococcus aureus* ATCC 29213). Each cranberry sample was analyzed to determine the minimum inhibitory concentration (MIC) and the maximal tolerated concentration (MTC) at neutral pH. The results, reported in µg phenol/mL, indicated that all the bacterial strains, both Gram-positive and Gram-negative, were selectively inhibited by the cranberry phenolic compounds. The extract rich in water-soluble phenolic compounds caused the most important growth inhibitions. The bacteria ERV, and to a lesser degree, *P. aeruginosa*, *S. aureus* and *E. coli* ATCC 25922, were the most sensitive to the antimicrobial activity of extract E1. The growth of *P. aeruginosa* and *E. coli* ATCC was also affected by the presence of the anthocyanin-rich cranberry extract E3, although the observed antibacterial effect was not as important as with E1. In general, *L. monocytogenes, E. coli* O157:H7 and *S.* Typhimurium were the most resistant to the antibacterial activity of the cranberry extracts. Within 30 minutes of exposure with pure neutralized cranberry juice, *L. monocytogenes* and ERV were completely inactivated.

Keywords: Cranberry juice; phenolic extracts; pathogenic bacteria; minimum inhibitory concentration (MIC); maximal tolerated concentration (MTC).

3.4. Introduction

In the United States during 1993-1997 period for which the etiology was determined, *Salmonella* sp., *Listeria monocytogenes* and *Escherichia coli* accounted for the largest number of foodborne outbreaks, cases, and deaths (Olsen *et al.*, 2000). *E. coli* O157:H7 is an emerging foodborne pathogen which can cause hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. *L. monocytogenes* can cause listeriosis which usually affects pregnant women, immunocompromised individuals, and the elderly. *Salmonella* sp. (i.e. *S.* Typhimurium) is one of the major strains causing salmonellosis (Jay *et al.*, 2005).

Addition of chemical preservatives is a conventional method of enhancing food safety. However, consumers today are increasingly concerned about chemical residues in food and tend to chose natural, healthful, and safe food (Gould, 1996). The addition of natural antimicrobial phenolic compounds or antimicrobial juice could be a good alternative to reduce health hazards and economic losses due to microbiological contaminations, and to extend the shelf life of processed food (Conner, 1993; Dorman & Deans, 2000).

Berry fruits are rich sources of bioactive compounds, such as phenolics and organic acids, which may hold antimicrobial activities (Puupponen-Pimiä *et al.*, 2001; Rauha *et al.*, 2000). The specific antimicrobial activity of cranberries was demonstrated toward numerous groups of illness-causing pathogenic bacteria, including *Helicobacter pylori*, *Salmonella*, *Staphylococcus aureus*, *E. coli*, and *Campylobacter*, to name a few. This would explain their apparent role in preventing certain infectious diseases, such as urinary tract disorders, dental decay, as well as stomach ulcers and cancers (Heinonen, 2007). Juice of the American cranberry (*Vaccinium macrocarpon*) in particular has long been consumed for the prevention of urinary tract infections. In 2008, Jepson and Craig concluded that there was some evidence that cranberry juice may decrease the number of

symptomatic urinary tract infections over a 12 month period, particularly for women with recurrent urinary tract infections. This knowledge has led to the first ever health claim on berry phenolics issued by the French Food Safety Authority in April 2004: "cranberry proanthocyanidins, in a daily dose of 36 mg, help reducing the adhesion of certain *E. coli* bacteria to the urinary tract" (Heinonen, 2007).

It is known that growth inhibition against microorganisms observed with cranberries is linked to low pH, but it was also hypothesized that other bioactive compounds in the cranberry, such as phenolics, may contribute to the observed antimicrobial actions (Wu *et al.,* 2008). Indeed the antiobiotic effect of the fruit has been associated with the high content of phenolic compounds which constitute a particularly diverse group in cranberry, including low-molecular-weight phenolic acids, condensed tannins, proanthocyanidins, and flavonoids such as anthocyanins (in high content) and flavonols (Häkkinen *et al.*, 1999; Leitao *et al.*, 2005; Puupponen-Pimiä *et al.*, 2005). The polymeric tannins and in particular, the proanthocyanidins consisting primarily of epicatechin tetramers and pentamers with at least one A-type linkage, seem to be a key protecting element of cranberries against pathogenic bacteria (Foo *et al.*, 2000b; Heinonen, 2007; Seeram & Heber, 2007). However, there are no available reports on antimicrobial activity of the different classes of cranberry phenolics against commonly occurring foodborne pathogens.

The objectives of the present study were (1) to evaluate the antimicrobial capabilities of cranberry juice and of three cranberry extracts (water-soluble and apolar phenolic compounds and anthocyanins) at neutral pH on *E. coli* O157:H7 EDL 933, *S.* Typhimurium SL1344, *L. monocytogenes* HPB 2812 1/2a, *Enterococcus faecium* resistant to vancomycin (ERV), *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 15442 and (2) determine the direct antibacterial effect of cranberry juice through growth inhibition kinetic monitoring of these bacteria.

3.5. Materials and methods

3.5.1. Raw material

Frozen cranberries and filtered cranberry juice (CJ) samples (*Vaccinium macrocarpon*) were used to determine the growth inhibition kinetic curves (for the CJ) and their MICs and MTCs. These samples were provided by Atoka Cranberries Inc. (Manseau, QC, Canada) and were stored at -80°C until used.

3.5.2. Extraction of phenolic compounds and samples preparation

The extraction conditions employed were as mild as possible to avoid oxidation, thermal degradation and other chemical and biochemical changes in the sample. The method consisted of the following steps: homogenization, maceration and evaporation. Extraction of phenolic compounds was done from frozen cranberries and was achieved according to three methods using solvents of different graded polarity for the recovery of specific classes of phenolics which have different solubility. The extract 1 (E1) containing mostly water soluble phenolics was recovered using water/methanol (85:15, v/v) (Seeram *et al.*, 2004), whereas the extract 2 (E2) containing the most apolar phenolics (flavonols, flavan-3-ols and proanthocyanidins) was recovered using acetone/methanol/water (40:40:20, v/v), a solvent combination modified from a method described by Neto *et al.* (2006). The extract 3 (E3) composed mainly of anthocyanins was recovered with methanol/water/acetic acid (85:15:0.5, v/v/v) as described by (Wu and Prior, 2005; Wu *et al.*, 2006). Frozen cranberries were crushed at 4°C for 40 s in a Waring commercial blender (Waring Laboratory, Torrington, CT) to obtain a fine puree. Immediately after crushing the fruit, extractions have been performed at 4°C under agitation and a constant flow of nitrogen for 40 min by macerating 300 g of

the fruit puree with the extracting solvents. Three successive extractions in each extracting solvent were performed using the same procedure. The first extraction was done using 700 ml of solvent, but for the two last ones, 500 ml was used. The solvent containing the phenolic compounds was recuperated after each extraction and the solvents from the successive extractions were combined, then filtered on Whatman paper n° 4 (Fisher Scientific, Nepean, ON, Canada). The filtrate was concentrated by evaporating the solvent using the SpeedVac automatic evaporation system (Savant System, Holbrook, NY), then dry matter was determined by freeze-drying the extracts for 48 h with a Virtis Freeze mobile 12 EL (The Virtis Co., Gardiner, N.Y), and stored at -80°C until used.

Prior to the experiment, the freeze-dried extracts were weighed and redissolved in their extracting solvent to a specified volume, then the cranberry juice (20 ml) and phenolic extracts were adjusted to pH 7 with 1.25 mol/l NaOH.

3.5.3. Total phenol concentration

The total phenolic compound (TP) content in each cranberry extract or CJ sample as gallic acid equivalents by Folin-Ciocalteu method (Singleton and Rossi, 1965; Waterhouse, 2004) with absorbance measured at 760 nm. The TP content of samples adjusted at pH 7.0 was then calculated using actual absorbance versus a gallic acid standard curve (10, 20, 40, 80, 100, and 500 µg/mL; r^2 = 0.9999) and expressed as grams of gallic acid equivalents (GAE) per g of dry weight (DW) sample.

3.5.4. Preparation of bacterial strains

E. coli O157:H7 EDL 933 (INRS-Institut Armand Frappier, Laval, QC, Canada), *S.* Typhimurium SL1344 (INRS-Institut Armand-Frappier, Laval, QC, Canada), *L. monocytogenes* HPB 2812 1/2a (Health Canada, St-Hyacinthe, QC, Canada), ERV (clinical isolate, CHUM, Montréal, QC,

Canada), *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 15442 were maintained at -80°C in brain heart infusion broth (BHI, Becton Dickinson-Difco, Sparks, MD) containing 10% glycerol. Prior to the experiment, the working cultures were prepared by subculturing 100 µL of each stock culture in 9 mL of BHI broth and incubated at 35 °C for 24 h. After incubation, 100 µL of bacteria from each working culture were individually inoculated in fresh BHI broth and incubated at 35 °C for 1 h (two *E. coli* strains and *Enterococcus faecium*) and 1 h 30 (other strains) in order to obtain inocula containing cultures in the exponential growth phase of approximately 1 x 10^7 Colony Forming Unit (CFU)/ml.

3.5.5. Determination of the minimum inhibitory concentration (MIC) and the maximal tolerated concentration (MTC)

The antimicrobial effects of CJ and the three phenolic extracts at neutral pH (pH 7) were determined by analyzing the MIC and MTC using 96 well microtiter plate method (broth dilution) according to the modified protocol described by Gutierrez *et al.* (2008). The first row of each plate contained 200 µl of Muller–Hilton broth (Becton Dickinson-Difco, Sparks, MD) and 50 µl of the CJ or each phenolic extract redissolved in extracting solvent at 200 mg/ml. From the second to the twelfth row, 125 µl of Muller–Hilton broth were added and wells containing CJ or each phenolic extract in the first row were then diluted two-fold along each column, until the eleventh column. Eleven final concentrations for phenolic extracts (40, 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 and 0.039 mg/ml) and cranberry juice (20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 and 0.019 % (v/v)) were tested. The twelfth column was used for growth control (positive control) and a row was used for control sample (negative control) at each dilution. These additional controls contained extracting solvents of each dilution and growth media inoculated with each microorganism

under investigation. Finally, 15 µl of each working culture of the bacterium ($1x10^7$ CFU/ml) or appropriate dilution was added to all the wells except the negative controls to obtain final concentrations of 10^6 and 10^3 CFU/ml. Plates were then incubated at 37 °C and after 24 h of incubation, they were placed in a microplate spectrophotometer (absorbance at 600 nm; ELx800 BioTech Instruments Inc., Vinooski, VT) to determine the presence or the absence of turbidity. For each treatment, the absence of turbidity in the wells compared to the control was considered as an inhibitory effect. The lowest concentration of CJ or phenolic extract required to completely inhibit the growth of the tested microorganism was designated as the MIC. The MTC was the highest concentration of CJ or phenolic extract which permitted bacterial growth. This paper includes MTC results to reproduce the food contamination event where a very low level of microorganisms can be present on the food.

As part of the method development, the possible antimicrobial effect of solvent on the growth of the selected pathogens was evaluated. The effect of various concentrations of solvents used for the dilution of the phenolic extracts, expressed in % (v/v) was determined on the MIC and MTC of the bacteria at 10^6 CFU/ml and 10^3 CFU/ml, respectively.

3.5.6. Growth inhibition kinetics

Growth inhibition kinetic curves for the seven bacteria were performed on CJ adjusted at pH 7.0. After filtration under sterile conditions through a 0.22-µm-pore-size filter, 20 ml of CJ samples were inoculated with each bacterium to obtain a final concentration of 10^3 CFU/ml. Growth controls were composed of sterile 0.85% NaCl (w/v) solutions at pH 7 and inoculated with each bacterium at the final concentration of 10^3 CFU/ml. Inoculated juices and growth controls were incubated at 37°C under agitation for 5h, and samples were collected at 0, 15, 30, 60, 180 and 300 min for microbial

analysis. From these CJ samples and growth control solutions, serial dilutions were prepared, and the selected ones were spread plated on sterile Petri plates containing tryptic soy agar (Difco, Becton Dickinson, Sparks, MD). Plates were incubated for 24 h at 37ºC, and results were expressed as log CFU/ml.

3.5.7. Analysis design

A random block consisting in a 3x3 factorial design was used: for each analysis, three separate replicates were analyzed and three samples were tested in each replicate.

3.6. Results and discussion

3.6.1. Total phenolics in cranberry extracts and cranberry juice

Since phenolic compounds found in cranberries play an important role in their antibacterial activity, TP reported GAE were measured in the three cranberry extracts containing respectively water-soluble (E1), apolar phenolic compounds (E2), and anthocyanins (E3), and in CJ by Folin-Ciocalteu method. The TP content in cranberry juice and in each cranberry extract is presented in Table 3.1. On a DW basis, cranberry juice had the highest amount of total phenolic compound, with a value of 19.583 mg GAE/g. This result is in agreement with the phenolic concentration range of 14.12-22.4 mg/g reported respectively by Wang and Stretch (2001) and Vison *et al.* (2008).

The cranberry extract obtained with 85/15 (v/v) water/methanol (E1) had the lowest concentration of total phenols (10.370 mg/g). In comparison, the phenolic concentrations in E2, obtained with acetone/methanol/water (40/40/20, v/v), and in E3, obtained with methanol/water/acetic acid (85/14.5/0.5, v/v/v), were 1.6 times higher than E1 with values of 16.490 and 16.050 mg/g, respectively.

3.6.2. Antimicrobial activity of cranberry juice and cranberry extracts

The antimicrobial activity of cranberry juice at concentrations of 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 and 0.019 % (v/v), and of three cranberry phenolic extracts at concentrations of 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 and 0.039 mg/mL, was determined on seven selected strains of pathogenic bacteria using a broth microdilution method. The pH of the samples was adjusted to 7.0 to ensure that the results would not be confounded by their natural acidity (Wu *et al.*, 2008). To compare the antimicrobial potential of different samples, the

Table 3.1. Total phenolics in cranberry juice and the three cranberry extracts.

Cranberry samples	Total phenolics (mg GAE/g)[1]
Juice	
	19,583
Extracts[2]	
E1	10,370
E2	16,490
E3	16,050

[1] Gallic acid equivalent, DW basis.

[2] E1: The most water-soluble phenolic compounds extracted with water/methanol (85/15, v/v);

E2: the most apolar phenolic. compounds extracted with acetone/methanol/water (40/40/20, v/v);

E3: anthocyanins extracted with methanol/water/acetic acid (85/14.5/0.5, v/v/v).

results of the lowest concentration of extract required to completely inhibit the growth of the microorganisms at concentrations of 10^6 CFU/mL (MIC) and the highest concentration of extract that was tolerated by the microorganisms at concentrations of 10^3 CFU/mL (MTC) were converted into µg phenol/well and are reported in Table 3.2.

As part of the method development, the possible antibacterial effect of the solvents used for the extract dilutions on the growth of the selected pathogens was evaluated. An inhibitory effect was observed only for the solvent composed of methanol/water/acetic acid (85/14.5/0.5, v/v/v) used for the dilution of E3 rich in anthocyanins. No apparent inhibitory effect on the pathogenic bacteria was observed with the other solvents - water/methanol (85/15, v/v) used for the dilution of E1 rich in the most water-soluble phenolic compounds and acetone/methanol/water (40/40/20, v/v) used for the dilution of E2 rich in apolar phenolic compounds. An inhibitory effect of methanol/water/acetic acid (85/14.5/0.5, v/v/v) was observed on *P. aeruginosa* ATCC 15442 (at 10%, v/v), *S. aureus* ATCC 29213 (at 20%, v/v), *L. monocytogenes* (at 20%, v/v), *E. coli* ATCC 25922 (at 10%, v/v), ERV (at 20%, v/v), and *S.* Typhimurium SL1344 (at 20%, v/v). These two solvent concentrations corresponded to extract concentrations of 20 and 40 mg/ml, respectively, in the broth dilution assay. Since the concentrations of methanol/water/acetic acid (85/14.5/0.5, v/v/v) for which a growth inhibition was observed were well above the observed MIC and MTC of the extracts (results not shown), it was possible to conclude that the observed growth inhibitions were not related to the presence of solvents used for the dilution of the extracts.

When comparing the MIC and MTC of the different extracts in µg phenol/well (Table 3.2), the extract rich in water-soluble phenolic compounds was the most efficient at inhibiting microbial growth. As per reports published previously (Cavanagh *et al.*, 2003; Leitao *et al*, 2005; Puupponen-

Table 3.2. Minimum inhibitory concentrations (MICs) and maximal tolerated concentration (MTCs) in µg of phenol per well of three cranberry phenolic extracts (E)[1] and cranberry juice (CJ) against seven bacteria.

	Concentrations (µg phenol/well)						
	P. aeruginosa ATCC 15442	*S. aureus* ATCC 29213	*L. monocytogenes* HPB 2812	*E. coli* ATCC 25922	*E. coli* O157:H7 EDL 933	*S.* Typhimurium SL 1344	ERV[2]
				MIC			
E1	25.20	25.20	50.4	25.20	50.40	50.40	12.60
E2	87.45	43.72	174.00	87.45	174.90	174.90	43.72
E3	31.07	62.12	124.3	31.07	62.12	62.12	62.12
CJ	NI[3]	NI	NI	NI	NI	NI	NI
				MTC			
E1	6.30	6.30	25.20	6.30	25.20	12.60	6.30
E2	21.86	21.86	43.72	21.86	87.45	21.86	21.86
E3	7.76	15.53	31.07	7.76	31.07	15.53	15.53
CJ	NI	44.50	NI	NI	NI	NI	NI

[1] E1: The most water-soluble phenolic compounds extracted with water/methanol (85/15, v/v); E2: the most apolar phenolic compounds extracted with acetone/methanol/water (40/40/20, v/v); E3: anthocyanins extracted with methanol/water/acetic acid (85/14.5/0.5, v/v/v).

[2] *Enterococcus faecium* resistant to vancomycin.

[3] NI: No Inhibition.

Pimiä *et al.*, 2005), our results did not indicate any correlation between the Gram-positive and Gram-negative bacteria status and their sensitivity to the cranberry phenolics.

The lowest MIC and MTC were obtained when the bacteria ERV was exposed to E1 (12.60 and 6.30 µg phenol/well). The bacteria *P. aeruginosa*, *S. aureus* and *E. coli* ATCC 25922 were also sensitive to the antimicrobial activity of E1, but to a lesser degree with MIC and MTC of 25.20 and 6.30 µg phenol/well. Although the antibacterial effect was not as important as with E1, *P. aeruginosa* and *E. coli* ATCC also appeared sensitive to the presence of the anthocyanin-rich cranberry extract. When these bacteria were in contact with E3, MIC and MTC of 31.07 and 7.76 µg phenol/well were obtained. These results differ slightly from those reported by Leitao *et al.* (2005), where among a wide range of pathogens, *S. aureus* was the only strain to exhibit some susceptibility to the anthocyanin-rich fraction isolated from cranberries. When comparing bacterial growth patterns in E2-rich media (cranberry extract obtained with a mixture of apolar solvents), *S. aureus* and ERV exhibited the most sensitivity; Puupponen-Pimiä *et al.* (2005) have also reported a significant inhibitory effect of a similar type of cranberry extract against *S. aureus*. In general, *L. monocytogenes, E. coli* O157:H7 and *S.* Typhimurium were the most resistant to the antibacterial effect of the cranberry extract E1 with a MIC of 50.40 µg phenol/well. These results are in accordance with recent literature reporting that cranberry phenolics and anthocyanins retain antimicrobial effects at neutral pH (Lacombe *et al.*, 2010).

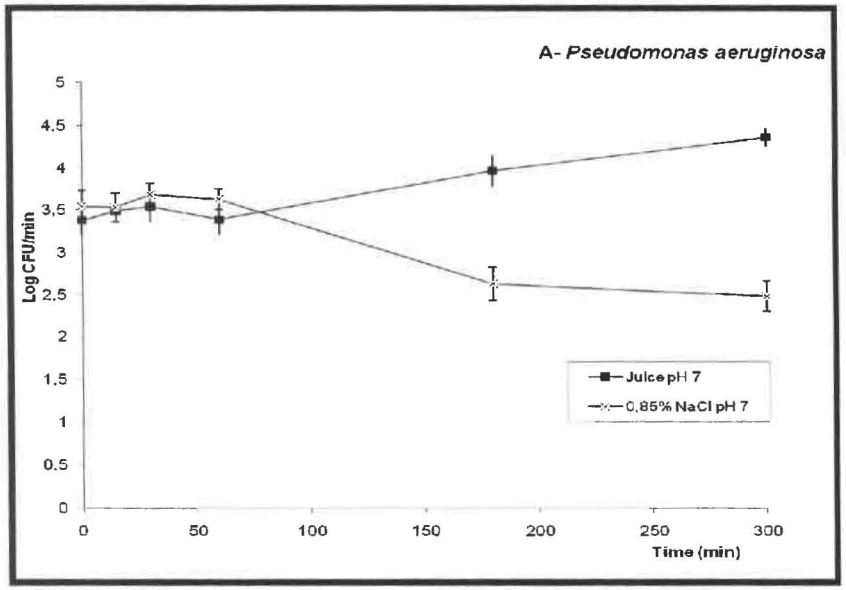
The majority of the CJ concentrations tested did not affect the growth pattern of the pathogens. Results presented in Table 3.2 indicate that at pH 7.0, none of the juice concentrations had an antimicrobial effect on the tested pathogens. These results differ somewhat from those of Leitao *et al.* (2005) and Magarinos *et al.* (2008) who, when using an agar-well diffusion method, did report an inhibitory activity against *S. aureus* with CJ. Differences among results may be related to many

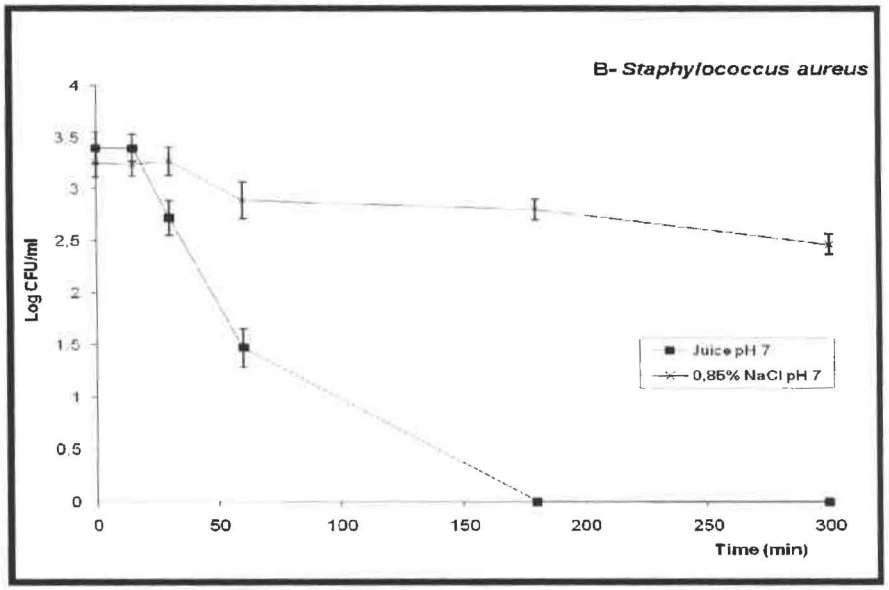
experimental variances such as bacterial strains, and source, as well as the methodology employed for the assay. The broth microdilution method is adequate for the generation of a quantitative result (the MIC) and the examination of a substantial number of bacterial cells (Jorgensen and Ferraro, 1998) and was used in this work for antibacterial screening purposes.

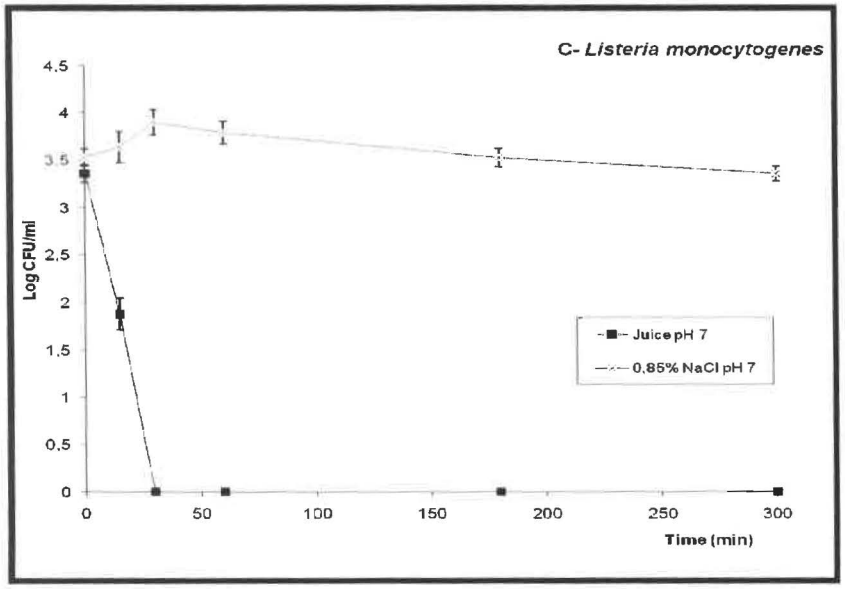
3.6.3. Effects of pure cranberry juice growth inhibition kinetics

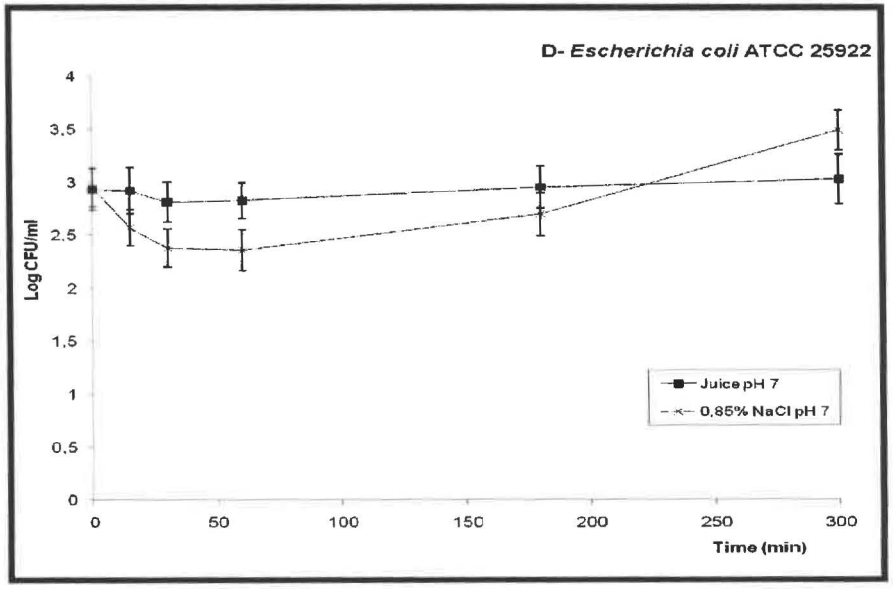
Considering the weak antimicrobial effect observed with CJ, growth inhibition kinetics of the microorganisms at low concentration of 10^3 CFU/ml was determined in CJ and the results are presented in Figure 3.1. Within 30 minutes of exposure with pure neutralized CJ, *L. monocytogenes* (C) and ERV (G) were inactivated. Compared to the growth control (0.85% NaCl solution adjusted to pH 7.0), the bacteria ERV was the most sensitive to pure CJ: the bacteria could not be recovered from the juice as early as 15 min after the inoculation. The exposure to neutralized juice for 30 min completely inactivated *L. monocytogenes*. A much weaker, but significant bactericidal effect of CJ phenols was observed on the growth kinetic obtained for *S. aureus* (B) where a 3-log CFU/mL reduction was obtained after 3 hours when compared with the control.

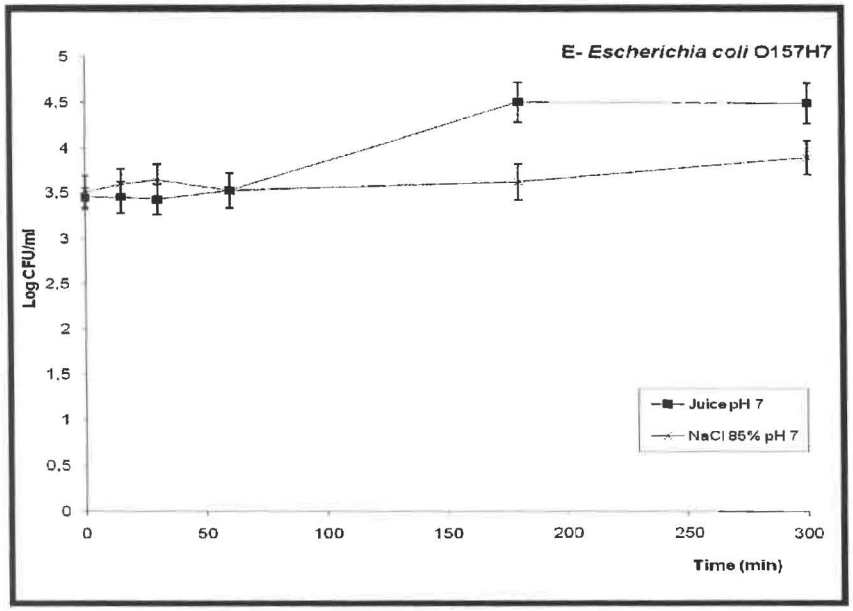
The growth inhibition kinetics obtained with *P. aeruginosa* (A), *E. coli* ATCC (D), *E. coli* O157:H7 (E), and *S.* Typhimurium (F) suggested that these pathogens are unaffected by the CJ phenolic compounds. For *E. coli* ATCC (D), *E. coli* O157:H7 (E), and *S.* Typhimurium (F) there were differences in the growth inhibition patterns between the mediums inoculated with the CJ and those inoculated with the growth control (0.85% saline water). The bacteria *P. aeruginosa* not only survived in CJ, but was also found at levels higher than the initial inoculation and than in the growth control after 5 hours. In their study, Wu *et al.* (2008) showed that cranberry juice concentrate had significant antibacterial effects against *L. monocytogenes* and *S.* Typhimurium. The Gram-negative

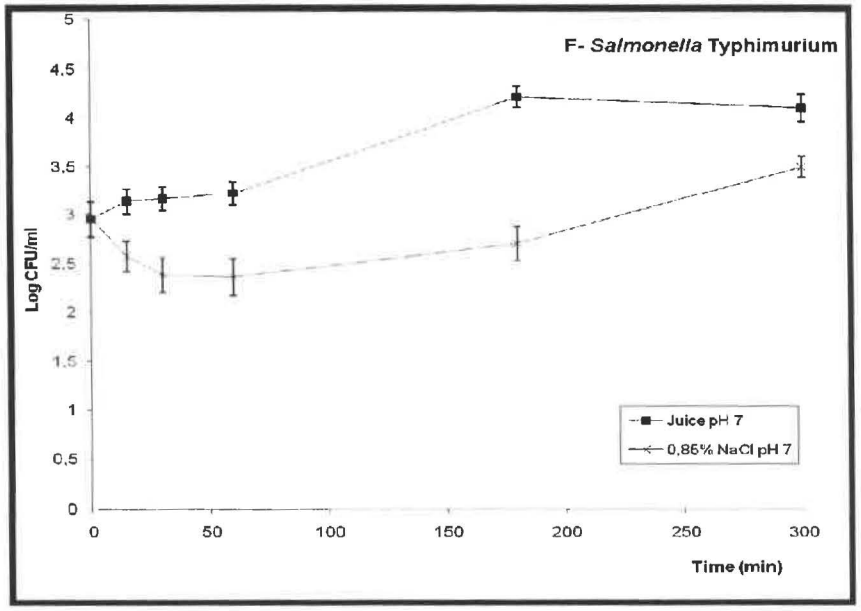

A- Pseudomonas aeruginosa
Log CFU/min
Time (min)
Juice pH 7
0,85% NaCl pH 7


B- Staphylococcus aureus
Log CFU/ml
Time (min)
Juice pH 7
0,85% NaCl pH 7


C- Listeria monocytogenes
Log CFU/ml
Time (min)
Juice pH 7
0,85% NaCl pH 7


D- Escherichia coli ATCC 25922
Log CFU/ml
Time (min)
Juice pH 7
0,85% NaCl pH 7





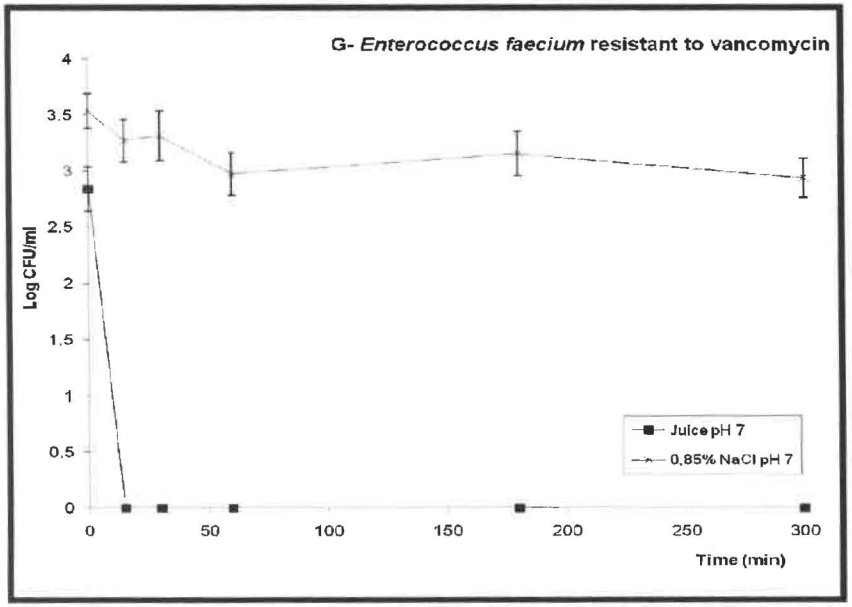


Figure 3.1. Growth inhibition kinetics of *P. aeruginosa* ATCC 15442 (A), *S. aureus* ATCC 29213 (B) *L. monocytogenes* HPB 2812 (C), *E. coli* ATCC 25922 (D); *E. coli* O157:H7 EDL 933 (E); *S.* Typhimurium SL1344 (F) and *Enterococcus faecium* resistant to vancomycin (G) in cranberry juice at pH 7.0 (♦) and in 0.85% NaCl solution at pH 7 (X). Threshold of detection (1 Log CFU/ml).

bacteria *S.* Typhimurium was the most sensitive and was reduced to non-detectable levels within 24 h treatment. The results presented in this study indicate that *S.* Typhimurium is unaffected by neutralized CJ, suggesting that this bacteria is more sensitive to acidity than to cranberry phenolics.

3.7. Conclusions

The results from this study demonstrate that ERV, and to a lesser degree, *P. aeruginosa*, *S. aureus,* and *E. coli.* are strongly inhibited by neutralized cranberry extract rich in water-soluble phenolic compounds. The growth of *P. aeruginosa* and *E. coli* was also affected by the presence of the neutralized anthocyanin-rich cranberry extract, although the observed antibacterial effect was not as important. In general, *L. monocytogenes, E. coli* O157:H7 and *S.* Typhimurium were more resistant to the antibacterial activity of the cranberry extracts adjusted at pH 7. The results confirm that cranberry antimicrobial properties are not just based upon a low pH. Over the last decade, specific bioactive components of cranberry were proposed to inhibit bacterial adherence to the epithelial cells. The polymeric tannins and in particular, the proanthocyanidins consisting primarily of epicatechin tetramers and pentamers with at least one A-type linkage, seem to be the protecting element against pathogenic bacteria (Foo *et al.*, 2000b; Heinonen, 2007; Seeram & Heber, 2007). However, our results indicate that the cranberry extract containing apolar phenolic compounds, such as the polymeric tannins, were not the most efficient at inhibiting the growth of the tested pathogens. On the contrary, it was the cranberry extract containing more water-soluble phenolic compounds, probably low-molecular-weight phenolic acids, and flavonoids such as anthocyanins and flavonols, had the showed the greatest antibacterial properties. When exposed to pure neutralized cranberry juice, *L. monocytogenes* and ERV were completely inactivated. Cranberry juice and cranberry extracts may be further investigated as a natural solution to the food industry by creating an additional barrier to inhibit the growth of foodborne pathogens while providing additional health benefits (Puupponen-Pimiä *et al.*, 2001; Vattem *et al.*, 2005b).

3.8. Acknowledgments

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CHAPITRE 4

Publication 4: Effects of juice processing on cranberry antimicrobial properties

Effects of juice processing on cranberry antibacterial properties

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Running title: Evaluation of cranberry antibacterial activity during juice processing

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4.1. Contributions des auteurs

J'ai contribué au développement des méthodes, la prise des échantillons et j'ai rédigé le manuscrit scientifique. Le Dr. Stéphane Caillet, codirecteur du projet et associé de recherche à l'INRS-Institut Armand-Frappier a supervisé les expériences et a participé aux discussions scientifiques et a corrigé le manuscrit. Le Dr. Gilles Doyon, chercheur scientifique au Centre de recherche et de développement sur les aliments de Saint-Hyacinthe (Québec), a participé aux discussions scientifiques. M. Dominique Dusseault, candidat au doctorat en biologie à l'INRS-Institut Armand-Frappier a participé à la correction du manuscrit. M. Jean-François Sylvain, Directeur technique de Canneberges Atoka Inc., a approuvé le projet et participé aux discussions scientifiques. Prof. Monique Lacroix était la responsable scientifique et la coordinatrice du projet de recherche. De plus, Prof. Lacroix a supervisé l'élaboration des protocoles et les discussions scientifiques entourant ce projet. Elle a révisé le manuscrit.

4.2. Résumé en français

Effets du procédé de jus sur les propriétés antibactériennes de la canneberge

Les effets du procédé de jus industriel sur la capacité des échantillons et des extraits de canneberges (polaire, apolaire et anthocyanique) neutralisés d'inhiber la croissance des bactéries *Enterococcus faecium* résistant à la vancomycine (ERV), *Escherichia coli* O157: H7 EDL 933, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* HPB 2812, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella* Typhimurium SL1344 et *Staphylococcus aureus* ATCC 29213) ont été investigués. De façon générale, le procédé de jus a augmenté le potentiel antibactérien des extraits de canneberges polaires et anthocyaniques. Les concentrations minimales inhibitrices (CMIs) les plus faibles (1,80 à 7,0 µg phénol / puits) ont été obtenues lorsque *S. aureus*, *S.* Typhimurium, et ERV étaient exposées au concentré de jus. *P.* aeruginosa, *L. monocytogenes*, *E. coli* ATCC et *E. coli* O157: H7 n'ont pas été inhibées par le concentré de jus, bien qu'elles aient démontré une certaine sensibilité (concentrations maximales tolérées de 0.007 à 0,4 µg phénol / puits). De faibles CMIs (22,6-90,5 phénol µg/puits) ont été observées lorsque *P. aeruginosa*, *S. aureus*, *S.* Typhimurium et ERV étaient exposées aux extraits anthocyanique du marc de canneberges. Le procédé de jus a nuit au pouvoir antibactérien des extraits de canneberges apolaires. Les bactéries testées ont démontré une plus grande résistance envers les extraits de canneberges obtenus à partir de la purée et de la purée macérée et dépectinisée.

4.3. Abstract

The effects of the industrial juice process on the ability of neutralized cranberry samples and extracts (polar, apolar and anthocyanins) to inhibit the growth of *Enterococcus faecium* resistant to vancomycin (ERV), *Escherichia coli* O157:H7 EDL 933, *E. coli* ATCC 25922, *Listeria monocytogenes* HPB 2812, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella* Typhimurium SL1344 and *Staphylococcus aureus* ATCC 29213 were investigated. The juice process appeared to have a general enhancing effect on the antibacterial properties of cranberry polar and anthocyanin extracts. The lowest minimum inhibitory concentrations (MICs) (1.80-7.0 µg phenol/well) were obtained when *S. aureus, S.* Typhimurium, and ERV were exposed to the juice concentrate. The growth of *P. aeruginosa, L. monocytogenes, E. coli* ATCC, and *E. coli* O157:H7 was not inhibited by the juice concentrate, but did show sensitivity (maximal tolerated concentrations of 0.007-0.4 µg phenol/well). The lowest MICs (22.6-90.5 µg phenol/well) for *P. aeruginosa, S. aureus, S.* Typhimurium, and ERV were observed when they were exposed to the cranberry anthocyanin extract obtained from the pomace. The results also showed a negative effect of the juice process on the antibacterial properties of the cranberry apolar extracts: the one obtained from frozen cranberries was most efficient against *P. aeruginosa, S. aureus, L. monocytogenes* and *S.* Typhimirium (MIC of 45.50 µg phenol/well). The tested bacteria showed the greatest resistance towards the cranberry extracts obtained from the mash and the macerated and depectinized mash.

Keywords: Cranberry juice processing; phenolic extracts; pathogenic bacteria; minimum inhibitory concentration (MIC); maximal tolerated concentration (MTC).

4.4. Introduction

Cranberry juice is a food product with an attractive red color and recognized health properties, two important attributes that have contributed to its success on the market place. The brilliant red color in cranberries is due mainly to anthocyanin pigments: galactosides and arabinosides of cyanidin and peonidin (Zapsalis and Francis, 1965). Two other malvidin and delphidin anthocyanin pigments are present but comprise less than 1% of the total red pigment (Fuleki and Francis, 1967; Wu *et al.*, 2006; USDA, 2007). Six yellow flavonols pigments: quercetin-3-galactoside, quercetin-3-rhamnoside, quercetin-3-arabinoside, quercetin, myricetin-3-arabinoside and myricetin-3-digalactoside also contribute to the color of the juice (Breeze, 1972). These compounds and other phytochemicals found in cranberries (flavanols and benzoic and cinnanmic acid derivatives) have also attracted a great deal of attention mainly because of their antioxidant, antibacterial, anti-inflammatory and antimutagen properties (Häkkinen *et al.*, 1999; Kaur and Kapoor, 2001; Rice-Evans, 2001; Dixon *et al.*, 2005; Leitao *et al.*, 2005; Puupponen-Pimia *et al.*, 2005a; Heinonen, 2007; Neto, 2007; Ruel & Couillard, 2007). The polymeric tannins and in particular, the proanthocyanidins consisting primarily of epicatechin tetramers and pentamers with at least one A-type linkage, seem to be a key protecting element of cranberries against bacteria (Foo *et al.*, 2000a and 2000b; Heinonen, 2007; Seeram & Heber, 2007). Through their antibacterial activity against *Helicobacter pylori*, *Salmonella*, *Staphylococcus aureus*, *Escherichia coli*, and *Campylobacter*, cranberries can play a role in preventing certain infectious diseases, such as urinary tract disorders, dental decay, as well as stomach ulcers and cancers (Heinonen, 2007). *Salmonella*, *Staphylococcus*, *Helicobacter* and *Bacillus* are the most sensitive bacteria for the berry phenolics (Puupponen-Pimiä *et al.*, 2001, 2005a & 2005b). In addition, the growth of *Escherichia*, *Clostridium* and *Campylobacter* species but not

Lactobacillus and *Listeria* species is inhibited by berry phenolics (Nohynek *et al.*, 2006, Puupponen-Pimia *et al.*, 2001, and 2005a and 2005b, Rauha *et al.*, 2000).

During juice processing, particular attention must be given to the conditions used to help and preserve the cranberry bioactive compounds. The stability of the cranberry phenolic compounds is known to be affected by heat, light, and dissolved oxygen (Francis & Servadio, 1963; Starr & Francis, 1968; Rein & Heinonen, 2004; Wrolstad *et al.,* 2005). The anthocyanins, as well as other phenolics, are easily oxidized, and are susceptible to degradative chain reactions. Enzymatic degradation and interactions with other food components (fibres and co-pigments) are other important factors that come into play. Understanding these factors, and putative degradation mechanisms, is imperative to determine the health properties that can be attributed to finished cranberry products (Macheix et Fleuriet, 1998).

Only few researchers have studied the effects of the juice process on the cranberry bioactive compounds or on their biological properties. According to Sapers *et al.* (1983), a freeze-thaw treatment prior to milling can increase the anthocyanin content of cranberry juice by as much as 15-fold. This treatment tends to facilitate the migration of anthocyanins from the exocarp into the mesocarp and endocarp and thus enhance pigment extraction. The cranberry tissues being firmer than that of other fruits, the use of pectinase was shown to enhance the rate and amount of juice recovery from batch presses (Holmes and Starr, 1993). Since the side activities of commercial pectinases can lead to browning and color degradation by enzymatic degradation of anthocyanins (Wrolstad *et al.*, 2005), Holmes and Starr (1993) have tested ways to minimize the amount of pectinase required. Their results have shown that a finer degree of grinding used during milling was also shown to increase the rate of depectinization during the mash maceration. Recently, White *et al.* (2010) characterized the polyphenolic profile of cranberry pomace, the press-cake residue of the juice

process. The pomace contained a total of 121.4 mg/100 g of dry weight anthocyanins, in comparison to 562 mg/100 g in fresh cranberries (Wu & Prior, 2005) and to 160 mg/100 g in a spray-dried cranberry extract (Prior *et al.,* 2001). The pomace also contained 358.4 mg/100 g of dry weight total flavonols, in comparison to 651.5 mg/100 g in fresh cranberries (Wu & Prior, 2005), and 167.3 mg/100 g of procyanidins with a degree of polymerization ranging from 1-6 on a dry pomace weight basis.

The aim of the present study was to evaluate the effects of the industrial juice process on the ability of neutralized cranberry samples and extracts (polar, apolar and anthocyanins) to inhibit the growth of *Enterococcus faecium* resistant to vancomycin (ERV), *Escherichia coli* O157:H7 EDL 933, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* HPB 2812, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella* Typhimurium SL1344 and *Staphylococcus aureus* ATCC 29213.

4.5. Materials and methods

4.5.1. Raw material

Processed cranberry samples were collected at each step of the juicing making process outlined in Figure 4.1. Fresh cranberries (*Vaccinium macrocarpon*) were provided by Atoka Cranberries Inc. (Manseau, QC, Canada) and stored at -80°C until used.

4.5.2. Extraction of phenolic compounds and samples preparation

The extraction conditions employed were as mild as possible to avoid oxidation, thermal degradation and other chemical and biochemical changes in the sample. The method consisted of the following steps: homogenization, maceration and evaporation. Extraction of phenolic compounds was done from frozen cranberries and achieved according to three methods using solvents of different graded polarity for the recovery of specific classes of phenolics which have different solubility. The cranberry polar extract was isolated using water/methanol (85:15, v/v) (Seeram *et al.*, 2004), whereas the apolar extract was recovered using acetone/methanol/water (40:40:20, v/v), a solvent combination modified from a method described by Neto *et al.* (2006). The anthocyanin extract was obtained with methanol/water/acetic acid (85:14.5:0.5, v/v/v) as described by (Wu and Prior, 2005; Wu *et al.*, 2006). Frozen cranberries were crushed at 4°C for 40 s in a Waring commercial blender (Waring Laboratory, Torrington, CT) to obtain a fine puree. Immediately after crushing the fruit, extractions have been performed at 4°C under agitation and a constant flow of nitrogen for 40 min by macerating 300 g of the fruit powder with the extracting solvents. Three successive extractions in each extracting solvent were performed using the same procedure. The first extraction was done using 700 ml of solvent, but for the two last ones, 500 ml was used. The solvent containing the phenolic compounds

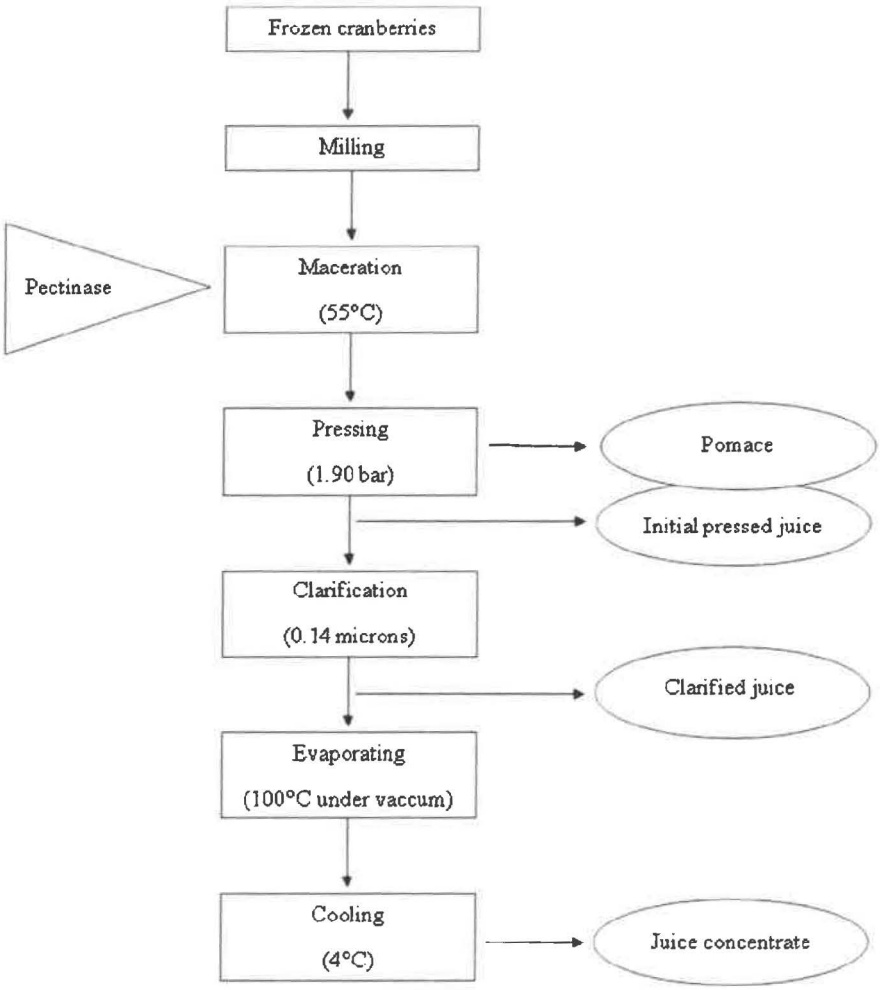

Figure 4.1. Flow chart for the juice processing of cranberry (*Vaccinium macrocarpon*) fruit.

was recuperated after each extraction and the solvents from the successive extractions were combined, then filtered on Whatman paper n° 4 (Fisher Scientific, Nepean, ON, Canada). The filtrate was concentrated by evaporating the solvent using the SpeedVac automatic evaporation system (Savant System, Holbrook, NY), then dry matter was determined by freeze-drying the extracts for 48 h with a Virtis Freeze mobile 12 EL (The Virtis Co., Gardiner, N.Y), and stored at -80°C until used.

Prior to the experiment, the freeze-dried extracts were weighed and redissolved in their extracting solvent to a specified concentration, then the cranberry liquid samples (20 ml) and the phenolic extracts were adjusted to pH 7 with 1.25 mol/l NaOH.

4.5.3. Total phenol concentration

The total phenolic compound (TP) content in each cranberry samples and extracts was determined by Folin-Ciocalteu method (Singleton and Rossi, 1965; Waterhouse, 2004) with absorbance measured at 760 nm. The TP of samples adjusted to pH 7.0 was then calculated using actual absorbance versus a gallic acid standard curve (10, 20, 40, 80, 100, and 500 µg/mL; r^2 = 0.9999) and expressed as grams of gallic acid equivalents (GAE) per g of dry sample.

4.5.4. Preparation of bacterial strains

E. coli O157:H7 EDL 933 (INRS-Institut Armand Frappier, Laval, QC, Canada), *S.* Typhimurium SL1344 (INRS-Institut Armand-Frappier, Laval, QC, Canada), *L. monocytogenes* HPB 2812 1/2a (Health Canada, St-Hyacinthe, QC, Canada), ERV (clinical isolate, CHUM, Montréal, QC, Canada), *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 15442 were maintained at -80°C in brain heart infusion broth (BHI, Becton Dickinson-Difco, Sparks, MD) containing 10% glycerol. Prior to the experiment, the working cultures were prepared by subculturing

100 µL of each stock culture in 9 mL of BHI broth and incubated at 35°C for 24 h. After incubation, 100 µL of bacteria from each working culture were individually inoculated in fresh BHI broth and incubated at 35°C for 1 h (two *E. coli* strains and *Enterococcus faecium*) and 1 h 30 (other strains) in order to obtain inocula containing cultures in the exponential growth phase of approximately 1 x 10^7CFU/ml.

4.5.5. Determination of the minimum inhibitory concentration (MIC) and of the maximal tolerated concentration (MTC)

The MIC of the tested cranberry samples and extracts at neutral (pH 7) were determined using 96-well microtitre plate 96 method (broth dilution) according to the modified protocol described by Gutierrez *et al.* (2008). The first row of each plate contained 200 µl of Muller–Hilton broth (Becton Dickinson-Difco, Sparks, MD) and 50 µl of cranberry liquid samples or of extract redissolved in extracting solvent at 200 mg/ml. From the second to the twelfth row, 125 µl of Muller–Hilton broth were added and the wells containing cranberry liquid samples or phenolic extract in the first row were then diluted two-fold along each column, until the eleventh column. Eleven final concentrations were tested, 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 and 0.039 mg/ml for the cranberry phenolic extracts, and 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 and 0.019 % (v/v) for the cranberry liquid samples. The twelfth column was used for growth control (positive control) and a row was used for control sample (negative control) at each dilution. These additional controls contained extracting solvents of each dilution and growth media inoculated with each microorganism under investigation. A working culture for each bacterial species was prepared at a concentration of 10^7 CFU/ml by diluting an overnight cultivated fermented broth using standardized procedures. Fermentation parameters and dilution ratios were determined and fixed prior to this experiment in

order to ensure accurate microorganism concentration during the assay. Finally, 15 µl of each working culture of the bacterium ($1x10^7$ CFU/ml) or appropriate dilution was added to all the wells except the negative controls to obtain final concentrations of 10^6 and 10^3 CFU/ml. Plates were then incubated at 37 °C and after 24 h of incubation, they were placed in a microplate spectrophotometer (absorbance at 600 nm; ELx800 BioTech Instruments Inc., Vinooski, VT) to determine the presence or the absence of turbidity. For each treatment, the absence of turbidity in the wells compared to the control was considered as an inhibitory effect. The lowest concentration of cranberry liquid sample or phenolic extract required to completely inhibit the growth of the tested microorganism was designated as the MIC.

This paper includes MTC results to reproduce the food contamination event where a very low level of microorganisms can be present on the food. The MTC was the highest concentration of cranberry liquid sample juice or phenolic extract which did not affect bacterial growth as determine by presence of turbidity compared to the control.

To allow a comparison of the antibacterial potential between the different samples, the results of the lowest concentration required to completely inhibit the growth of the microorganisms at concentrations of 10^6 CFU/mL (MIC) and the highest concentration tolerated for bacterial growth of the microorganisms at concentrations of 10^3 CFU/mL (MTC) were converted into µg phenol/well.
As part of the method development, the possible antibacterial effect of solvent on the growth of the selected pathogens was evaluated. The effect of various concentrations of solvents used for the dilution of the phenolic extracts, expressed in % (v/v) was determined on the MIC and the MTC of the bacteria at 10^6 CFU/ml and 10^3 CFU/ml, respectively.

4.5.6. Analysis design

A random block consisting in a 3x3 factorial design was used: for each analysis, three separate replicates were analyzed and three samples were tested in each replicate. Analysis of variance and Student *t* test (effect of pH) were employed to statistically analyze the TP results. Differences between means were considered significant when $p \leq 0.05$. PASW Statistics 18 software (SPSS Inc., Chicago, IL, USA) was used for the analysis.

4.6. Results and discussion

4.6.1. Effect of the cranberry juice process on total phenolic content

The TP content (mg GAE/g DW) measured in each collected sample to report the MICs and MTCs in µg phenol/well were also utilized to determine the effects of the juice process on the TP content of cranberry. The TP results are presented in Table 4.1. In the cranberry extracts obtained from solid-containing intermediary juice samples (frozen fruit, mash, macerated and depectinized mash, pomace), the TP content ranged from 29.11 to 324.00 mg GAE/g DW. The TP content in the cranberry polar and apolar extracts ranged from 29.11 to 196.90, and from 105.50 to 283.20 mg GAE/g DW, respectively. The anthocyanin extract had a TP content ranging between 68.92 and 324.00 mg GAE/g DW. Among the the liquid intermediary juice samples (initial pressed juice and clarified juice, and cranberry juice concentrate), the TP content ranged from 1.63 to 26.80 mg GAE/g.

When comparing the TP content between the extracts, except for those isolated from macerated and depectinized cranberry mash, the apolar extracts obtained with acetone/methanol/water (40/40/20, v/v) had the highest TP concentrations. In the macerated and depectinized cranberry mash, the highest TP level was found in the anthocyanin extract (324.00 mg GAE/g DW). These results suggest a relative stability of the proportions in which the different classes of phenolic compounds are found in cranberries.

The transformation steps involved in the juice process had the effect of increasing the TP concentrations in the cranberry extracts, which then peaked in the extracts isolated from macerated and depectinized cranberry mash. Indeed, the three extracts obtained from frozen cranberries exhibited the lowest total phenolic levels (29.11, 142.20, and 98.80 mg GAE/g DW, respectively),

Table 4.1. Total phenolics measured in cranberry samples (and cranberry extracts) collected at each step of the industrial juice process.

Cranberry samples	Total phenolics (mg GAE/g)[1]
Frozen fruit	
Polar extract[2]	29.11 ± 0.06
Apolar extract[3]	142.20 ± 0.2
Anthocyanin extract[4]	98.80 ± 2.2
Mash	
Polar extract	102.70 ± 0.09
Apolar extract	200.70 ± 0.2
Anthocyanin extract	179.60 ± 9.1
Macerated and depectinized mash	
Polar extract	196.90 ± 0.5
Apolar extract	283.20 ± 0.5
Anthocyanin extract	324.00 ± 5.5
Initial pressed juice	
	14.80 ± 0.3
Pomace	
Polar extract	66.00 ± 0.2
Apolar extract	105.50 ± 0.4
Anthocyanin extract	68.92 ± 4.59
Clarified juice	
	26.80 ± 0.9
Juice concentrate	
	1.63 ± 0.05

[1] Gallic acid equivalent, dry weight (DW) basis.

[2] Polar extract obtained with water/methanol (85/15, v/v).

[3] Apolar extract obtained with acetone/methanol/water (40/40/20, v/v).

[4] Anthocyanin extract obtained with methanol/water/acetic acid (85/14.5/0.5, v/v/v).

whereas the ones isolated from macerated and depectinized cranberry mash contained the highest levels of total phenolics (196.90, 283.20, and 324.00 mg GAE/g DW, respectively). The clarified cranberry juice was the liquid intermediary juice sample with the highest amount of TP, with 26.80 mg GAE/g DW, whereas the most transformed sample, cranberry juice concentrate, had the lowest TP level. Following the milling step, the addition of pectolytic enzymes and the heating of the mash both contribute to extracting the red color pigments (anthocyanins), through an effect called plasmolysis (Sapers *et al.*, 1983; Grassin & Fauquembergue, 1996), but it may also influence the content of other phenolic compounds. Versari *et al.* (1997) evaluated the effect of commercial pectolytic enzymes on the content of phenolic compounds (anthocyanins, flavonols and ellagic acids) in strawberry and raspberry juices under enzymatic pectinase treatment. Versari *et al.* (1997) reported that a 6-hr pectinase treatment caused a 20% loss of anthocyanins in raspberry juice, and for strawberry juice, a 35% decrease in the flavonol content.

During the juice pressing step, high amounts of press cake are obtained: cranberry pomace is the main by-product. It is composed primarily of skin, seeds, and stems left over after pressing the fruit for juice production. Even when juice extraction conditions are optimal, the content in phenolic compounds of pomace remains significant (Lu & Foo, 1999). In this study, the TP concentrations of the three extracts obtained from cranberry pomace were, in most cases, equivalent or greater to those obtained from frozen cranberries. On a dry pomace weight basis, the cranberry apolar extract had the highest level of TP (105.50 mg GAE/g). The cranberry polar and anthocyanin extracts had similar TP levels: 66.00 and 68.92 mg GAE/g DW, respectively. The stability of the cranberry phenolics is known to be affected by heat, light, and dissolved oxygen (Francis & Servadio, 1963; Starr & Francis, 1968; Rein & Heinonen, 2004; Wrolstad *et al.*, 2005). Despite these negative factors at play throughout this juice process, this study reveals that a processed cranberry juice intermediary

(depectinized cranberry mash) contained concentrations of phenolics higher than the unprocessed frozen cranberry.

4.6.2. Effect of juice processing on cranberry antibacterial activity

The effects of the industrial juice process on the ability of neutralized cranberry samples and extracts (polar, apolar and anthocyanins) to inhibit the growth of *Enterococcus faecium* resistant to vancomycin (ERV), *Escherichia coli* O157:H7 EDL 933, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* HPB 2812, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella* Typhimurium SL1344 and *Staphylococcus aureus* ATCC 29213 was evaluated using a broth microdilution method. The pH of the cranberry samples and extracts was neutralized before the MIC and MTC determination to ensure that the results would not be confounded by the natural acidity of the cranberry (Wu, Qiu, Bushway, & Harper, 2008). To allow a comparison of the antibacterial activities between the different samples, the results of the lowest concentration required to completely inhibit the growth of the microorganisms at concentrations of 10^6 CFU/mL (MIC) and of the highest concentration tolerated for bacterial growth of the microorganisms at concentrations of 10^3 CFU/mL (MTC) were converted into µg phenol/well. The MICs and MTCs of the different cranberry samples (and extracts) collected throughout the juice process are presented in Tables 4.2, 4.3, and 4.4.

As part of the method development, the possible antibacterial effect of the solvents used for the extract dilutions on the growth of the selected pathogens was evaluated. Some inhibitory effects were observed when the solvents for the dilution of the cranberry apolar extracts (acetone/methanol/water, 40/40/20, v/v/v) and anthocyanin extracts (methanol/water/acetic acid, 85/14.5/0.5, v/v/v) were used. The solvent mixture used to dilute the cranberry polar extracts (water/methanol, 85/15, v/v) did not cause any inhibitory effect on the tested bacteria. An inhibitory

Table 4.2. Minimum inhibitory concentrations (MICs) and maximal tolerated concentrations (MTCs) in µg of phenol per well of cranberry polar extract obtained with water/methanol (85/15, v/v) from samples collected at each step of the industrial juice process against seven bacteria.

Cranberry samples	Concentrations (µg phenol/well)						
	P. aeruginosa	*S. aureus*	*L. monocytogenes*	*E. coli* ATCC	*E. coli* O157:H7	*S.* Typhimurium	ERV[1]
	MIC						
Frozen fruit	46.4	46.4	92.8	92.8	46.4	46.6	23.2
Mash	165.8	41.4	165.8	165.8	165.8	165.8	82.9
Macerated and depectinized mash	464.0	29.0	116.0	464.0	232.0	232.0	116.0
Initial pressed juice	> 5.9	5.9	> 5.9	> 5.9	> 5.9	> 5.9	> 5.9
Pomace	> 844.5	> 844.5	> 844.5	> 844.5	> 844.5	844.5	52.8
Clarified juice	> 6.4	3.2	> 6.4	> 6.4	> 6.4	> 6.4	> 6.4
Juice concentrate	> 7.0	1.8	> 7.0	> 7.0	> 7.0	7.0	7.0
	MTC						
Frozen fruit	11.6	2.9	23.2	2.9	5.8	5.8	5.8
Mash	20.7	10.4	17.6	20.7	20.7	20.7	35.2
Macerated and depectinized mash	14.5	14.5	14.5	7.3	14.5	14.5	14.5
Initial pressed juice	1.5	0.7	<0.006	1.5	<0.006	<0.006	1.5
Pomace	13.2	6.6	6.6	26.4	13.2	6.6	13.2
Clarified juice	0.4	0.2	3.2	0.4	0.2	0.4	0.02
Juice concentrate	0.03	0.4	<0.007	0.01	0.05	0.05	0.007

[1] *Enterococcus faecium* resistant to vancomycin.

Table 4.3. Minimum inhibitory concentrations (MICs) and maximal tolerated concentrations (MTCs) in µg of phenol per well of cranberry apolar extract obtained with acetone/methanol/water (40/40/20, v/v) from samples collected at each step of the industrial juice process against seven bacteria.

Cranberry samples	Concentrations (µg phenol/well)						
	P. aeruginosa	*S. aureus*	*L. monocytogenes*	*E. coli* ATCC	*E. coli* O157:H7	*S.* Typhimurium	ERV[1]
			MIC				
Frozen fruit	106.1	53.0	53.0	212.3	91.0	728.0	53.0
Mash	1184.4	74.0	296.1	296.1	296.1	1184.4	74.0
Macerated and depectinized mash	152.5[2] (10%)	76.3	152.5	610.2	305.1	305.1[2] (20%)	1220.4[2] (20%)
Pomace	154.3	1234.0	308.6	617.1[2] (20%)	617.1	154.3	38.6
			MTC				
Frozen fruit	13.3	6.6	13.3	13.3	5.7	6.6	6.6
Mash	74.0	2.3	18.5	18.5	18.5	4.6	37.0
Macerated and depectinized mash	19.1	19.1	19.1	38.1	4.8	38.1	19.1
Pomace	4.8	4.8	9.6	19.3	9.6	9.6	3.6

[1] *Enterococcus faecium* resistant to vancomycin.

[2] Inhibition effect due to the presence of solvent used for extract dilution when the MIC obtained in presence of the solvent, expressed in percentage, is equal or inferior to the MIC obtained in presence of cranberry apolar extract.

Table 4.4. Minimum inhibitory concentrations (MICs) and maximal tolerated concentrations (MTCs) in µg of phenol per well of cranberry anthocyanin extract obtained with methanol/water/acetic acid (85/14.5/0.5, v/v/v) from samples collected at each step of the industrial juice process against seven bacteria.

Cranberry samples	Concentrations (µg phenol/well)						
	P. aeruginosa	*S. aureus*	*L. monocytogenes*	*E. coli* ATCC	*E. coli* O157:H7	*S.* Typhimurium	ERV[1]
			MIC				
Frozen fruit	63.8[2] (10%)	127.6[2] (20%)	63.8	> 255.2	63.8	127.6[2] (10%)	63.8
Mash	296.1	59.5	119.0	119.0	119.0	237.9[2] (10%)	59.5
Macerated and depectinized mash	78.3	78.3	156.6	1252.8[2] (20%)	156.6	156.6[2] (10%)	156.6
Pomace	45.2	22.6	723.8	265.5[2] (20%)	181.0	90.5	45.2
			MTC				
Frozen fruit	16.0	4.0	8.0	16.0	16.0	16.0	4.0
Mash	9.3	7.4	14.9	29.7	29.7	29.7	7.4
Macerated and depectinized mash	19.6	19.6	19.6	19.6	39.2	19.6	9.8
Pomace	5.6	5.6	5.6	22.6	11.3	5.6	2.8

[1] *Enterococcus faecium* resistant to vancomycin.

[2] Inhibition effect due to the presence of solvent used for extract dilution when the MIC obtained in presence of the solvent, expressed in percentage, is equal or inferior to the MIC obtained in presence of cranberry anthocyanin extract.

effect of acetone/methanol/water (40/40/20, v/v/v) used to dilute the cranberry apolar extracts was observed on *P. aeruginisa*, *S. aureus*, *L monocytogenes, E. coli* ATCC, *S.* Typhimurium, and ERV. The solvent methanol/water/acetic acid (85/14.5/0.5, v/v/v) used to dilute the cranberry anthocyanin extracts also had an inhibitory effect against *P. aeruginisa, S.* Typhimurium, *S. aureus*, *L. monocytogenes*, *E. coli* ATCC, *E. coli* O157:H7, and ERV. In most cases, the MICs obtained in presence of the solvent mixture were well above the MICs obtained in presence of the extracts, and it was possible to conclude that the observed growth inhibitions were not related to the presence of solvents used for the dilution of the extracts. In the few cases where the MICs obtained in presence of the solvent mixture were equal or inferior to the MICs obtained in presence of the extracts, the results presented in Tables 4.2, 4.3, and 4.4 were marked as such with the MIC obtained in presence of the solvent, expressed in percentage, and were not taken into consideration for the discussion.

4.6.2.1. Cranberry polar extract

For a complete overview of the effects of the juice process on the antibacterial properties of the cranberry polar extract, the MIC and MTC results obtained from the solid-containing intermediary juice samples were compared to the results of the liquid intermediary juice samples (Table 4.2). These two types of samples were comparable because the extraction solvent used to isolate the cranberry polar extract (water/methanol, 85/15, v/v) resembled the most the aqueous juice extraction occurring in a normal industrial juice process.

The cranberry juice process appeared to enhance the antibacterial properties of cranberry water-soluble phenolic compounds. The lowest MICs (1.80-7.0 µg phenol/well) were obtained when *S. aureus, S.* Typhimurium, and ERV were exposed to neutralized juice concentrate. The growth of *P. aeruginosa, L. monocytogenes, E. coli* ATCC, and *E. coli* O157:H7, initially at a concentration of 10^6 CFU/mL, was not inhibited by the highest tested concentration of cranberry juice concentrate (MIC

>20%, v/v, or the equivalent of > 7 µg phenol/well). When an initial bacterial concentration of 10^3 CFU/mL was exposed to the juice concentrate, these bacteria could however not proliferate (MTCs at 0.02-1.25%, v/v, or the equivalent of 0.007-0.4 µg phenol/well), suggesting a great sensitivity toward the most processed cranberry sample.

The cranberry polar extract obtained from the mash was the least effective against *P. aeruginosa, E. coli* O157:H7, *S.* Typhimurium and ERV with MTC values ranging from 20.7 to 35.2 µg phenol/well. Depending on the origin of the cranberry polar extract, the bacteria *S. aureus, L. monocytogenes,* and *E. coli* ATCC showed variable resistance. The highest MTC for *S. aureus* (14.5 µg phenol/well) was observed when it was exposed to the cranberry polar extract obtained from macerated and depectinized mash. The cranberry polar extract obtained from frozen cranberries were the least efficient against *L. monocytogenes* (MTC of 23.2 µg phenol/well), and the one obtained from cranberry pomace was the least efficient against *E. coli* ATCC (MTC of 26.4 µg phenol/well).

Leitao *et al.* (2005) reported a loss of cranberry antibacterial properties when *E. coli* ATCC 10538, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 6358 and 25923 were exposed to the neutralized juice. The MTC results presented in Table 2 suggest that neutralized cranberry juice can prevent these bacteria from proliferating when present at concentration of 10^3 CFU/mL. Differences among results may be related to many experimental variances, such as the bacterial source and the methodology employed for the assay (in 2005, Leitao *et al.* reported using an agar-well diffusion method).

The changes in structural integrity of the plant material that occur during juice processing were shown to impact, both negatively and positively, the putative phenolic composition as well as the properties of berries. The milling and depectinization steps in blueberry juice processing, by activating the native polyphenol oxidase (PPO) activity caused the greatest losses of anthocyanins,

flavonols, and procyanidins (Kader *et al.*, 1997; Skrede *et al.*, 2000; Lee *et al.*, 2002). The conversion of certain compounds also occurs during juice processing; for example, the caffeic-quinic acids conjugate called chlorogenic acid is found in the juice, but has not been found in whole cranberries (Chen *et al.*, 2001; Harnly *et al.*, 2006). These changes could explain the effects the juice process had on the antibacterial properties of the cranberry polar extracts.

4.6.2.2. Cranberry apolar extract

To determine the effect of the juice process, from the beginning to the end, on the antibacterial properties of the cranberry apolar extracts, the MIC and the MTC of the cranberry extract obtained with acetone/methanol/water (40/40/20, v/v) from solid-containing intermediary juice samples were compared (Table 4.3).

The cranberry apolar extract obtained from frozen cranberry had the greatest effect on the growth of *P. aeruginosa, S. aureus, L. monocytogenes, E. coli* ATCC and *E. coli* O157:H7 (MIC of 53.0 to 212.3 µg phenol/well), whereas the growth *S.* Typhimirium and ERV was more affected by apolar extract from cranberry pomace (MIC of 38.6 to 154.3 µg phenol/well). The bacteria *P. aeruginosa, E. coli* O157:H7 and ERV showed the greatest resistance towards the apolar extract obtained from cranberry mash with MTC values of 18.5-74.0 µg phenol/well, whereas the bacteria *S. aureus, L. monocytogenes, E. coli* ATCC, and *S.* Typhimurium were more tolerant to the cranberry apolar extract obtained from macerated and depectinized mash (MTCs of 19.1-38.1 µg phenol/well).

Numerous studies have identified the procyanidins, consisting primarily of epicatechin tetramers and pentamers with at least one A-type linkage, as one of key protecting element of cranberries against pathogenic bacteria (Foo *et al.*, 2000a & 2000b; Heinonen, 2007; and Seeram & Heber, 2007). Prior *et al.* (2001) showed how cranberry processing could affect the procyanidin content: compared to fresh cranberries, the commercially pressed cranberry juice had a higher

proportion of procyanidin monomers, dimers, and A-type trimers. In 2004, a report issued by the USDA showed that more than 85% of the proanthocyanidins found in raw cranberries had a degree of polymerization of 4-10 or higher. These literature data suggest that the decrease in the antibacterial activity against *P. aeruginosa, S. aureus, L. monocytogenes*, *E. coli* ATCC and *E. coli* O157:H7 observed with the cranberry apolar extracts may have been caused by a change in the proanthocyanidin profile, and that these bacteria are more susceptible to proanthocyanidins that have a high degree of polymerization. The presence of proanthocyanidins (White *et al.*, 2010), and more specifically of anthocyanin-polyflavan-3-ol oligomers (Reed *et al.*, 2005), was detected in cranberry pomace. More recently, White *et al.* (2011) reported that cranberry pomace retained 22-31% of the oligomers, 43-52% of the polymeric procyanidins, approximately 40% of the total procyanidins, and with a total polymer concentration averaging 122.6 mg/100g of frozen berry. Based on this information, we propose that the cranberry apolar extract obtained from the pomace with acetone/methanol/water (40/40/20, v/v/v), contained some of these compounds that exhibited a greater antibacterial activity towards *P. aeruginosa, S.* Typhimirium and ERV.

4.6.2.3. Cranberry anthocyanin extract

To determine the effect of the juice process, from the beginning to the end, on the antibacterial properties of the cranberry anthocyanin extract, the MIC and the MTC of the cranberry extract obtained with methanol/water/acetic acid (85/14.5/0.5, v/v) from solid-containing intermediary juice samples were compared (Table 4.5).

The greatest growth inhibition (MICs of 22.6-90.5 µg phenol/well) was observed when *P. aeruginosa, S. aureus, S.* Typhimurium, and ERV were exposed to the cranberry anthocyanin extract obtained from the pomace. Growth of *L. monocytogenes* and *E. coli* O157:H7 was the most inhibited by the presence of cranberry anthocyanin extract from the frozen fruit (MIC of 63.8 µg phenol/mL).

The cranberry anthocyanin extract isolated from the mash was the least effective against *E. coli* ATCC, and *S.* Typhimurium (MIC and MTC of 119-237.9 and of 29.7 µg phenol/well, respectively). The bacteria *P. aeruginosa, S. aureus, L. monocytogenes, E. coli* O157:H7 and ERV showed the greatest resistance towards macerated and depectinized cranberry mash anthocyanin extracts with MIC and MTC values of 78.3-156.6 and of 9.8-39.2 µg phenol/well, respectively.

Many factors involved in juice processing could explain the antibacterial activity results obtained with the cranberry anthocyanin extracts. First of all, the structural transformation of anthocyanins as function of pH is fundamental to their color and stability. They can exist in a variety of forms, in relative proportions depending on pH. At a pH of approximately 3 or lower, the color of the anthocyanins is orange or red and exists as a flavylium cation. As the pH is raised, kinetic and thermodynamic competition occurs between the hydration reaction of the flavylium cation and the proton transfer reactions related to the acidic hydroxyl groups of the aglycone. While the first reaction gives a colorless carbinol pseudo-base, which can undergo ring opening to a chalcone pseudo-base, the latter reaction gives rise to quinonoidal bases. Further deprotonation of the quinonoidal bases can take place at pH 6-7 with the formation of purplish, resonance-stabilized quinonoid anions (Fossen *et al.*, 1998). Our results show that despite the fact that all anthocyanin extracts were neutralized before testing, implying a greater proportion of anthocyanins existing in the quinonoidal form, they maintained their ability to inhibit microbial growth. Against *P. aeruginosa, L. monocytogenes, E. coli* O157:H7 and ERV, the neutralized cranberry anthocyanin extract isolated from the least processed sample (frozen cranberries) displayed the greatest antibacterial properties, with MIC and MTC of 63.8 and of 4-16 µg phenol/well, respectively.

Apart from their pH sensitivity, anthocyanins are extremely heat labile, can easily be oxidized when exposed to light or oxygen and when subjected to endogenous enzymatic activity

(polyphenoloxidase, peroxidase), they can undergo degradation or molecular reorganizations (copigmentation) (Attoe & Von Elbe, 1981; Lee *et al.*, 2002; Worlstad *et al.*, 2005). Wightman and Wrolstad (1995) found that enzyme preparations used in cranberry juicing contain glycosidase activity, specifically β-galactosidase activity, capable of destabilizing the anthocyanins by producing aglycones. The polyphenoloxidase activity, catalyzed by the presence of oxygen or chlorogenic acid, also favors the formation of highly reactive compounds, such as o-quinones that can react with anthocyanins and promote their polymerization (Kader *et al.*, 1997 and 1999, Santos-Buelga and Scalbert, 2000). White *et al.* (2011) have reported retention levels of 13-17% of total anthocyanins in cranberry pomace, but a significantly different anthocyanin profile with higher proportions of galactosides of cyanidin and peonidin. Our results show that despite the possible molecular degradation that took place, cranberry anthocyanin extracts maintained some antibacterial activity: the lowest MICs for *P. aeruginosa, S. aureus*, *S.* Typhimurium and ERV were obtained with the anthocyanin extract from cranberry pomace.

4.7. Conclusions

This study revealed the possible effects the juice process can have on the antibacterial properties of cranberries and various cranberry extracts against seven different bacterial strains. The process reduced the ability of cranberry apolar and anthocyanin extracts to inhibit bacterial growth. The cranberry anthocyanin extract from the pomace did show antibacterial activity towards certain bacteria; this property can be useful to processors wishing to improve the marketing of this by-product. Cranberry extracts obtained from intermediary juice process products (mash and macerated and depectinized mash) demonstrated the lowest antibacterial activity. Determination of biological activities of specific cranberry phenolic constituents is important for evidence-based selection of cranberry variants with favorable phenolic profiles and improved health benefits. Growth inhibition against microorganisms observed with cranberries has been linked to low pH (Wu *et al.,* 2008), but our experiments performed with neutralized cranberry samples confirms that other bioactive compounds in the cranberry, such as phenolics, contribute to the observed antibacterial actions. Moreover these results suggest that the observed antibacterial effect of cranberries would persist even after the alkalization of the bolus in the small intestine (Sherwood, 1993). Cranberry juice and cranberry extracts may thus be used as a natural solution in the food industry by creating an additional barrier to inhibit the growth of foodborne pathogens while providing additional health benefits (Puupponen-Pimiä *et al.,* 2001; Vattem *et al.,* 2005). More research should be conducted to characterize and monitor the evolution of the profile of phenolic compounds, to determine which individual phenolic compounds can better resist the juice process conditions and contribute to the antibacterial activity of the juice and juice concentrate.

4.8. Acknowledgments

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CHAPITRE 5

Publication 5: Effects of juice processing on cranberry antioxidant properties

Effects of juice processing on cranberry antioxidant properties

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Running title: Evolution of cranberry antioxidant capacity during juice processing

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5.1. Contributions des auteurs

J'ai contribué au développement des méthodes, la prise des échantillons et j'ai rédigé le manuscrit scientifique. Le Dr. Stéphane Caillet, codirecteur du projet et associé de recherche à l'INRS-Institut Armand-Frappier a supervisé les expériences et a participé aux discussions scientifiques et a corrigé le manuscrit. Le Dr. Gilles Doyon, chercheur scientifique au Centre de recherche et de développement sur les aliments de Saint-Hyacinthe (Québec), a participé aux discussions scientifiques. M. Dominique Dusseault, candidat au doctorat en biologie à l'INRS-Institut Armand-Frappier a participé à la correction du manuscrit. Guillaume Lorenzo, stagiaire à l'INRS-Institut Armand-Frappier en visite de l'Université de Perpignan Via Domitia a participé aux expériences. M. Jean-François Sylvain, Directeur technique de Canneberges Atoka Inc., a approuvé le projet et participé aux discussions scientifiques. Prof. Monique Lacroix était la responsable scientifique et la coordinatrice du projet de recherche. De plus, Prof. Lacroix a supervisé l'élaboration des protocoles et les discussions scientifiques entourant ce projet. Elle a révisé le manuscrit.

5.2. Résumé en français

Effets du procédé de jus sur les propriétés antioxydantes de la canneberge

Les effets du procédé de jus sur le potentiel antioxidant des échantillons et des extraits de canneberges (polaire (E1), apolaire (E2) et anthocyanique (E3)) ajustés à pH 2.5 et à 7.0 ont été déterminés. Le pouvoir de piégeage des radicaux libres (PRL) et la capacité d'inhibition de la peroxydation des lipides (IPL) de chaque échantillon et extrait ont été déterminés à l'aide de méthodes spectrophotométriques dosant respectivement, le N,N-diéthyl-p-phénylènediamine et les substances d'acide thiobarbiturique réactives. Les capacités de PRL et d'IPL, exprimés respectivement, en mM équivalent Trolox® (ET)/mg de phénol et mg ET/mL/mg de phénol, ont démontré les effets négatifs du procédé de jus sur les propriétés antioxydantes de l'extrait polaire. Le pouvoir antioxidant des extraits apolaire et anthocyanique ont également été affectées par les conditions du procédé, mais moins fortement. Le broyage des fruits a augmenté la capacité de PRL de E2 et E3, mais a diminué leur capacité d'IPL. L'évaporation du jus n'a pas affecté sa capacité de PRL, mais a diminué sa capacité d'IPL. Au début et après chaque étape du procédé, la capacité de PRL des extraits était supérieure à la capacité IPL. De façon constante, les résultats de capacité de PRL des extraits étaient classés dans l'ordre de polarité suivant: E1> E3> E2, et leurs teneur en composés phénoliques totaux dans l'ordre suivant: E2> E3> E1. De manière générale, la neutralisation du pH des extraits n'a pas eu d'influence significative sur les capacités de PRL et d'IPL observés au cours du procédé de jus, à l'exception de l'extrait riche en anthocyanes pour lequel une diminution de la capacité d'IPL a été observée.

5.3. Abstract

The effects of the industrial juice process on the antioxidant capacities of cranberry samples and of three phenolic extracts (polar phenolics (E1), apolar phenolics (E2) and anthocyanins (E3)) adjusted at pH 2.5 and 7.0 were investigated. The free radical-scavenging (FRS) and the lipid peroxidation inhibition (LPI) activities of each sample and extract, were studied using a N,N-diethyl-p-phenylenediamine discoloration test and the thiobarbituric acid reactive substances assay, respectively. The FRS and the LPI results, expressed in mM Trolox® equivalent (TE)/mg phenol and mg TE /mL /mg phenol, respectively, showed a negative effect of the juice process steps on E1. The E2 and E3 extracts were also affected by the process but not as much. The milling step increased the FRS capacity of E2 and E3 extracts, but decreased their LPI capacity. The evaporation of the juice did not have a significant effect on the FRS capacity, but lowered the LPI capacity. Before and after each step of the juice process, the FRS capacity of the cranberry extracts was greater than the LPI capacity. The results were consistent in classifying the FRS capacity of the extracts in the following order of polarity: E1 > E3 > E2; and their total phenolic (TP) content in the following order: E3 > E2 > E1. In general, the neutralization of the pH of the extracts did not significantly influence the changes observed during the juice process in the FRS and LPI capacity, except for E3 for which a decrease in the LPI capacity was observed.

Keywords: Cranberry juice processing; phenolic extracts; free radical scavenging activity; lipid peroxidation inhibition activity.

5.4. Introduction

Oxidative stress by free radicals is an important event in the cell as it can alter the integrity of large biomolecules such as lipids, proteins, and DNA, resulting in an increased risk of inflammatory disease, cardiovascular disease, some cancers, diabetes, Alzheimer's disease, cataracts, and age-related functional decline. Free radicals or reactive oxygen species (ROS) are generated as by-products of normal metabolism. They are also found in atmospheric pollution and tobacco smoke. Oxidative stress will occur when the rate at which the intracellular content of ROS increases relative to the capacity of the cell to dispose of these free radicals (Ruel & Couillard, 2007; Seeram & Heber, 2007).

Biological compounds that can delay or prevent the effects of oxidation have been broadly considered as antioxidants, including compounds that either inhibit specific oxidizing enzymes or react with oxidants before they damage critical biological molecules (Ruel & Couillard, 2007). Effective antioxidants are radical scavengers that break down radical chain reactions caused by reactive oxygen/nitrogen species or that can inhibit the reactive oxidants from being formed in the first place (Huang *et al.*, 2005). Compounds occurring in berry fruits are marked by varied antioxidant activity which relies on various mechanisms, subject to their structure (Borowska *et al.*, 2005; Szajdek & Borowska, 2008).

Phenolic compounds can protect the easily oxidizable food compounds; they inhibit the oxidation of vitamin C, carotenoids and unsaturated fatty acids. In the group of phenolic acids, hydroxycinnamic acid derivatives, such as caffeic acid, chlorogenic acid, ferulic acid, sinapic acid and p-coumaric acid are more active antioxidants than hydroxybenzoic acid derivatives, such as p-hydroxybenzoic acid and vanilic acid (Zheng & Zhang, 2003). Meyer *et al.* (1998) demonstrated that hydroxycinnamic acids are capable of inhibiting LDL oxidation *in vitro*. Flavonoids inhibit lipid

oxidation, chelate metals and scavenge the active forms of oxygen. Anthocyanins—which are a flavonoid subgroup—inhibit the oxidation of human LDL and liposomes, and scavenge free radicals. They also protect ascorbic acid against oxidation. Tannins are an important component of berry fruits. They comprise both condensed non-hydrolysable tannins, known as proanthocyanidins, and esters of gallic acid and ellagic acid—defined as hydrolysable tannins (Shahidi & Naczk, 2004). They are characterized by particularly high antioxidant properties (Hagerman *et al.*, 1998). Ellagic acid derivatives and condensed tannins have a much greater free radical scavenging ability than ascorbic acid, tocopherols and low-molecular weight polyphenols (Szajdek & Borowska, 2008).

The antioxidant properties of cranberries are well documented in the literature and the fruit ranks as one of the highest antioxidant activities among many other fruits (Sun *et al.*, 2002; Heinonen, 2007). The anthocyanin pigments are responsible for the brilliant red color of the fruit and are among the principal antioxidant constituents. They exhibit good free radical-scavenging activities and are powerful antioxidants in lipid-containing hydrophilic environments (Heinonen *et al.*, 1998; Prior *et al.*, 1998; Kähkönen *et al.*, 2001; Kähkönen *et al.*, 2003; Zheng & Wang, 2003; Viljanen *et al.*, 2004). Other phytochemicals found in cranberries (flavonols, flavanols and benzoic and cinnamic acid derivatives) have attracted a great deal of attention because of their antioxidant activity (Häkkinen *et al.*, 1999; Kaur and Kapoor, 2001; Rice-Evans, 2001; Dixon *et al.*, 2005; Heinonen, 2007; Neto, 2007; Ruel & Couillard, 2007). Cranberry phenolics have been shown to have free radical-scavenging properties against superoxide radical ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$), and they can also inhibit lipid peroxidation, as well as protein and lipid oxidation in liposomes (Wang & Jiao, 2000; Wu *et al.*, 2004; Seeram & Heber, 2007). Porter *et al.* (2001) demonstrated that proanthocyanidins found in cranberry inhibit the oxidation of human LDL catalyzed by copper ions *in vitro*. This could explain how they exhibit such

strong physiological activities that help prevent cancerous mutations and cancers, as well as against cardiovascular and neurodegenerative diseases (Kroon & Williamson, 2005; Williamson & Manach, 2005; Lala *et al.*, 2006; Lau *et al.*, 2006; Stoner *et al.*, 2006; Heinonen, 2007; Neto *et al.*, 2008; Basu *et al.*, 2010).

The conditions used during juice processing (temperature, light and oxygen), as well as enzymatic reactions and interactions with other food components (fibres and co-pigments), can affect the stability of the cranberry bioactive compounds. Understanding these factors, and putative degradation mechanisms, is imperative to determine the health properties that can be attributed to finished cranberry products (Macheix and Fleuriet, 1998).

The aim of the present study was to evaluate the effects of the juice process on the antioxidant properties of cranberry samples and extracts adjusted at pH 2.5 and 7.0. Because no single antioxidant assay can accurately reflect the antioxidant potency, two tests were used to measure the antioxidant properties: the N,N-diethyl-p-phenylenediamine (DPD) discoloration free radical-scavenging test and the thiobarbituric acid reactive substances (TBARS) lipid peroxidation inhibition assay. In addition, the samples and extracts were also evaluated for total phenolic compound (TP) content and reported as gallic acid equivalents (GAE).

5.5. Materials and methods

5.5.1. Raw material

Cranberry (*Vaccinium macrocarpon*) samples were collected at each step of the juice process which is illustrated in Figure 5.1. These samples were provided by Atoka Cranberries Inc. (Manseau, QC, Canada) and were stored at -80°C until used.

5.5.2. Extraction of phenolic compounds and sample preparation

The extraction conditions employed were as mild as possible to avoid oxidation, thermal degradation and other chemical and biochemical changes in the sample. The method consisted of the following steps: homogenization, maceration and evaporation. Extraction of phenolic compounds was done from frozen cranberries and achieved according to three methods using solvents of different graded polarity for the recovery of specific classes of phenolics which have different solubility. The polar extract (E1) was recovered using water/methanol (85:15, v/v) (Seeram *et al.*, 2004), whereas the apolar extract (E2) was recovered using acetone/methanol/water (40:40:20, v/v/v), a solvent combination modified from a method described by Neto *et al.* (2006). The anthocyanin extract (E3) was recovered with methanol/water/acetic acid (85:15:0.5, v/v/v) as described by (Wu and Prior, 2005; Wu *et al.*, 2006). Frozen cranberries were crushed at 4°C for 40 s in a Waring commercial blender (Waring Laboratory, Torrington, CT) to obtain a fine puree. Immediately after crushing the fruit, extractions were performed at 4°C under agitation and a constant flow of nitrogen for 40 min by macerating 300 g of the fruit powder with the extracting solvents. Three successive extractions in each extracting solvent were performed using the same procedure.

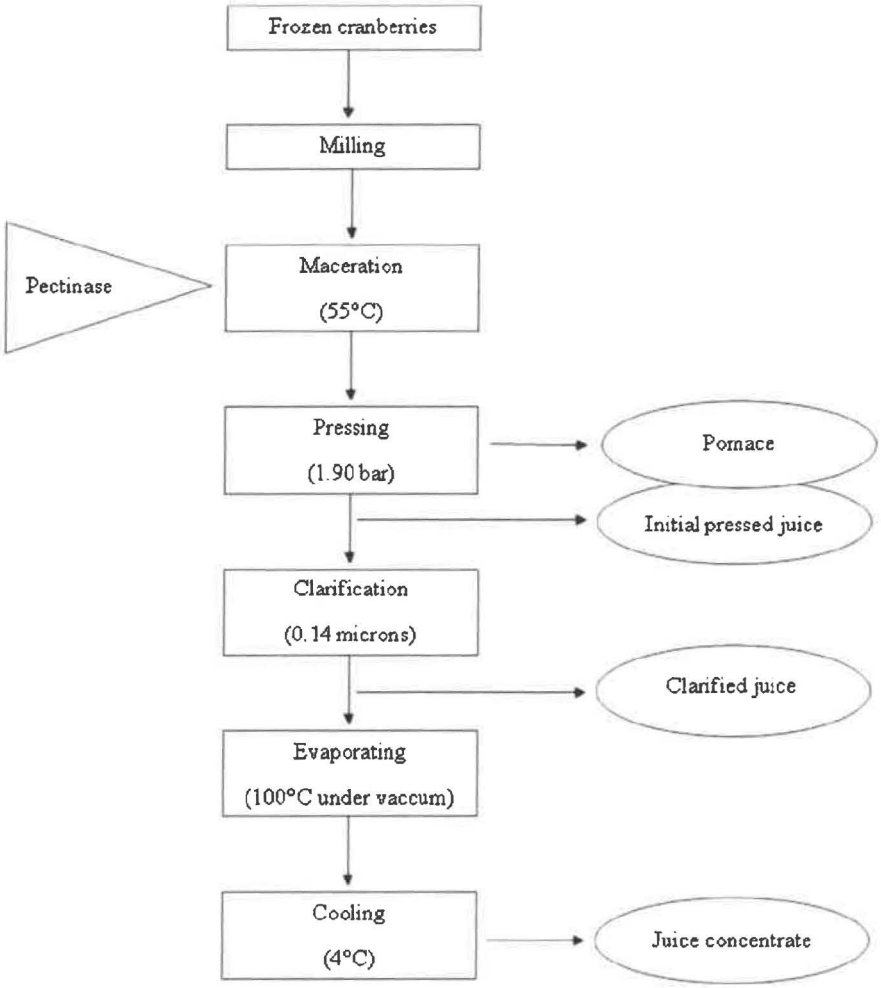

Figure 5.1. Flow chart for the juice processing of cranberry (*Vaccinium macrocarpon*) fruit.

The first extraction was done using 700 ml of solvent, but for the two last ones, 500 ml were used. The solvent containing the phenolic compounds was recuperated after each extraction and the solvents from the successive extractions were combined, then filtered on Whatman paper n° 4 (Fisher Scientific, Nepean, ON, Canada). The filtrate was concentrated by evaporating the solvent using the SpeedVac automatic evaporation system (Savant System, Holbrook, NY), then dry matter was determined by freeze-drying the extracts for 48 h with a Virtis Freeze mobile 12 EL (The Virtis Co., Gardiner, N.Y), and stored at -80°C until used.

Prior to the experiment, the freeze-dried extracts were weighed and dissolved a second time in their extracting solvent to a specified volume, then the cranberry liquid samples (20 ml) and the phenolic extracts were adjusted to pH 7 with 1.25 mol/l NaOH.

5.5.3. Total phenol concentration

The content of total phenolic compounds (TP) in each cranberry sample and extract was determined by Folin-Ciocalteu method (Singleton and Rossi, 1965; Waterhouse, 2004) with absorbance measured at 760 nm. The TP of samples adjusted to pH 7.0 was then calculated using actual absorbance versus a gallic acid standard curve (10, 20, 40, 80, 100, and 500 µg/mL; r^2 = 0.9999) and expressed as mg of gallic acid equivalent (GAE) per g of dry weight (DW) sample.

5.5.4. Determination of free radical-scavenging capacity

The free radical scavenging (FRS) capacity of phenolic compounds in cranberry extract or juice samples adjusted at natural fruit pH (pH 2.5) and neutral pH (pH 7.0) was analyzed using a procedure of DPD (N,N-diethyl-p-phenylenediamine; Sigma-Aldrich Ltd, Oakville, ON, Canada) colorimetric assay, modified by others (Oussalah *et al.*, 2004; Caillet *et al.*, 2006). Two hundred

(200) µL of sample (1.25 mg/mL) were added to a cell containing 3 mL of 0.15 M NaCl and submitted to electrolysis for 1 min (continuous current, 400 V, 10 mA DC) using a power supply (Bio-Rad, model 1000/500, Mississauga, ON, Canada). After electrolysis, an aliquot of 200 µL was added to 2 mL of DPD solution (25 mg/mL). The generated oxidative species (superoxide anion ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), and hydroxyl radicals ($OH^{\cdot}$) and their by-products (hydrogen peroxide (H_2O_2) and hypochlorite ion (OCl^-)) react instantly with DPD, producing a red coloration that can be measured at 515 nm using a DMS 100S spectrophotometer (Varian Canada Inc., Mississauga, ON, Canada). The FRS activity is equivalent to the capacity of phenolic compounds to inhibit the accumulation of oxidative species (able to oxidize DPD) and consequently the red coloration at 515 nm. The reaction was calibrated using the non-electrolyzed NaCl solution (no oxidative species, ascribed to 100 % scavenging) and the electrolyzed NaCl solution (0 % scavenging, in the absence of any antioxydants). The FRS percentage was calculated according to the following equation:

$$FRS\ (\%) = [1 - [(OD_{sample} - OD_{0\%})/(OD_{100\%} - OD_{0\%})]] \times 100,$$

where OD_{sample} represents the optical density (OD) of the cranberry sample, $OD_{0\%}$, the OD of the cranberry sample in the non-electrolyzed NaCl solution, and $OD_{100\%}$,the OD of the electrolyzed NaCl solution in the absence of the cranberry sample. The degree of discoloration indicates the FRS capacity of the sample. The FRS activity of a substance is usually ranked with the following scale: i) FRS activity $\geq$ 70%: elevated, ii) FRS activity between 40-70%: average, iii) FRS activity $\leq$ 40%: weak or absent.

The reaction was standardized with Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma-Aldrich Ltd) whose FRS activity was 100%. The antiradical activity of the samples was estimated from a calibration curve (r^2 = 0.9916) by plotting known solutions of Trolox® (0.8, 1.6, 2.4, 3.2, and 4.0 mM) against % scavenging capacity. Then, data were reported to TP

concentrations and results were expressed as mM Trolox® Equivalent (TE)/mg of phenol.

5.5.5. Determination of the lipid peroxidation inhibition capacity

The lipid peroxidation inhibition (LPI) activity of cranberry extracts or juice at pH 2.5 and 7 was determined with the thiobarbituric acid reactive substances (TBARS) assay. This was done using a microtechnique based on the non-enzymatic peroxidation of rat liver microsomes method (Esterbauer *et al.*, 1982) modified by others (Lessard, 1995; Caillet *et al.*, 2007) where artificial membranes were used instead of rat liver microsomes, in order to obtain a more stable and reproducible system. This spectrophotometric test indirectly measures the TBARS concentration produced during the peroxidation of liposomes exposed to iron ions in 20 mM phosphate buffer solution in the presence of ascorbate. The antioxidant activity is equivalent to the LPI capacity.

5.5.5.1. Liposomes preparation

Liposomes were formed by an injection method, as described by Batzri & Korn (1973). Linoleic acid (Sigma-Aldrich, Oakville, ON, Canada) was dissolved in 95% ethanol. The mixture was injected into phosphate buffer (20 mM, pH 7.4) in a proportion of 1:9 (v/v), using a hypodermic syringe fitted with a fine needle (G26).

5.5.5.2. *Microplate preparation*

Twenty five µL of samples (1.25 mg/mL), positive control, negative control and color controls were added to a microplate (96 wells). The reaction mixture containing 4 mL of liposomes solution, 2.25 mL of phosphate buffer (20 mM, pH 7.4), 250 µL of sodium ascorbate (3.1 mg/mL), 10 µL EDTA (104 µM), and 10 µL H_2O_2 (1 mM) was prepared, and then 65µL was added to the microplate using a multichannel pipette. Finally, 10 µL of $FeCl_3$ solution (Sigma-Aldrich, 4.3 mg/mL) were added to the wells. The microplate was then incubated at 37°C for 15 minutes. A

volume of 150 μL of a fresh SDS solution (Sigma-Aldrich, 10% v/v) and of thiobarbituric acid (Sigma-Aldrich, 0.67% v/v) in a 1:2 ratio was added in the microplate. The colorimetric reaction was produced at 80 °C for 30 min. The LPI capacity of the controls and samples was evaluated at 540 nm using a Microplate Autoreader (model EL 309, Biotek Instruments, Winooski, VT). The positive control was represented by the reaction mixture in presence of Trolox without the sample, and the optical density of the chromogene formed indicated the complete peroxidation (assigned to 100% of LPI capacity). The negative control contained reaction mixture without the sample (assigned to 0% of LPI capacity). Color controls were prepared to offset the color interference between the sample and the reaction mixture. The relative LPI capacity was calculated using the following equation:

$$LPI\ (\%) = [OD_{(negative\ control)} - OD_{(sample)}/OD_{(negative\ control)} - OD_{(positive\ control)}] \times 100,$$

Then, data were reported to TP concentrations and results were expressed as mg TE /mL /mg of phenol relative to the positive control (100%).

5.5.6. Statistical analysis

Analysis of variance, Ducan's multiple-range test and Student *t* test (effect of pH) were employed to statistically analyze all results. Differences between means were considered significant when $p \leq 0.05$. PASW Statistics 18 software (SPSS Inc., Chicago, IL, USA) was used for the analysis. For each measurement, three samples in each replicate were tested.

5.6. Results and discussion

5.6.1. Effect of the cranberry juice process on total phenolic content

The TP content of the cranberry samples collected at each step of the juice process is shown in Table 5.1. In the cranberry extracts obtained from solid-containing intermediary juice samples (frozen fruit, mash, macerated and depectinized mash, pomace), the TP concentration ranged from 21.65 (E1; frozen fruits) to 274.90 mg GAE/g dry weight (DW) (E3; macerated and depectinized mash). The TP content in the polar (E1) and apolar (E2) extracts ranged from 21.65 to 177.10, and from 122.46 to 250.90 mg GAE/g DW, respectively. The anthocyanin extract (E3) had a TP content ranging between 87.49 and 274.90 mg GAE/g DW. For each solid-containing intermediary cranberry juice sample, except for the macerated and depectinized mash sample, the E2 extracts had the highest TP concentrations. In the macerated and depectinized cranberry mash, the highest TP level was found in the E3 extract. These results suggest a relative stability of the proportions in which the different classes of phenolic compounds are found in cranberries. In the liquid intermediary juice samples (initial pressed juice, clarified juice and concentrate), the TP content ranged from 1.65 to 26.06 mg GAE/g; the clarified juice had the highest content. Seeram *et al.* (2008) reported an average phenolic content of 1.7 mg GAE/g in American 100% cranberry juice, an amount that is much lower than the 26.06 mg GAE/g DW reported in this study.

The transformation steps involved in the juice process had the effect of increasing the TP concentrations in the cranberry extracts which peaked to a maximum level in extracts isolated from macerated and depectinized cranberry mash. The extracts obtained from frozen cranberries and cranberry pomace exhibited the lowest levels of TP. Following the milling step, the addition of

Table 5.1. Total phenolic content measured in cranberry samples (and cranberry extracts) collected at each step of the industrial juice process.

Cranberry samples	Total phenols (mg GAE/g)[1,2]
Frozen fruit	
E1[3]	21.65±0.98c
E2[4]	122.46±0.94h
E3[5]	87.49±0.58f
Mash	
E1	97.26±2.30g
E2	188.69±3.66j
E3	179.09±4.18i
Macerated and depectinized mash	
E1	177.10±5.80i
E2	250.90±10.20k
E3	274.90±5.20l
Initial pressed juice	
	17.25±0.36b
Pomace	
E1	80.11±5.40e
E2	123.50±2.11h
E3	87.71±5.56ef
Clarified juice	
	26.06±0.87d
Juice concentrate	
	1.65±0.05a

[1] Gallic acid equivalent/ g of dry weight basis.

[2] Within each column, means bearing the same letter are not significantly different ($P > 0.05$).

[3] Cranberry water/methanol (85/15, v/v) extract.

[4] Cranberry acetone/methanol/water (40/40/20, v/v) extract.

[5] Cranberry methanol/water/acetic acid (85/14.5/0.5, v/v/v) extract.

pectolytic enzymes and the heating of the mash both contribute to extracting the red color pigments (anthocyanins), through an effect called plasmolysis (Sapers *et al.*, 1983; Grassin & Fauquembergue, 1996). But it may also influence the content of other phenolic compounds. Versari *et al.* (1997) evaluated the effect of commercial pectolytic enzymes on the content of phenolic compounds (anthocyanins, flavonols and ellagic acids) in strawberry and raspberry juices under enzymatic pectinase treatment. They found that a 6-hr pectinase treatment caused a 20% loss of anthocyanins in raspberry juice, and for strawberry juice, a 35% decrease in the flavonol content. Among the liquid intermediary juice samples, the clarified cranberry juice was the sample with the highest amount of TP, whereas the most transformed sample (juice concentrate) had the lowest TP level. Phenolic stability and the rate of their degradation are markedly influenced by temperature (Hrazdina *et al.*, 1982). When studying the effects of the blueberry juice process, Skrede *et al.* (2000) did not report significant losses of anthocyanin, chlorogenic acid and flavonol glycosides during the evaporation of blueberry juice, but it lowered nearly by half the procyanidin level. In this study, the juice evaporation caused a significant change in the TP content, which decreased from 26.06 to 1.65 mg GAE/g DW.

During the juice pressing step, high amounts of press cake are obtained: cranberry pomace is the main by-product. It is composed primarily of skin, seeds, and stems left over after pressing the fruit for juice production. Even when juice extraction conditions are optimal, the content in phenolic compounds of pomace remains significant (Lu & Foo, 1999). In this study, the TP concentrations in the three extracts obtained from cranberry pomace was, in most cases, equivalent or greater than in the extracts obtained from frozen cranberries. On a dry pomace weight basis, the E2 extract had the highest level of TP ($p \leq 0.05$) whereas the E1 and E3 extracts had similar TP levels ($p > 0.05$). According to these results, the efficiency of the phenolic extraction of this juice process, through

milling and depectinization, balances the negative effects of the juice process conditions (temperature, oxidation, and enzymatic degradation) known to affect negatively the stability of cranberry phenolic compounds (Macheix et Fleuriet, 1998).

5.6.2. Effect of the cranberry juice processing on FRS and LPI

5.6.2.1. Cranberry water/methanol (85:15, v/v) extract (E1)

The effects of the juice process on the FRS and the LPI capacities of the E1 extracts are presented in Table 5.2. The FRS and LPI capacity results of E1 obtained from the solid-containing intermediary juice samples were compared to the results of the liquid intermediary juice samples because the extraction solvent used to isolate E1 (water/methanol, 85/15, v/v) resembled the most the aqueous juice extraction occurring in a normal industrial juice process. At each step of the juice process, the FRS capacity of cranberry water-soluble phenolics was greater than their LPI capacity. The FRS capacities of E1 varied from 6.25 to 52.96 and from 6.56 to 48.17 mM TE/mg phenol, respectively at pH 2.5 and 7.0, whereas the LPI capacities ranged from 2.16 to 8.02 and from 1.39 to 7.72 mg TE /mL /mg phenol, respectively at pH 2.5 and 7.0.

Overall, the juice process reduced significantly the antioxidant capacity of the extract E1.Neutralizing the pH of the initial pressed and the clarified juice samples, and of E1 from the pomace had a significant ($p \leq 0.05$) influence on their FRS capacity: at pH 7.0, it was higher in the initial pressed juice and the pomace, but lower in the clarified juice. However, the pH difference did not have an influence on the overall effects of the juice process on the FRS capacity of the E1 extract and the liquid intermediary juice samples.

The most important change in FRS capacity was observed when the cranberries were mashed. Indeed, the milling, heating and depectinization conditions caused a significant ($p \leq 0.05$) change in

Table 5.2. Antioxidant activity of cranberry polar extract obtained with water/methanol (85/15, v/v; E1) from samples collected at each step of the industrial juice process.

Cranberry samples	pH[1]	
	2.5	7.0
Free radical-scavenging capacity (mM TE/mg phenol)[2]		
Frozen fruit	52.96 ± 1.90f	48.17 ± 3.27g
Mash	20.05 ± 1.53e	18.43 ± 0.52f
Macerated and depectinized mash	15.20 ± 1.22d	13.84 ± 0.83e
Initial pressed juice	6.62 ± 0.04a	7.85 ± 0.03c*
Pomace	8.76 ± 0.06c	10.11 ± 0.18d*
Clarified juice	6.90 ± 0.05ab	6.56 ± 0.05a*
Juice concentrate	6.25 ± 1.13a	7.00 ± 0.35b
Lipid peroxidation inhibition capacity (mgTE/mL/mg phenol)[3]		
Frozen fruit	8.02 ± 1.98b	7.72 ± 1.88c
Mash	ND[5]	0.42 ± 0.15a*
Macerated and depectinized mash	ND	ND
Initial pressed juice	ND	ND
Pomace	2.16 ± 0.36a	1.39 ± 0.21b*
Clarified juice	ND	ND
Juice concentrate	ND	ND

[1] Within each column, means bearing the same letter are not significantly different ($P > 0.05$).

[2] Data presented as mean mM values of Trolox® Equivalent ± SD (n=3) per mg of phenol.

[3] Data presented as mean values of mg Trolox® Equivalent ± SD (n=3) per mL per mg of phenol.

[4] None detected.

* Difference between means results at pH 2.5 and 7.0 is significant ($P \leq 0.05$) according to Student *t* test.

the FRS capacity of E1: it decreased from 52.96 to 20.05 and from 48.17 to 18.43 mM TE/mg phenols, at pH 2.5 and 7.0 respectively. Following that step, FRS capacity of the cranberry water-soluble phenolics decreased slightly during the heating, maceration and depectinization of the mash. After the juice extraction, the FRS capacity remained stable in the liquid intermediary juice samples at values ranging from 6.25 to 6.90 and from 6.56 to 7.85 mM TE/mg phenol at pH 2.5 and 7.0, respectively. Similar patterns were observed in the changes of LPI capacity results, although for numerous samples, the values obtained were below the limit of detection of the method. The most important decrease in LPI capacity was observed when the cranberries were mashed; the level went from 8.02 to ND and from 7.72 to 0.42 mg TE /mL /mg phenol at pH 2.5 and 7.0, respectively. The level of LPI capacities of the liquid intermediary juice were also below the detection limit of the method and thus did not provide meaningful LPI values.

The changes in structural integrity of the plant material that occur during juice processing have been shown to impact negatively the putative phenolic composition as well as the properties of berries. The milling and depectinization steps in blueberry juice processing, by activating the native polyphenol peroxidase (PPO) activity caused the greatest losses of anthocyanins, flavonols, and procyanidins (Kader *et al.*, 1997; Skrede *et al.*, 2000; Lee *et al.*, 2002). The conversion of certain compounds also occurs during juice processing; for example, the caffeic-quinic acids conjugate called chlorogenic acid was found in the juice, but not in whole cranberries (Chen *et al.*, 2001; Harnly *et al.*, 2006). Although flavonol stability has not been measured, according to Pappas & Schaich (2009), given their known concentrations in cranberries and commercially available juices, flavonol retention could be estimated at between 40 and 70 %, compared to 2-30% for anthocyanins. In summary, these changes could explain the effects the juice process had on the antioxidant properties of cranberry water-soluble phenolics.

Phenolic compounds with a catechol unit (phenolic acids with dihydroxylation in the 3,4 positions and flavonoids with a adjacent dihydroxy substituents on the B ring) have been shown to be effective hydrogen donors with good antioxidant properties (Zheng &Wang, 2003). In a study on cacao, the number of catechol units in the reaction mixture was found to positively correlate with the ability of catechins and procyanidins to protect against oxidation of LDL, independently of monomer or chain structure (Steinberg *et al.,* 2002). Määttä-Riihinen *et al.,* (2005) have hypothesized that phenolics with a lower content of catechol units cannot achieve the saturation point for the antioxidant effect in the LDL model. In this study, the lower LPI capacity of the liquid intermediary juice samples could be attributed to juice process-induced degradation or oxidative polymerization of the catechol unit-containing cranberry phenolics which lowers their ability to chelate the iron ions and prevent the Fenton chemical reaction and the lipid peroxidation (Robards *et al.,* 1999; Mira *et al.,* 2002; Cheng & Breen, 2000; Caillet *et al.*, 2007).

5.6.2.2. Cranberry acetone/methanol/water (40:40:20, v/v/v) extract (E2)

The effects of the juice process on the antioxidant properties of the cranberry apolar extracts (E2) isolated from solid-containing intermediary cranberry juice samples, adjusted at pH 2.5 and pH 7.0 are presented in Table 5.3. At each step of the juice process, the FRS capacity of cranberry apolar extracts was greater than their LPI capacity and in general, their pH neutralization did not significantly affect the patterns of changes in FRS and LPI capacities observed throughout the juice process. The FRS and the LPI capacities varied from 6.13 to 9.71 mM TE/mg phenol and from 1.48 to 3.44 mg TE /mL /mg phenol, respectively when E2 extracts were adjusted at pH 2.5. The most important change in E2 FRS capacity occurred during the juice extraction from the macerated and depectinized cranberry mash: it decreased from 12.76 to 6.13 mM TE/mg phenol at

Table 5.3. Antioxidant activity of cranberry apolar extract obtained with acetone/methanol/water (40/40/20, v/v; E2) from samples collected at each step of the industrial juice process.

Cranberry samples	pH[1]	
	2.5	7.0
Free radical-scavenging capacity (mM TE/mg phenol)[2]		
Frozen fruit	9.71 ± 2.42b	8.07 ± 1.08a
Mash	14.46 ± 0.33c	13.58 ± 0.60c
Macerated and depectinized mash	12.76 ± 1.73bc	12.02 ± 0.44b
Pomace	6.13 ± 0.11a	7.31 ± 0.09a*
Lipid peroxidation inhibition capacity (mg TE/mL/mg phenol)[3]		
Frozen fruit	3.44 ± 0.78b	2.94 ± 0.30c
Mash	1.75 ± 0.29a	1.87 ± 0.22b
Macerated and depectinized mash	1.66 ± 0.11a	0.74 ± 0.25a*
Pomace	1.48 ± 0.15a	1.15 ± 0.22a

[1] Within each column, means bearing the same letter are not significantly different ($P > 0.05$).

[2] Data presented as mean mM values of Trolox® Equivalent ± SD (n=3) per mg of phenol.

[3] Data presented as mean values of mg Trolox® Equivalent ± SD (n=3) per mL per mg of phenol.

* Difference between means results at pH 2.5 and 7.0 is significant ($P \leq 0.05$) according to Student *t* test.

pH 2.5. The milling of the cranberries caused the most significant ($p \leq 0.05$) change in LPI capacity, which level went from 3.44 to 1.75 mg TE /mL /mg phenol, at pH 2.5.

Compared to E1, the juice process did not affect the antioxidant capacity of E2 to the same extent nor in same the fashion. These results concur with previous reports which showed that polymerized chains of catechin monomers forming procyanidin trimers usually had a decreased ability to prevent free radical damage in a lipid system versus an aqueous phase (Plumb *et al.,* 1998; Cao & Prior, 1999). The milling step increased the FRS capacity of E2 extract from 9.71 to 14.46 mM TE/mg phenol at pH 2.5, which represented the highest FRS capacity level for that extract. The frozen cranberries had the highest LPI capacity (3.44 mg TE /mL /mg phenol) and the milling was the most damaging step to E2 LPI capacity, which decreased from 3.44 to 1.75 mg TE /mL /mg phenol at pH 2.5. Prior *et al.* (2001) showed that compared to fresh cranberries, commercially pressed cranberry juice had a higher proportion of procyanidin monomers, dimers, and A-type trimers. These reports suggest that the cranberry apolar phenolics underwent a certain level of polymerization during the milling step which, in turn, affected their overall antioxidant properties.

5.6.2.3. Cranberry methanol/water/acetic acid (85:14.5:0.5, v/v/v) extract (E3)

The effects of the juice process on the antioxidant properties of the anthocyanin extracts (E3) isolated from solid-containing intermediary cranberry juice samples, adjusted at pH 2.5 and pH 7.0 are presented in Table 5.4. At each step of the juice process, the FRS capacity of cranberry anthocyanin extract was greater than their LPI capacity. The FRS capacities of E3 varied from 9.99 to 16.61 and from 11.27 to 17.50 mM TE/mg phenol, respectively at pH 2.5 and 7.0; the highest FRS capacity being obtained with the mash. The LPI capacities of E3 ranged from 1.06 to 7.95 and from 0.91 to

Table 5.4. Antioxidant activity of cranberry anthocyanin extract obtained with methanol/water/acetic acid (85/14.5/0.5, v/v/v; E3) from samples collected at each step of the industrial juice process.

Cranberry samples	pH[1]	
	2.5	7.0
Free radical-scavenging Capacity (mM TE/mg phenol)[2]		
Frozen fruit	13.28 ± 2.50b	17.50 ± 1.86c
Mash	16.61 ± 0.18c	16.63 ± 0.10c
Macerated and depectinized mash	13.04 ± 0.40b	11.27 ± 0.50a*
Pomace	9.99 ± 0.09a	12.87 ± 0.04b*
Lipid peroxidation inhibition Capacity (mg TE/mL/mg phenol)[3]		
Frozen fruit	7.95 ± 1.12c	3.19 ± 0.27c*
Mash	2.43 ± 0.14b	1.66 ± 0.39b*
Macerated and depectinized mash	1.06 ± 0.18a	0.91 ± 0.13a
Pomace	2.73 ± 0.19b	3.28 ± 0.19c*

[1] Within each column, means bearing the same letter are not significantly different ($P > 0.05$).

[2] Data presented as mean mM values of Trolox® Equivalent ± SD (n=3) per mg of phenol.

[3] Data presented as mean values of mg Trolox® Equivalent ± SD (n=3) per mL per mg of phenol.

* Difference between means results at pH 2.5 and 7.0 is significant ($P \leq 0.05$) according to Student *t* test.

3.28 mg TE/mL/mg phenol, respectively at pH 2.5 and 7.0; the highest LPI capacity result was obtained from the least processed cranberry sample (frozen cranberries).

Compared to E1, the juice process did not affect to the same extent or in the way, the antioxidant capacity of E3. The most important change in the FRS capacity of E3 adjusted at pH 2.5 occurred when the juice was pressed out from the macerated and depectinized cranberry mash: it decreased from 13.04 to 9.99 mM TE/mg phenol when at pH 2.5. At pH 7.0, the most significant change was caused by the heating and maceration of the mash with pectinase: it decreased from 16.63 to 11.27 mM TE/mg phenol. The milling step had the most important lowering effect on the LPI capacity of E3, which decreased from 7.95 to 2.43 and from 3.19 to 1.66 mg TE/mL/mg phenol, respectively at pH 2.5 and 7.0.

Pappas & Schaich (2009) have reported that temperature may be the most important factor in cranberry anthocyanin stability, particularly during thermal pasteurization necessary for microbial stabilization. Anthocyanins can also easily be oxidized when exposed to light or oxygen, and can undergo degradation or molecular reorganizations (copigmentation) when subjected to endogenous enzymatic activity (polyphenoloxidase, peroxidase) (Attoe & Von Elbe, 1981; Kader *et al.*, 1997 and 1999, Santos-Buelga and Scalbert, 2000; Lee *et al.*, 2002; Worlstad *et al.*, 2005). Our results indicate that the breakdown cranberry cell walls during milling increased the FRS capacity of E3. The heating and maceration of the mash with pectinase brought E3 FRS capacity back to its initial level. The milling, heating and maceration steps only had negative impacts on the LPI capacity of cranberry anthocyanins. These results suggest that cranberry anthocyanins lose their metal chelating properties when exposed to heat and subjected to pectinase and endogenous enzymes, but manage to retain free radical scavenging properties through molecular reorganization. Viskelis *et al.* (2009) reported that cranberry anthocynins extracted from frozen berries and pomace had similar radical scavenging

activities. In our study, there was a significant ($p \leq 0.05$) difference between the FRS capacity of E3 isolated from frozen cranberries and cranberry pomace.

Increasing instability of anthocyanins at higher pH is another factor widely known and attributed to their conversion to equilibrium forms that are more sensitive to oxidation and subsequent degradation (Pappas & Schaich, 2009). Anthocyanins are known to exist in a variety of forms, in relative proportions depending on pH. At a pH of approximately 3 or lower, the color of the anthocyanins is orange or red and exists as a flavylium cation. As the pH is raised, kinetic and thermodynamic competition occurs between the hydration reaction of the flavylium cation and the proton transfer reactions related to the acidic hydroxyl groups of the aglycone. While the first reaction gives a colorless carbinol pseudo-base, which can undergo ring opening to a chalcone pseudo-base, the latter reaction gives rise to quinonoidal bases. Further deprotonation of the quinonoidal bases can take place at pH 6-7 with the formation of purplish, resonance-stabilized quinonoid anions (Fossen *et al.*, 1998). Our results indicate that pH neutralization had conflicting but significant effects on the FRS capacity of E3 isolated from macerated and depectinized mash (decrease), and from pomace (increase). The change in pH had a more significant and systematic influence on E3 LPI capacity: values at pH 7.0 were generally lower than at pH 2.5. When the pH of the anthocyanin-rich cranberry extract was adjusted to 7.0, it most certainly caused a shift in the equilibrium of the pH-dependent forms of anthocyanins, and favored the quinonoidal forms which consistently decreased the observed LPI activities.

5.7. Conclusions

This study exposed the complex and contrasting effects of the juice process on the antioxidant properties of specific classes of cranberry phenolic compounds. Despite the fact that all three cranberry extracts differed in their polarity (polar, apolar and anthocyanins), their ability to scavenge free radicals was always greater than their capacity to inhibit lipid peroxidation. In general, the antioxidant capacity of cranberry polar extracts was more affected by the juice process than that of the apolar and the anthocyanin extracts. Removal of the juice from the macerated and depectinized mash diminished the FRS capacity of cranberry apolar phenolics by more than half. The LPI capacity of E2 and E3 were similarly affected by the juice process: the most significant decrease was caused by the milling step. The milling, heating and maceration steps had negative impacts on the LPI capacity of cranberry anthocyanins. The evaporation of the juice did not have a significant effect on its FRS capacity. The antioxidant and antiradical activities of E1, isolated from cranberries using an aqueous solvent mixture (85%), differed greatly from those of E2 and E3 obtained with organic solvent mixtures (80 or 85%). Indeed the cranberry polar extracts were more potent antioxidant than the apolar and the anthocyanin extracts, confirming the determinant role that polarity plays in the antioxidant and antiradical activities of phenolic compounds. Overall, pH neutralization did not have a significant effect on the changes observed during the juice process in the FRS and LPI capacity of the extracts, except for the anthocyanin extract for which it lowered the LPI capacity. The cranberry phenolic compounds in the cranberry pomace showed promising antioxidant activities; this property could be exploited by processor wishing to improve the marketing of this by-product. Various assays with different reference antioxidants such as Trolox, L-ascobic acid, and α-tocopherol, have been used for the determination of antioxidant activities of berries; therefore, our results were difficult to compare to the literature. Determination of biological activities of specific cranberry phenolic

constituents is important for evidence-based selection of cranberry variants with favorable phenolic profiles and improved health benefits. More research should be conducted to characterize and monitor the evolution of the profile of phenolic compounds, to determine which individual phenolic compounds can better resist the juice process conditions and contribute to the total antioxidant activity of the juice and juice concentrate. In general, the results showed that the cranberry juice pressing by-product (pomace) contain significant amounts of antioxidatively active compounds, which may be regarded as a promising natural additive for health beneficial functional foods and nutraceuticals.

5.8. Acknowledgments

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CHAPITRE 6

Conclusions générales

À cause de sa saveur amère et son goût acidulé, la canneberge est rarement consommée à l'état naturel. Elle est souvent transformée en jus ou en concentré de jus, et ensuite mélangée à de l'eau et du sucre pour en faire une boisson de jus de fruit. Malheureusement, la couleur rouge de la canneberge est extrêmement instable; elle peut rapidement se dégrader et brunir. En effet, ses anthocyanes, ainsi que ses autres composés phénoliques et flavonoïdes s'oxydent facilement au cours des procédés de transformation (Macheix *et al.*, 1990). C'est pourquoi il est pertinent pour les manufacturiers de jus de comprendre quels sont les effets des différentes conditions de leur procédé sur ces composés bioactifs. Ils peuvent ainsi maximiser la qualité et la durée de vie de leurs produits, mais aussi leurs propriétés biologiques. Cette étude avait pour objectif global de démontrer les effets du procédé de jus sur le taux de récupération et la conservation des phénols, les propriétés antimicrobiennes et les propriétés antioxydantes de la canneberge car très peu d'études ont tenté d'évaluer les effets cumulatifs des conditions du procédé de transformation de jus sur les composés bioactifs et les propriétés de la canneberge. De plus, en déterminant les propriétés biologiques des produits intermédiaires et le sous-produit (marc ou moût) du procédé en question, nous allions pouvoir confirmer et faire valoir leur potentiel comme agent de conservation naturel. Les consommateurs d'aujourd'hui sont de plus en plus préoccupés par les résidus chimiques dans les aliments et ont tendance à choisir des aliments naturels, sains et sans danger (Gould, 1996). L'addition de composés phénoliques naturels ou de jus antimicrobiens et antioxydants pourrait être une bonne alternative pour réduire les risques pour la santé et les pertes économiques dues à des contaminations microbiologiques, et d'étendre la durée de conservation des produits alimentaires transformés (Conner, 1993; Dorman et Deans, 2000). Durant plus d'un demi-siècle, l'inhibition de la croissance microbienne par la canneberge et le jus de canneberges a été liée à son faible pH (2,5). Le perfectionnement des techniques d'analyse pour la caractérisation des composés chimiques au début

du XXI^{e} siècle a permis à plusieurs chercheurs de démontrer que des composés bioactifs de la canneberge, tels que les acides phénoliques de faible poids moléculaire, les tannins condensés, les proanthocyanidines constitués principalement de tétramères épicatéchine et pentamères avec au moins une liaison de type-A, et les flavonoïdes tels que les anthocyanines (en haute teneur) et les flavonols, contribuent également au pouvoir antimicrobien observé (Häkkinen *et al.*, 1999; Foo *et al.*, 2000; Leitao *et al.*, 2005; Puupponen-Pimiä *et al.*, 2005; Heinonen, 2007; Seeram & Heber, 2007; Wu *et al.*, 2008). C'est pourquoi l'un des objectifs du projet visait à déterminer les effets antimicrobiens de la canneberge exclusivement lié à la présence de ses composés bioactifs. Pour ce faire, le pH des échantillons a été ajusté à 7,0 afin de ne pas confondre l'effet antimicrobien des phénols de la canneberge avec celui de son acidité naturelle. Les propriétés antioxydantes de la canneberge sont bien documentées dans la littérature et le fruit se classerait parmi les meilleurs antioxydants (Sun *et al.*, 2002; Heinonen, 2007). Toutefois, les composés biologiques que l'on retrouve dans les baies sont reconnus pour avoir une activité antioxydante diversifiée basée sur plusieurs mécanismes qui dépendent de leur structure moléculaire (Borowska *et al.*, 2005; Szajdek & Borowska, 2008). Comme aucun test unique ne peut refléter le plein potentiel antioxydant d'un produit, deux méthodes spectrophotométriques dosant le N, N-diéthyl-p-phénylènediamine (piégeage des radicaux libres) et les substances d'acide thiobarbiturique réactives (inhibition de la peroxydation des lipides) ont été utilisées pour mesurer la capacité antioxydante des échantillons collectés tout au long du procédé de jus.

L'étude présentée dans le chapitre 3 avait pour objectif de valider la capacité de la méthode de Gutierrez *et al.* (2008) à mesurer l'activité antimicrobienne d'extraits et du jus de canneberges. Pour ce faire, sept souches bactériennes (*Enterococcus faecium* résistant à la vancomycine (ERV), *Escherichia coli* O157: H7 EDL 933, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* HPB

2812, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella* Typhimurium SL1344 et *Staphylococcus aureus* ATCC 29213) ont été exposées à 11 concentrations différentes des trois extraits (polaire (E1), apolaires (E2) et anthocyanique (E3)) et du jus de canneberges, ajustés à pH 7,0, afin de déterminer les concentrations minimale inhibitrice (CMI) et maximale tolérée (CMT). Dans le cadre de l'évaluation de la méthode, l'effet antimicrobien des solvants de dilution des extraits a été vérifié. Seul le solvant composé de méthanol/eau/acide acétique (85/14,5/0,5, v/v/v) utilisé pour la dilution de E3 a inhibé la croissance des bactéries *P. aeruginosa* ATCC 15442 (à 10%, v/v), *S. aureus* ATCC 29213 (à 20% , v/v), *L. monocytogenes* (à 20%, v/v), *E. coli* ATCC 25922 (à 10%, v/v), *E*RV (à 20%, v/v) et *S.* Typhimurium SL1344 (à 20%, v/v). Ces deux concentrations de méthanol/eau/acide acétique (85/14,5/0,5, v/v/v) correspondaient toutefois à des concentrations d'extraits supérieures aux CMI et CMT obtenues. Il a donc été possible de conclure que les effets antimicrobiens observés en présence des extraits n'étaient pas dus à la présence de leur solvant de dilution. Les composés phénoliques de la canneberge ont démontré des effets antimicrobiens sur toutes les souches bactériennes testées, mais avec des taux d'inhibition variables. Tel que démontré précédemment (Cavanagh *et al.*, 2003; Leitao *et al.*, 2005; Puupponen-Pimiä *et al.*, 2005), les résultats n'ont révélé aucune corrélation entre le statut Gram positif et négatif des bactéries et leur sensibilité aux composés phénoliques de la canneberge. L'extrait de canneberges polaire avait le plus grand pouvoir antimicrobien. Les meilleures CMI et CMT ont été obtenues avec les bactéries *E*RV, *P. aeruginosa*, *S. aureus* et *E. coli* ATCC 25922. L'extrait anthocyanique a également démontré un effet antimicrobien contre *P. aeruginosa* et d'*E. coli* ATCC, mais moindre que celui observé avec E1. Leitao *et al.* (2005) pour leur part, ont démontré que seule la bactérie *S. aureus* était sensible aux anthocyanes de la canneberge. Les bactéries *L. monocytogenes*, *E. coli* O157: H7 et *S.* Typhimurium ont montré la meilleure résistance aux extraits de canneberge neutralisés. Puisque la majorité des

concentrations de jus de canneberges testée n'a pas affectée la croissance des bactéries, des cinétiques d'inhibition de croissance du jus pur neutralisé ont été faites sur des concentrations microbiennes plus faibles (10^3 UFC/mL). En moins de 30 minutes, les bactéries *L. monocytogenes* et *E*RV ont été complètement inactivés par le jus. En 2008, Wu *et al.* ont démontré des résultats semblables en exposant *L. monocytogenes* et *S.* Typhimurium au concentré de jus de canneberge.

Dans le cadre des études des effets du procédé de jus sur les propriétés antimicrobiennes et antioxydantes de la canneberge (chapitres 4 et 5), les effets du procédé sur le taux de récupération et la conservation des phénols totaux (PT) de la canneberge ont été évalués. La teneur en PT des extraits de canneberges isolés des échantillons intermédiaires solides (fruits surgelés, purée, purée macérée et dépectinisée, marc) variait entre 21,65-29,11 et 274,90-324,00 mg équivalent d'acide gallique (EAG)/g matières sèches (MS). En comparant la teneur en PT entre les trois types d'extrait, dans tous les cas sauf avec la purée de canneberges macérée et dépectinisée, les extraits apolaires obtenus avec le mélange d'acétone/méthanol/eau (40/40/20, v/v/v) avaient les plus fortes concentrations en PT (105,50-283,20 EAG/g MS). L'extrait anthocyanique de la purée de canneberges macérée et dépectinisée s'est avérée être le plus riche en PT (274,90-324,00 mg EAG/g MS). Ces résultats suggèrent une stabilité relative des proportions dans lesquelles les différentes classes de composés phénoliques sont représentées dans les canneberges. Dans les échantillons intermédiaires liquides (jus pressé, jus filtré et concentré de jus), la teneur en PT variait entre 1,63 et 26,80 mg EAG/g MS. L'ajout d'enzymes pectolytiques et le chauffage de la purée, par un effet de plasmolyse, favorisent l'extraction des pigments rouge (anthocyanes) (Sapers *et al.*, 1983; Grassin & Fauquembergue, 1996), mais peuvent également avoir un impact négatif sur la teneur des autres composés phénoliques. Versari *et al.* (1997) ont constaté qu'une enzymation de six heures pouvait entraîner une perte de 20% des anthocyanes dans les jus de framboises et une diminution de 35% de la teneur en

flavonols dans le jus de fraises. Le broyage, le chauffage et la dépectinisation des canneberges ont eu pour effet d'augmenter la concentration de PT des trois extraits. Parmi les échantillons intermédiaires liquides, le jus pressé était le plus riche en PT (26,80 mg EAG/g MS), tandis que le concentré de jus avait la plus faible teneur. Hrazdina *et al.* (1982) ont démontré comment les hautes températures affectaient la stabilité des phénols. Lors du pressage de la purée pour en extraire le jus, d'importantes quantités de marc sont générées; ce résidu renferme principalement la pelure du fruit, les graines, et parfois même quelques tiges. Même lorsque les conditions d'extraction de jus sont optimales, la teneur en composés phénoliques du marc demeure importante (Lu & Foo, 1999). Dans cette étude, les concentrations en PT des trois extraits de marc étaient, dans la plupart des cas, soit équivalentes ou supérieures à celles des extraits obtenus à partir de canneberges congelées. Bien que les conditions (température, oxydation et dégradation enzymatique) employées lors du broyage et de la macération peuvent affecter négativement la stabilité des composés phénoliques de la canneberge (Macheix et Fleuriet, 1998), les résultats ont démontré que ces effets peuvent être contrebalancés par l'augmentation du taux de récupération résultant de la dépectinisation de la purée.

Les effets du procédé du jus sur les propriétés antimicrobiennes de la canneberge ont ensuite été évalués et les résultats sont présentés sous forme d'article dans le chapitre 4. Les effets du procédé de jus industriel sur les propriétés antibactériennes de la canneberge et de trois extraits de canneberges (polaire (E1), apolaires (E2) et anthocyanique (E3)) ont été étudiés sur sept souches bactériennes (*E*RV, *E. coli* O157: H7 EDL 933, *E. coli* ATCC 25922, *L. monocytogenes* HPB 2812, *P. aeruginosa* ATCC 15442, *S.* Typhimurium SL1344 et *S. aureus* ATCC 29213). Pour éviter de confondre les effets antimicrobiens des phénols de la canneberges de son acidité naturelle (Wu *et al.*, 2008), le pH de l'ensemble des échantillons a été ajusté à pH 7,0 avant de mesurer les concentrations minimale inhibitrice (CMI) et maximale tolérée (CMT). Dans certains cas, l'effet inhibiteur observé

en présence des mélanges de solvants de dilution d'E2 (acétone/méthanol/eau, 40/40/20, v/v/v) et d'E3 (méthanol/eau/acide acétique, 85/14,5/0,5, v/v/v) ont dû être pris en considération; les résultats marqués comme tels n'ont pas été utilisés pour la discussion. Le procédé de jus a amélioré de façon générale les propriétés antibactériennes des composés phénoliques hydrophiles et des anthocyanes de la canneberge. Les meilleures CMIs (1,80 à 7,0 µg phénol / puits) pour les bactéries *S. aureus*, *S.* Typhimurium, et ERV ont été obtenues lorsque qu'elles étaient exposées au concentré de jus. Le concentré de jus n'a toutefois pas réussi à inhiber la croissance de *P. aeruginosa*, *L. monocytogenes*, *E. coli* ATCC et *E. coli* O157: H7, bien que ces bactéries aient démontré une certaine sensibilité (CMT de 0.007 à 0,4 µg phénol / puits). Leitao *et al.* (2005) ont rapporté que les bactéries *E. coli* ATCC 10538, *P. aeruginosa* ATCC 27853, ainsi que *S. aureus* et 6358 et 25923 pouvaient résister à l'effet antimicrobien du jus de canneberges neutralisé. Les meilleures CMIs (22,6-90,5 phénol µg/mL) pour les bactéries *P. aeruginosa*, *S. aureus*, *S.* Typhimurium et ERV ont été observées lorsque qu'elles étaient exposées à l'extrait anthocyanique du marc de canneberges, suggérant un effet bénéfique du procédé sur la structure moléculaire des composés de cet extrait. La transformation structurelle des anthocyanes en fonction du pH est essentielle à leur couleur et à leur stabilité : à pH 6-7, le transfert de proton des groupes hydroxyles de l'anthocyanidines donne lieu à la formation d'anions quinoniques stabilisés par résonance (Fossen et *al.*, 1998). Malgré cela, les anthocyanes sont extrêmement sensibles à la chaleur, l'oxygène et la lumière, et lorsque soumis à l'activité enzymatique endogène (polyphénoloxydase, peroxydase), ils peuvent se dégrader ou se réorganiser sur le plan moléculaire (copigmentation) (Attoe & Von Elbe, 1981). Nos résultats suggèerent que les extraits anthocyaniques neutralisés peuvent être sous forme de bases quinoniques ou subir une dégradation ou une réorganisation moléculaire, et conserver leur capacité d'inhiber la croissance microbienne. Les résultats ont toutefois démontré que le broyage, le chauffage et la dépectinisation

diminuaient le pouvoir antimicrobiens des composés phénoliques apolaires de la canneberge. En effet, l'extrait apolaire de la canneberge congelée était le plus efficace contre *P. aeruginosa*, *S. aureus*, *L. monocytogenes* et *S.* Typhimurium. De nombreuses études ont identifié les procyanidines de type A (des composés phénoliques apolaires) comme étant l'un des éléments clés de la protection de la canneberge contre les bactéries pathogènes (Foo *et al.*, 2000a & 2000b; Heinonen, 2007; Seeram & Heber, 2007). Prior *et al.* (2001) a également rapporté qu'en comparaison à la canneberge fraîche, le jus de canneberge renferme une plus grande proportion de procyanidines de type A. Ces données suggèrent que la diminution de l'activité antibactérienne de l'extrait apolaire au cours du procédé peut être attribuée aux changements au niveau du profil de proanthocyanidines. Parmi tous les échantillons, le potentiel antimicrobien de la purée et la purée macérée et dépectinisée s'est avéré être le moins intéressant.

Les effets du procédé du jus sur les propriétés antioxydantes de la canneberge ont ensuite été évalués et les résultats sont présentés sous forme d'article dans le chapitre 5. Les effets du procédé de jus industriel sur les capacités de piégeage des radicaux libres (PRL) et d'inhibition de la peroxydation des lipides (IPL) ont été déterminés à l'aide de méthodes spectrophotométriques dosant respectivement, le N,N-diéthyl-p-phénylènediamine et les substances d'acide thiobarbituriques réactives. Tout d'abord, les extraits isolés des échantillons intermédiaires solides (polaire (E1), apolaire (E2) et anthocyanique (E3)) et des échantillons intermédiaires liquides ont été ajustés à deux pHs : 2,5 et 7,0. Ensuite, leur capacité de PRL et d'IPL a été déterminée; les résultats étaient exprimés, respectivement en mM équivalent Trolox® (ET)/mg de phénol et mg ET/mL/mg de phénol. Les étapes du procédé ayant causé affecté l'intégrité structurale des matières végétales du fruit semble avoir nuit aux propriétés antioxydantes de l'extrait de canneberges polaire. Le broyage du bleuet, ainsi que la dépectinisation de la purée de bleuets, en activant la peroxydase des polyphénols (PPO),

sont reconnus pour entraîner des pertes significatives d'anthocyanes, de flavonols, et de procyanidines (Kader *et al.*, 1997; Skrede *et al.*, 2000; Lee *et al.* , 2002). Certains composés phénoliques peuvent également se complexer pour former des composés ayant différentes propriétés biologiques. C'est entre autres le cas de l'acide caféique dans la canneberge qui se conjugue à l'acide quinique pour former l'acide chlorogénique lorsque celle-ci est transformée en jus (Chen *et al.*, 2001;. Harnly *et al.*, 2006). Bien que la stabilité spécifique des flavonols de la canneberge n'ait jamais été étudiée, selon Pappas & Schaich (2009), compte tenu de leurs concentrations connues dans le fruit et le jus de fruits, on peut estimer que la conservation des flavonols se situe entre 40 et 70%, comparativement à 2-30% pour les anthocyanines. Ces pertes et ces réorganisations moléculaires pourraient expliquer les effets du processus de jus qui ont été observés au niveau des propriétés antioxydantes de l'extrait polaire. Les capacités de PRL et d'IPL de E2 et E3 ont également été affectées par les conditions du procédé, mais moins fortement. L'étape du broyage a augmenté la capacité de PRL de E2 et E3, mais a diminué leur capacité d'IPL. Les composés phénoliques dont la structure moléculaire incorpore une ou plusieurs unités de catéchol (les acides phénoliques avec une dihydroxylation en positions 3,4 et les flavonoïdes avec un substitut adjacent dihydroxy sur l'anneau B) se sont révélés être de bons donneurs d'hydrogène avec de bonnes propriétés anti-oxydantes (Zheng & Wang, 2003). Prior et al. (2001) ont démontré que, comparativement au fruit frais, le jus de canneberges renfermait une plus grande proportion de procyanidines sous forme de monomères, de dimères et de trimères de type A. Ces études suggèrent donc qu'au cours du broyage des canneberges, un processus de polymérisation des composés phénoliques apolaires pourrait augmenter la capacité de piéger les radicaux libres, et limiterait leur habilité à inhiber la peroxidation des lipides. Pour les extraits de canneberges anthocyanique, nos résultats indiquent que la dégradation des parois cellulaires causée par le broyage augmente leur capacité de PRL; le chauffage et la dépectinisation de la purée a toutefois pour effet de

rabaisser les taux de PRL à leur niveau initial. La capacité d'IPL des anthocyanes a été diminuée par le broyage, le chauffage et la dépectinisation de la purée de canneberges. Ces résultats laissent croire que la diminution du pouvoir de chélation des anthocyanes observée après leur exposition à la chaleur et la dégradation enzymatique, est compensée par leur réorganisation moléculaire qui augmente leur pouvoir de piégeage des radicaux libres. Les résultats ont démontré que l'évaporation du jus n'avait pas d'impact sur la capacité de PRL du jus, mais diminuait son pouvoir d'IPL. Au début et après chaque étape du procédé, la capacité de PRL des extraits était supérieure à leur capacité d'IPL. Les activités antioxydantes et antiradicalaires d'E1, extrait des canneberges en utilisant un mélange de solvants aqueux (85%), étaient très différentes de celles de E2 et E3 obtenus avec des mélanges de solvants organiques (80 ou 85%). En effet, les phénols hydrophiles de la canneberge ont démontré un pouvoir antioxydant supérieur à celui des composés phénoliques apolaires et des anthocyanes, confirmant le rôle déterminant que joue la polarité des composés phénoliques dans leurs activités antioxydantes et antiradicalaires. De manière générale, la neutralisation du pH des extraits n'a pas eu d'influences significatives sur les changements de la capacité de PRL et de l'IPL observés au cours du procédé de jus, à l'exception de l'extrait riche en anthocyanes pour lequel une diminution de la capacité IPL a été observée.

Dans un contexte où très peu d'études ayant tenté d'évaluer les effets des conditions de procédés de transformation sur les composés bioactifs et les propriétés de la canneberge ont été publiées, ce projet aura permis d'nnover en isolant les composés bioactifs de la canneberge en fonction de leur polarité afin d'étudier leur propriétés biologiques séparément. Les résultats obtenus nous ont permis de confirmer que le broyage et la dépectinisation du fruit augmentait le taux de récupération des phénols totaux. Malgré les traitements de chaleur, d'hydrolyse enzymatique et d'oxydation, des quantités importantes de phénols ont été mesurées dans le marc (résidu de pressage

du jus). Nous avons également confirmé le pouvoir antimicrobien des phénols de la canneberge neutralisés et constaté que les composés phénoliques hydrophiles et anthocyaniques extraits des échantillons des canneberges les plus transformées sont les plus efficaces pour inhiber la croissance des bactéries *P. aeruginosa, S. aureus, S.* Typhimurium et *E*RV. Le procédé a toutefois nuit aux propriétés antimicrobiennes des composés phénoliques apolaires de la canneberge contre les bactéries *S. aureus, L. monocytogenes, E. coli* O157:H7 *et E*RV résistant à la vancomycine. Dans l'ensemble, le procédé de jus a diminué les capacités antiradicalaires et inhibitrices de peroxydation des lipides des composés phénoliques hydrophiles de la canneberge. La capacité inhibitrice de peroxydation des lipides des composés phénoliques apolaires et des anthocyanes a été réduite lors du broyage, mais a augmenté leur capacité antiradicalaire. L'évaporation ne semble pas avoir nui au pouvoir antiradicalaire du jus de canneberges.

Une méthode d'analyse LC-DAD-MS a été développée et validée au laboratoire du partenaire industriel du projet, Canneberges Atoka Inc., dans le but de surveiller les changements dans le profil des composés phénoliques prédominants de la canneberge. Cette analyse devait servir à déterminer quelles étapes du processus de jus et les mécanismes impliqués dans la dégradation des composés phénoliques de canneberge et de la perte de leurs propriétés. Malheureusement, cet objectif n'a pu être réalisé dans le cadre de ce projet, car l'étudiant à du quitter la compagnie pour des raisons personnelles. Une étude menée par White *et al.* (2011) a démontré l'impact des différentes étapes du procédé de production de jus sur les anthocyanines, les flavonols et les procyanidines de la canneberge. Les analyses ont toutefois portées sur des échantillons obtenus d'un procédé pilote. Il aurait été intéressant de comparer les résultats obtenus par White *et al.* à ceux obtenus à partir du procédé industriel afin de déterminer la validité d'un procédé pilote comme outil de travail. De plus, les résultats de cette recherche pourraient sevir à développer une gamme de produits nouveaux à

cibles spécifiques et donner une valeur ajoutée à une marque commerciale québécoise. Ils pourraient également contribuer au développement de suppléments alimentaires fait à partir du sous-produit, le marc, dont les proprités biologiques ont été démontré dans cette étude.

Ce projet de recherche comportait plusieurs aspects novateurs, dans la mesure où il a permis de démontrer 1) les propriétés biologiques de trois extraits de canneberges différents : polaire, apolaire et anthocyanique, 2) la stabilité des propriétés antibactériennes de la canneberge neutralisée. Il a également permis de comparer les effets des conditions du procédé de jus sur les propriétés antibactérienne et antioxydante de trois extraits de canneberges, ainsi que de démontrer l'effet du pH sur les propriétés antioxydantes de trois extraits de canneberges.

Certaines avenues de recherches futures mériteraient d'être explorées, telles que 1) la caractérisation du profile phénolique des trois extraits de canneberges étudiés, 2) les effets des conditions du procédé de jus sur les composés bioactifs de la canneberge prédominants, 3) les effets de différents moyens de conservation sur les propriétés biologiques du marc de canneberges, et finalement, les effets de la dépectinisation (agitation, activité enzymatique secondaire) sur la propriété antiradicalaire de la canneberge.

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Annexe I – Biographie

Articles récents

1. **CÔTÉ, J**, Caillet, S., Doyon, G., Dussault, D., Sylvain, J.-F. et Lacroix, M. (2011). Antimicrobial Effects of Cranberry Juice and Extracts. Food Control. In press.
2. CAILLET, S., **Côté, J.**, Doyon, G. Sylvain, J.-F. et Lacroix, M. (2011). Antioxidant and antiradical properties of cranberry juice and extracts. Food Research International. In press.
3. **CÔTÉ, J.**, Caillet, S., Doyon, G., Sylvain, J.-F. et Lacroix, M. (2010). Analyzing Cranberry Bioactive Compounds. Critical Reviews in Food Science and Nutrition, vol. 50. p. 872–888.
4. **CÔTÉ, J.**, Caillet, S., Doyon, G., Sylvain, J.-F. et Lacroix, M. (2010). Bioactive Compounds in Cranberries and their Biological Properties. Critical Reviews in Food Science and Nutrition. vol. 50. p. 666–679.
5. **CÔTÉ, J.** (2003). Separation and characterization of glycosylated phenolic compounds and flavonoids from maple products. M.Sc. Thesis, McGill University, Montreal, QC, Canada.

Annexe II – Limites des resultats présentés

Chapitre 1

Définition des flavonoïdes

Dans l'article publié, on peut lire: "Flavonoids are defined as naturally-occurring organic species that possess two six-carbon aromatic centers, called the A and B rings, and a three-carbon bridge, called the C ring which forms a phenol bridge with oxygen (Robards & Antolovich, 1997; Haslam, 1998; Gee and Johnson, 2001)". Il n'est pas exacte de parler d'un pont phénol avec l'oxygène, mais plutôt d'un pont éther.

Propriété antibactérienne des extraits de la canneberge

En lisant cet extrait dans l'article publié: "Dental caries are caused principally by *Streptococcus mutans* infection [...] During the formation of dental caries by *Steptococcus mutans*, the microorganism synthesizes extracellular glucans from sucrose using glucosyltransferases. The glucans can then promote the accumulation of the streptococci and other microorganism on the tooth surface and help in forming a biofilm to eventually carry out glycolysis of multiple carbohydrates efficiently. [...] compared to the fractions (flavonols, proanthocyanidins, anthocyanins), the crude cranberry extract showed the highest percentage of inhibition of glucosyltransferase activity", le lecteur peut se demander si la canneberge peut avoir un effet antibactérien direct sur la bactérie *Streptococcus mutans*, et si oui, par quel le mécanisme d'action. La suite de cet extrait de la publication présentée au chapitre 1 répond à cette question :"Certain extracts obtained from cranberry juice were shown to be effective in decreasing the congregation and salivary concentration of *Streptococcus mutans* which causes tooth decay (Weiss *et al.*, 1998 and 2002) [...] Its polymeric tannins and in particular, the proanthocyanidins consisting primarily of epicatechin tetramers and pentamers with at least one A-type linkage, seem to be the protecting element against pathogenic

bacteria (Foo *et al.*, 2000b; Heinonen, 2007; Seeram & Heber, 2007). Several mechanisms could explain the effect of the A-type proanthocyanidin in the growth inhibition of bacteria: the destabilization of cytoplasmic membrane, the permeabilization of cell membrane, the inhibition of extracellular microbial enzymes, the direct actions on microbial metabolism and the deprivation of the substrates required for microbial growth (Heinonen, 2007). Proanthocyanidins are known to bind metals and form complexes involving their *o*-diphenol groups, a property often viewed as imparting negative traits with regards to bioavailability of essential mineral micronutrients, especially iron and zinc (House, 1999). Iron depletion can severely limit bacterial growth, and it has been suggested that the ability to bind iron represents another mechanism through which proanthocyanidins inhibit bacterial activity (Dixon et al., 2005)."

Propriété antioxydante des composés phénoliques

Les deux phrases extraites dans l'article publié: "...the relative contribution of cranberry phenolics to plasma and tissue antioxidant function *in vivo* is likely to be relatively minor (Higdon, 2007) [...] Many studies have also shown that they (phenolics) can activate detoxification enzymatic systems (Phase II) and prevent oxidative damage to the DNA [...] semblent contradictoires. Je crois toutefois que la suite de cet extrait de la publication présentée au chapitre 1 peut l'expliquer: "...even with very high flavonoid intakes, plasma and intracellular flavonoid concentrations in humans tend to be 100 to 1000 times lower than concentrations of other antioxidants, such as ascorbate or glutathione. Moreover, most circulating flavonoids are actually flavonoid metabolites, some of which have lower antioxidant activity than the parent flavonoids."

Chapitre 2

Méthode de dosage des phénols totaux par le réactif Folin Ciocalteu

Cette publication aurait du inclure des détails sur la méthode de dosage des phénols totaux par le réactif Folin-Ciocalteu puisque c'est la méthode de dosage des composés phénoliques retenue par l'auteur pour doser les différents extraits phénoliques lors de ses travaux. Voici donc un court survol de cette méthode d'analyse.

La méthode avec le réactif Folin-Ciocalteu a été initialement développée pour analyser les protéines, mais a plus tard servie à mesurer les phénols totaux dans le vin et d'autres produits. Il s'agit d'une réaction colorimétrique d'oxydation du réactif Folin-Ciocalteu fait à base de sel de molybdène et de tungstène. Initialement de couleur jaune intense à pH basique, celui-ci est réduit pour former un complexe de couleur bleu en présence de phénols (Singleton, 1965). Ce réactif a toutefois le désavantage de réagir avec d'autres composés non-phénoliques (vitamine C, Cu^{1+}). La présence d'autres agents réducteurs, tels que les glucides et les lipides peuvent causer des interférences et nuire la quantification des phénols effectuée avec cette méthode. Finalement d'autres produits tels que l'hydroxylamine, la guanidine, les bases azotées, certains groupements thiols, et l'hydroxyacétone peuvent également interférer. C'est malgré tout un test simple et répétable qui a été employé pour déterminer la teneur en phénols des fruits, des bleuets, des baies (Vinson *et al.*, 2001; Wu *et al.*, 2004b; Huang *et al.* 2005; Schmidt *et al.*, 2005; Taruscio *et al.*, 2005).

Chapitre 3

Matériels et méthodes - Préparation des souches bactériennes et détermination des CMI/CMT

Selon la méthode de préparation des souches bactériennes pour la détermination des CMI/CMT, un milieu de croissance très riche et complexe (agar Muller-Hinton) est utilisé. L'usage de l'agar Muller-Hinton est fréquent dans les tests standards d'antibiotiques, car il est mieux adapté pour supporter la croissance de microorganismes fastidieux qui ont des besoins nutritifs plus complexes et permet donc une croissance plus rapide et en plus grande densité. L'utilisation d'un milieu de croissance moins nutritif aurait pu influencer les résultats en ralentissant la croissance bactérienne, et donner des CMI/CMT plus faibles. Cela aurait faussé les résultats en surestimant le pouvoir antibactérien des composés phénoliques de la canneberge. L'aspect plus clair de l'agar Muller-Hilton, comparativement à d'autres milieux de croissance moins riche tel que l'agar de soja tryptique, facilite également la lecture des résultats de croissance (turbidité) détecté par spectrophotométrie. À ce sujet, il y avait une différence de milieu de croissance utilisé pour la préparation des souches bactériennes (bouillon BHI, brain heart infusion) et la détermination des CMI/CMT (agar Muller-Hilton). À l'avenir, il serait judicieux de choisir le même milieu de croissance pour la préparation des souches et la détermination des CMI/CMT.

La durée d'incubation utilisée pour la préparation des souches de bactéries de 1h30 peut paraître courte pour atteindre la phase de croissance exponentielle. Cependant, le temps d'incubation a été validé par des courbes de croissance afin d'atteindre juste le début de la phase de croissance exponentielle (et avoir obtenu seulement un cycle de division cellulaire) et ainsi garantir la même concentration finale de bactéries par puit, soit 10^3 UFC/mL pour la détermination des CMT et 10^6 UFC/mL pour la détermination des CMI.

Matériels et méthodes - Détermination des CMI/CMT et courbes de la cinétique de destruction

Les méthodes d'agitation utilisées pour la détermination des CMI/CMT et pour les courbes de la cinétique de destruction étaient différentes: les microplaques ne sont pas agitées tandis que les jus inoculés sont agités pendant 5h. ÈA ce sujet, il est important de mentionner que bien que différents, ces deux tests sont complémentaires. Les résultats de CMI/CMT permettent d'évaluer rapidement le potentiel antibactérien de plusieurs concentrations différentes (11 concentrations dans ce cas-ci) de composés phénoliques de la canneberge pour déterminer à quel niveau l'effet se fait ressentir. Puisqu'on surveille le niveau de tolérance et limitant la croissance, il faut toutefois travailler dans un milieu qui maintient les bactéries en vie, ce qui limite parfois les concentrations pouvant être testées. L'obtention de courbes de la cinétique de destruction requiert beaucoup plus de manutention, mais permet toutefois de tester l'effet antibactérien d'extrait pur ou de produits non dilués. Cela-dit, il pourrait être pertinent d'agiter le microplaque durant la période d'incubation afin de favoriser une interaction bactéries-phénols semblable à celle obtenue par la méthode de courbes de la cinétique de destruction, toutefois il faudrait utiliser des couvercles hermétiques qui préviendrait la contamination des puits voisin pouvant fausser les résultats.

Discussion des résultats - Comparaison des CMI/CMT et des courbes de la cinétique de destruction

Il semble surprenant que le jus de canneberges qui n'inhibe aucune bactérie (selon les résultats de CMI présentées dans le tableau 3.2), démontre un pouvoir antibactérien contre *S. aureus, L. monocytogenes* et ERV (selon les courbes de la cinétique de destruction dans la figure 3.1). Les CMI/CMT ont étés déterminées en utilisant onze concentrations différentes de jus (20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 et 0.019 % (v/v)), mais nous n'avons pas observée d'inhibition de la croissance à la plus forte concentration de jus testée (20%). C'est pourquoi nous

avons décidé de faire une courbe de la cinétique de destruction pour vérifier le potentiel antibactérien du jus pur à 100%, non dilué et neutralisé à pH 7. À cette concentration, les courbes ont démontré que le jus pouvait détruire *S. aureus, L. monocytogenes* et ERV.

Conclusion

L'extrait suivant de la conclusion: "In general *L. monocytogenes* [...] were more resistant to the antibacterial activity of the cranberry extracts adjusted at pH 7 [...] When exposed to pure neutralized cranberry juice, *L. monocytogenes* [...] were completely inactivated", souligne la divergence des résultats obtenus avec les deux méthodes d'analyse. Toutefois il faut noter que la détermination des CMI/CMT a été effectuée en utilisant différentes concentrations d'extraits (40, 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 et 0.039 mg/ml) et de jus (20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 et 0.019 % (v/v)), tandis que les courbes ont étés obtenues avec un jus pur à 100%. Il est donc possible d'affirmer que les concentrations d'extraits et de jus testés étaient trop faibles pour voir un effet antibactérien sur cette bactérie. De plus, il se peut que *L. monocytogenes* soit plus sensible à l'effet antibactérien provenant de la synergie de tous composés phénoliques de la canneberge, qu'à celui des composés isolés (polaires, apolaires et anthocyanes).

Chapitre 4

Matériels et méthodes - Interaction du solvant dans la réaction Folin-Ciocalteu

L'effet possible des mélanges de solvant sur la réponse de la réaction Folin-Ciocalteu n'a pas été évalué dans le cadre d'expériences préliminaires. Toutefois, afin d'éviter une interférence possible dans les résultats obtenus, trois courbes de calibration avec l'acide gallique ont été utilisée, chacune préparée avec l'un des trois mélanges de solvant, soit le mélange d'eau/méthanol (85:15, v/v), d'acétone/méthanol/eau (40:40:20, v/v) et de méthanol/eau/acide acétique (85:14.5:0.5, v/v/v). De cette manière il a été possible de prendre en considération les interférences qui auraient pu biaiser les résultats de phénols totaux obtenus.

Matériels et méthodes - Température d'évaporation

Étant donné la nature coopérative du projet de recherche avec l'industrie, et pour des raisons de confidentialité il a été jugé nécessaire de ne pas entrer dans les détails de l'évolution des températures lors de l'évaporation du jus de canneberges. Une température d'évaporation aléatoire de 100°C a donc été donné afin d'illustrer le fait que cette étape peut-être considérée comme une pasteurisation bien que dans les faits ce n'est pas exactement cette température qui est utilisée par l'industriel. Une température aussi élevée peut contribuer à la perte de phénols observée.

Discussion des résultats – Analyse des composés bioactifs

Il aurait été approprié d'inclure dans cette publication des résultats d'analyse par HPLC-MS des composés phénoliques des différents extraits effectuée au moins une fois pour s'assurer que chaque extrait soit bien des composés phénoliques différents et qu'il n'y a pas duplication du dosage de certains phénols. Une méthode d'analyse HPLC-DAD a été développée et validée à l'INRS Institut Armand-Frappier. Elle a servie à comparer les extraits entre eux et s'assurer qu'ils avaient bien un profile phénolique distinct. Les résultats de ces analyses n'ont toutefois pas été publiés. De

plus, une méthode d'analyse LC-DAD-MS a été développée et validée au laboratoire du partenaire industriel du projet, Canneberges Atoka Inc., dans le but de surveiller les changements de profil des composés phénoliques prédominants de la canneberge. Cette analyse devait servir à déterminer quelles étapes du processus de jus et les mécanismes impliqués dans la dégradation des composés phénoliques de canneberge et de la perte de leurs propriétés. Malheureusement, cet objectif n'a pu être réalisé dans le cadre de ce projet, car l'étudiant a quitté la compagnie pour des raisons personnelles.

Discussion des résultats – Calcul du bilan massique

Un bilan de masse permettant de connaître réellement les pertes dues au procédé et possiblement de cibler les étapes les plus critiques n'a pas été effectué lors du projet. Un bilan massique est un calcul qui cherche à comparer la masse des produits initiaux à celle des produits finaux afin de développer une équation prenant en compte les entrées et les ajouts associées au procédé de production, ainsi que les sorties et les rejets. Ce calcul permet donc de normaliser tous les échantillons afin de les comparer les uns aux autres sur une base équivalente et de déterminer les effets de procédés testés. Pour les échantillons de canneberges solides et liquides, les concentrations en phénols obtenues sont rapportées en poids de matières sèches. Elles auraient pu être rapportées en poids de canneberges initial en les multipliant par le ratio : masse volumique$_{\text{produit finis}}$/masse volumique$_{\text{canneberge initial}}$. Cette conversion aurait permis de mieux prendre en considération les effets de concentration et de dilution sur les valeurs en phénols totaux obtenus.

Discussion des résultats – Pouvoir antibactérien de l'acide chlorogénique

L'évolution de la teneur en acide chlorogénique aurait du être analysé tout au long du procédé afin de déterminer sa contribution au pouvoir antibactérien de la canneberge. C'était d'ailleurs un des objectifs initiaux qui aurait été atteint en utilisant la méthode d'analyse LC-DAD-MS développée et

validée au laboratoire du partenaire industriel du projet, Canneberges Atoka Inc. L'acide chlorogénique est un composé phénolique important tant pour sa contribution à la teneur en phénols totaux des échantillons de canneberges étudiés que pour rôle dans des réactions de polymérisation impliquant les enzymes peroxydase et polyphénol oxydase. La polyphénol oxydase et la peroxydase ont un effet indirect sur la teneur anthocyanes des fruits (Worlstad *et al.*, 2005). Leur activité, catalysée par la présence d'oxygène ou d'acide chlorogénique, semblerait favoriser la formation de composés polymérisés ayant une structure moléculaire semblable aux tanins du vin qui sont astringent et ont un poids moléculaire élevé (Haslam, 1998).

Discussion des résultats –Autres méthodes d'analyse de la propriété antibactérienne

Des tests mesurant des effets autres que la toxicité bactérienne (effet sur la mobilité ou l'adhésion au tissu hôte) ou des tests commerciaux qui mesurent l'effet toxique plus spécifiquement (perméabilité membranaire ou stress métabolique) n'ont pas été utilisés dans ce projet. L'un des objectifs de ce projet était de comparer le pouvoir antibactérien des différentes classes de composés phénoliques que la canneberge et voir l'évolution de leur potentiel antibactérien tout au long du procédé de jus. Pour ce faire nous avons eu recours à des méthodes d'analyse quantitative (détermination CMI/CMT, courbe de la cinétique de destruction). Ces tests pourraient servir à mieux caractériser les effets antibactériens observés avec les extraits de canneberges riches en composés phénoliques hydrophiles et en anthocyanes, car ce sont eux qui ont démontré le plus grand potentiel antibactérien. En parallèle à ces tests, une caractérisation du profile de composés phénoliques de ces extrait pourrait également être faite par HPLC-MS.

Chapitre 5

Matériels et méthodes - pH de la réaction de Fenton pour l'analyse de l'inhibition de la peroxydation des lipides

Les analyses de la capacité de peroxydation des lipides des jus et des extraits de canneberges ont été effectuées avec des échantillons ajustés à pH 2,5 (pH naturel de la canneberge) et à pH 7,0 (pH physiologique). La méthode utilisée est basée sur la réaction de Fenton au cours de laquelle le chlorure de fer s'auto-oxyde en milieu aqueux pour former des radicaux $O_2^{\bullet-}$ qui à leur tour vont former du peroxyde qui va réagir avec le Fe^{2+} et former des radicaux $^{\bullet}OH$ en présence d'acide ascorbique (le catalyseur). C'est la présence des radicaux $^{\bullet}OH$ qui va entrainer l'oxydation des liposomes. L'effet du pH des échantillons sur le milieu de la réaction doit été vérifié afin de s'assurer d'avoir un milieu acide (pH habituellement < 3) permettant de générer une quantité suffisante d'oxydant (Fe^{2+}). Sinon, à pH neutre, le Fe^{3+} tend à précipiter et les radicaux $^{\bullet}OH$ ne sont pas génère (Lee and Sedlak 2009).

La méthode utilisée dans cette étude est basée sur la méthode validée par Caillet *et al.* (2007) et qui fait référence à une méthode d'analyse validée par cinétique par Kwon et Lee (2004) dans le but déterminer la concentration de radicaux produits. Cette méthode est basée sur une réduction de Fe^{3+}-ethylenediamine d'acétate (EDTA) en Fe^{2+}-EDTA par les radicaux $HO_2^{\bullet}$ et $O_2^{\bullet-}$ et sur la réaction de Fenton du H_2O_2 et de Fe^{3+}-EDTA produisant des radicaux $^{\bullet}OH$. Les concentrations de radicaux $HO_2^{\bullet}$ et $O_2^{\bullet-}$ et la limite de détection les plus basses ont étés obtenues à pH 5, et ces valeurs augmentaient graduellement au-delà et en-dessous de ce pH. Cette étude démontrait donc la possibilité d'obtenir une bonne réaction de Fenton à différents pH en ayant recours à l'EDTA comme agent de complexation au fer. Toutefois, par mégarde, la méthode décrite dans le chapitre 5 n'incluait pas l'EDTA. La correction nécessaire a donc été apportée.

Question philosophique

Si les extraits de canneberges peuvent protéger du stress oxydatif, pourquoi sont-ils « mauvais » pour les bactéries? L'oxydation est une réaction caractérisée par la perte d'un ou plusieurs électrons par un atome ou une molécule. Durant le processus d'oxydation, la substance oxydante va accepter des électrons et ainsi causer l'oxydation d'un autre composé (Prior et Cao, 1999; Cancalon, 2007). La présence d'oxygène, de peroxydes lipidiques et des métaux de transition (Cu^{2+}, Fe^{2+}) dans l'environnement, ainsi que l'activité de certaines enzymes (lipoxygénase, peroxydases) dans les organismes vivant, contribuent à la formation, en cascade, de radicaux libres qui sont des molécules extrêmement instables. Ces radicaux libres vont aussi interagir avec d'autres molécules ou radicaux libres pour se stabiliser (Cancalon, 2007). Les mécanismes responsables du pouvoir antioxydant des composés phénoliques de la canneberge sont nombreux. Parmi ceux-ci, on note l'interruption dans la cascade de réactions radicalaires pouvant mener à la peroxydation des lipides, l'inhibition de la formation de radicaux par la chélation des métaux de transition (Fe^{3+}, Cu^{2+}), la neutralisation des substances réactives oxygénées et l'inhibition de l'activité enzymatique associée à la peroxydation (Frankel et Meyer, 2000; Santos-Buelga, 2000; Huang *et al.*, 2005).

À des concentrations élevées, les composés phénoliques, comme ceux présents dans la canneberge et ses extraits, ont également des propriétés cytotoxiques qui pourraient contribuer à l'effet antibactérien rapporté dans cette étude et dans la littérature. La structure moléculaire de ces composés ferait en sorte qu'ils peuvent entraîner une déstabilisation de la membrane cytoplasmique, une perméabilisation de la membrane cellulaire, une inhibition des enzymes extracellulaires, et même avoir des actions directes sur le métabolisme cellulaire et priver les cellules de substrats (fer et zinc) nécessaires à leur croissance (House, 1999 ; Dixon et al., 2005; Heinonen, 2007).