

**Pharmacokinetics of Cyanidin and Anti-Influenza Phytonutrients in an Elder Berry Extract Determined by LC-MS and DART TOF-MS**

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## Abstract

Pharmacokinetic analyses were conducted on flavonoid phytonutrients in a Standardized Elder Berry Extract (SEBX) to determine bioavailability and uptake kinetics, and to compare LC-MS and DART TOF-MS for pharmacokinetic analyses. In the first study, serum and urine levels of Cyanidin from an SEBX lozenge were monitored by LC-MS. In the second study, DART TOF-MS was used to compare the serum pharmacokinetics and bioavailability of Cyanidin and other flavonoids in SEBX when delivered as a slow-dissolve lozenge and as a drink. When the SEBX lozenge was consumed, serum concentrations of Cyanidin were between 3.1 (LC-MS) and 5.4 nmol L<sup>-1</sup> (DART TOF-MS), equivalent to 2.7 and 4.7% bioavailability (BA), respectively. Averionol (methylated flavonoid) reached a C<sub>max</sub> of 23 nmol L<sup>-1</sup> (10.5% BA), while Tristenonol (esterified flavonoid) and Istrocyanidin (A-type proanthocyanidin) reached C<sub>max</sub> values of 3.9 nmol L<sup>-1</sup> (8.6% BA) and 7.5 nmol L<sup>-1</sup> (19.7% BA), respectively. When the SEBX was consumed as a drink, the bioavailability of Cyanidin decreased 20-fold (0.2% BA), while Averionol and Istrocyanidin decreased 2-fold (4.6 and 10.8% BA, respectively) compared to the lozenge ingestion, indicating primary uptake in the oral cavity. The bioavailability of Tristenonol increased by ca. 2-fold (18.8% BA) when the SEBX drink was consumed compared to the lozenge indicating the small intestine as the primary uptake site.

Key Words: Elder berry, DART TOF-MS, Cyanidin, Influenza

## Introduction

Though fairly routine for pharmaceuticals, pharmacokinetic investigations on the complex chemicals found in botanicals present significant analytical challenges. These challenges include the lack of availability of standards and/or biomarkers for many molecules of interest, the detection limitations of the analytical methods, and the chemical complexity of a botanical extract requiring multiple sample preparation steps. Typically liquid chromatography (LC) alone, or combined with mass spectrometry (MS), have been used to detect and quantify chemicals of interest in pharmacokinetic studies. These technologies allow for the separation of small molecules in body fluids such as serum or urine as well as their identification and quantification as long as authentic chemical standards are available. New mass spectrometric capabilities like DART (Direct Analysis in Real Time) time-of-flight mass spectrometry (TOF-MS) can overcome these limitations, and was explored here to examine the pharmacokinetics of Cyanidin and key anti-influenza flavonoid chemicals in a standardized elder berry fruit extract (SEBX).

Flavonoids are a class of phytonutrients that are particularly abundant in a diversity of fruits and vegetables (Cook and Samman 1996). These chemicals have a broad range of health and nutritional functions/activities that include anti-oxidant, immune enhancement, anti-inflammation and protection from certain infectious diseases (Kowalczyk *et al.* 2003). This major class of phenolic compounds includes flavonols, flavononols, proanthocyanidins and cyanidins which are sometimes called anthocyanins. The bioavailability of these flavonoid phytonutrients has been questioned (Ross and Kasum 2002; Tapiero *et al.* 2002). Flavonoids may occur naturally in glycosylated or non-glycosylated (aglycone) forms, esterified, and/or methylated, and studies in humans and animals have shown that these modified forms are the most bioavailable (Cao and Prior 1999; Felgines *et al.* 2002; Frank *et al.* 2002; Mulleder *et al.* 2002; El Mohsen *et al.* 2006) Previous investigations have shown that methylated flavonoids are not metabolized (glucuronidated and/or sulfated) by the kidneys and are more bioavailable than their unmethylated counterparts for this reason (Wen and Walle 2006). Proanthocyanidins can be taken up in the small intestine in humans, but are degraded after 48 hours into low-molecular-weight phenolic acids (Deprez *et al.* 2000; Baba *et al.* 2002).

Milbury *et al.* (2002) showed that glycosylated cyanidins appear in serum within 20 minutes after ingestion, reaching levels of  $96 \text{ nmol L}^{-1}$  within about 1 hour, and decline slowly over 6 hours. Significant levels of similar compounds were also seen in urine 4 hours after ingestion (Wu *et al.* 2002; Bitsch *et al.* 2004). However, cyanidins were not detectable in the human serum using the LC-MS methods described by Wu *et al.*, 2002. Matsumoto *et al.* (2001) reported that specific anthocyanidins (delivered as a liquid) were present in plasma within an hour after intake and reached maximum levels in urine between 1.25 and 1.75 hours. Studies on anthocyanidins in red grape juice and wine by Bitsch *et al.* (2004) revealed that these compounds reached maximum serum concentrations of 100 and  $43 \text{ nmol L}^{-1}$  at 30 and 60 minutes respectively, after consumption of 400 mL of each liquid, representing 0.2% bioavailability. These authors

and others (Cao and Prior 1999; Wu *et al.* 2002; Bitsch *et al.* 2004), have concluded that anthocyanidins have low bioavailability. Therefore, we sought to determine whether an extract rich in esterified, methylated and glycosylated forms of flavonoids would show enhanced bioavailability, and if the primary site of uptake would influence the bioavailability of these types of flavonoid phytonutrients.

The SEBX used here was shown to be highly effective in blocking Human Influenza A virus (H1N1) infection *in vitro* and this activity was attributed to the presence of three novel flavonoids, Averionol, Tristenonol, and Istrocyanidin (Roschek Jr. *et al.* 2008). These three compounds bind to H1N1 virions and mask their ability to infect target cells. We utilized DART TOF-MS technology to monitor the bioavailability of these novel flavonoids in serum without the need for specific reference standards because DART TOF-MS analysis yields the exact mass of each chemical species detected and allows for accurate identification (Cody *et al.* 2005), and because small molecules can be quantified directly in serum without sample manipulation (Cody *et al.* 2005).

The bioavailability of anthocyanidins are typically analyzed by LC-MS of urine and/or serum samples using only Cyanidin (glycoside and aglycone forms) as a reference standard. DART TOF-MS provides the ability to quantify multiple chemicals in a given sample at the same time with only a single internal standard for calibration (Cody *et al.* 2005). In the present study, we also sought to examine the uptake kinetics and bioavailability of these chemicals when the SEBX was administered as a slow-dissolve lozenge (SEBX lozenge), where primary uptake would be in the oral cavity and when it was administered as a drink (SEBX drink), where the primary uptake would be in the lower GI tract (stomach and small intestine).

## Materials and Methods

**Elder berry fruit extract:** A standardized super critical CO<sub>2</sub> and affinity adsorbent extract of elder berry (*Sambucus nigra* L.) fruits (SEBX) was utilized. The SEBX contained over 1000 chemicals and was enriched in phenolic acids, polyphenolics, and a broad diversity of other flavonoids (Roschek *et al.*, in review). The SEBX was formulated into a 175 mg slow-dissolve lozenge.

**Study design:** The study was designed in two parts. In the first part, six healthy consenting adults ranging in age from 23 to 50 were hospitalized 24 hr prior to the initiation of the study and provided a diet free of flavonoids. A certified individual collected blood samples at several time intervals between 0 and 480 minutes after SEBX lozenge ingestion. Immediately after the time zero blood samples were collected, a single 175-mg dose of the SEBX lozenge was administered and allowed to dissolve slowly in the oral cavity of the subjects. Blood samples were handled with approved protocols and precautions, centrifuged to remove cells and the serum fraction was collected and frozen. Blood was not treated with heparin to avoid any analytical interference. Blood samples were processed for LC-MS analysis as described below. Urine samples were collected from the same six subjects on a time course (0 to 720 minutes) and processed for LC-MS analyses as described below.

In the second part of this study, a single healthy adult male (age 50) was recruited. A certified individual collected blood samples over a prescribed time course. Blood samples were handled with approved protocols and precautions. The subject fasted for 24 hours prior to the initiation of blood collection, and received only water and foods absent in flavonoids during the course of the study. Blood samples were taken at several time intervals between 0 and 360 minutes. Immediately after the time-zero blood samples was collected, a single 175-mg dose of the SEBX lozenge was administered and allowed to dissolve slowly in the oral cavity of the subject. A month later, this procedure was repeated except that, immediately after the time-zero blood sample was collected, a 350-mg SEBX dose (2 lozenges) that was dissolved in 8 oz of water was administered in the form of a drink, whereby the bulk of the EB extract was delivered directly to the stomach and lower GI tract. Blood samples were collected and processed as above until analysis.

**LC-MS calibration:** A neat methanol stock solution of Cyanidin-3-glucoside (Cyanidin) was prepared. This stock solution was diluted with DI water to yield a 100 ng mL<sup>-1</sup> standard solution of Cyanidin. The calibration curves for blood and urine Cyanidin quantification were developed using serial dilutions of the stock solutions over the range of 10-200 ng mL<sup>-1</sup>. The calibration solutions were vortexed, processed and analyzed according to the methods for preparing the samples described below.

**LC-MS sample preparation:** Urine samples were prepared for LC-MS analysis according to Milbury *et al.* (2002). Briefly, the samples were collected, concentrated, and 0.44 M trifluoroacetic acid (TFA) was added to the samples. The samples were stored at -80°C until LC-MS analysis. Blood samples were handled in a similar manner except that the serum was collected after centrifugation and treated with 0.44 M TFA and stored at -80°C until LC-MS analysis.

**LC-MS analysis:** A Waters UPLC/Quattro Premier LC-MS system equipped with an Acquity<sup>®</sup> UPLC BEH C<sub>18</sub> (2.1x50 mm, 1.7µm) column (Waters, Milford, MA) was used for HPLC analysis. The column temperature was held to 35°C and the mobile phase consisted of solvent A (0.5% [v/v] formic acid/water) and B (0.5% [v/v] formic acid/acetonitrile). The following solution gradient was applied: t = 0 min, 5% B; t = 5 min, 90% B; t = 5.5 min, 90% B; t = 5.6 min, 5% B; t = 5.7 min, 5% B. The flow rate was 0.4 mL min<sup>-1</sup> and the injection volume was 10 µL. Positive-ion electrospray ionization was used for mass spectrometric analysis. The capillary voltage was set to 3.44 kV, the cone voltage was set to 25 V, RF lens was set to 0.2 V, the ion source temperature was 100°C, the dry gas temperature was 450°C, the cone hole blowing airflow was 100 L h<sup>-1</sup>, the dry gas flow rate was 770 L h<sup>-1</sup>, the dwell time was 0.2 sec, and the collision energy was 22 eV. The ions monitored were  $m/z [M]^+ = 449$  (Cyanidin glucoside) and  $m/z [M]^+ = 287$  (Cyanidin aglycone).

**DART TOF-MS calibration:** A calibration curve was developed using demethoxycurcumin (DMC;  $m/z [M+H]^+ = 339.1$ ) as an internal standard (>97% purity). The internal standard was chosen because it has similar physical properties to the

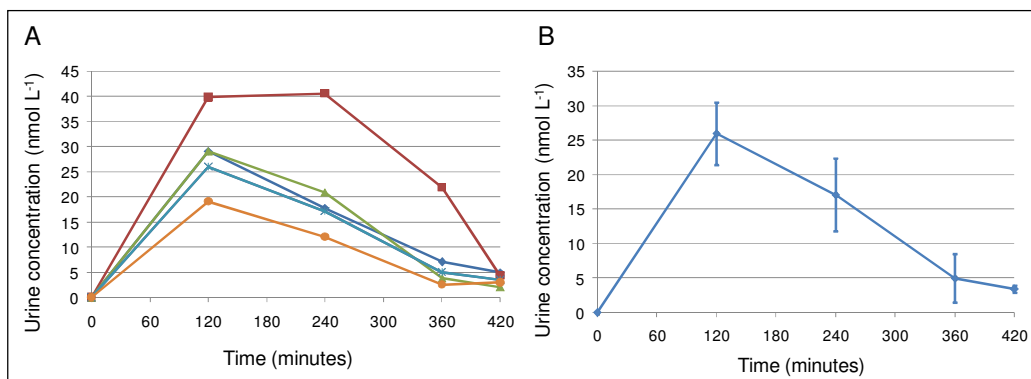
SEBX anti-influenza flavonoids. A 5  $\mu\text{L}$  aliquot of the internal standard, DMC, in 100% (v/v) ethanol (USP) ( $200\ \mu\text{g mL}^{-1}$ ) was analyzed in the presence of varying concentrations of a secondary standard, methyl linoleate ( $m/z\ [M+H]^+ = 295.2$ ; >97% purity). The calibration was developed comparing the DART TOF-MS intensities of DMC and methyl linoleate. The DART TOF-MS intensities of the SEBX anti-influenza chemicals (Averionol,  $m/z\ [M=H]^+ = 359.3$ ; Tristenonol,  $m/z\ [M=H]^+ = 479.2$ , and Istrocyanidin,  $m/z\ [M=H]^+ = 607.4$ ), as measured in the serum samples spiked with a known concentration of DMC as an internal standard, were converted to molar concentrations using established methods for development of quantitative analysis of drug metabolites in urine using DART TOF-MS (JEOL, 2007).

**DART TOF-MS sample preparation:** Serum samples were stored frozen until analysis. The serum was extracted with an equal volume of neat ethanol (USP) to minimize background of proteins, peptides, and polysaccharides present in serum. The ethanol extract was centrifuged for 10 minutes at  $4^\circ\text{C}$ , the supernatant was removed, concentrated to 200  $\mu\text{L}$  volume, and 50  $\mu\text{L}$  of an internal standard was added. This material was used for DART TOF-MS analyses.

**DART TOF-MS analysis:** A JEOL DART™ AccuTOF-mass spectrometer (JMS-T100LC; Jeol USA, Peabody, MA) was used for analysis of the serum samples and was executed in positive ion mode ( $[M+H]^+$ ). The needle voltage was set to 3500 V, heating element to  $250^\circ\text{C}$ , electrode 1 to 150 V, electrode 2 to 250 V, and helium gas flow to  $2.42\ \text{L min}^{-1}$ . For the mass spectrometer, the following settings were used: Orifice 1 set to 10 V, ring lens voltage set to 5 V, and orifice 2 set to 5 V. The peak voltage was set to 1000 V in order to give peak resolution beginning at 100  $m/z$ . The microchannel plate detector voltage was set at 2500 V. Mass calibrations were performed internally with each sample using a 10% (w/v) solution of PEG 600 (Ultra Chemical, North Kingston, RI) that provided mass markers throughout the required mass range 100-1000  $m/z$ . Calibration tolerances were held to 10 mmu. Samples (10  $\mu\text{L}$ ) were pipetted onto the closed end of a borosilicate glass melting point capillary tube and introduced into the DART helium plasma by holding the tube in the He plasma stream until the entire sample was consumed. The next sample was introduced when the Total Ion Chromatograph returned to baseline levels, which occurred within seconds of sample consumption.

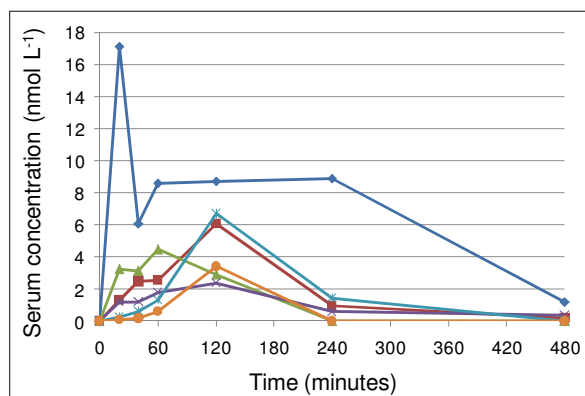
## Results

**Urine levels of Cyanidin determined by LC-MS from SEBX lozenge (n=6):** The concentration of urine Cyanidin monitored as the glycoside and aglycone reached  $27.5\ \text{nmol L}^{-1}$  within 2 hours of consumption. This urine concentration is equivalent to 34% wt/wt (89.1  $\mu\text{g}$ ) of the consumed Cyanidin from the 175-mg dose of the EB extract. The urine Cyanidin followed first-order kinetics and virtually all of the detectable Cyanidin was excreted within 7 hours of ingestion of the SEBX lozenge (Figure 1).



**Figure 1. Cyanidin urine concentrations determined by LC-MS from a 175-mg dose of SEBX lozenge.** (A) Urine concentrations of Cyanidin are plotted as a function of time over 7 hours (420 minutes) for each subject indicated in different colors. (B) The median urine concentration of Cyanidin is plotted as a function of time over 7 hours (420 minutes) for each subject. Error bars represent  $\pm 1$  S.D; n = 6.

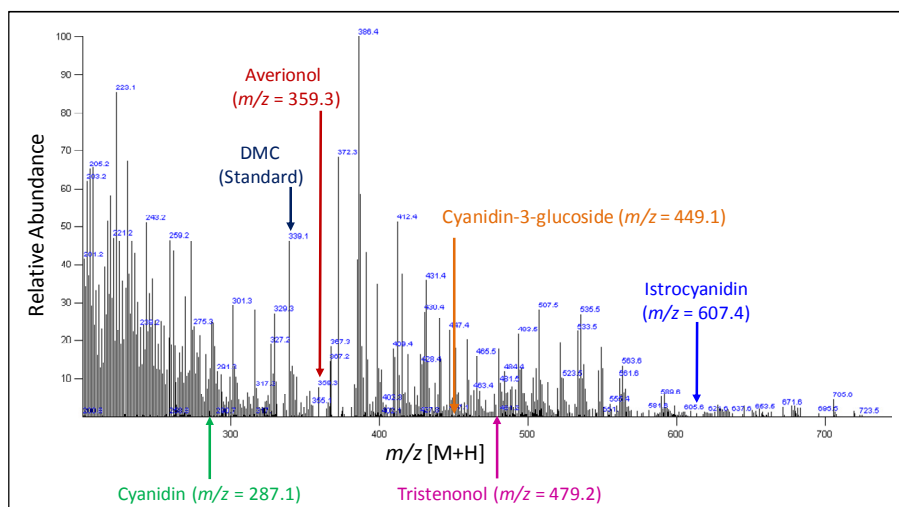
**Serum levels of Cyanidin determined by LC-MS from SEBX lozenge:** The serum levels of Cyanidin determined from 6 subjects who received a single 175-mg SEBX lozenge are shown in Figure 2. For all subjects, Cyanidin was first detected between 20 and 40 minutes after ingestion. Serum levels, which varied among individuals, were maintained among the subjects through 240 minutes (see Table 1).



**Figure 2. Cyanidin serum concentrations determined by LC-MS from a 175-mg dose SEBX lozenge.** Serum concentrations of Cyanidin are plotted as a function of time over 8 hours (480 minutes) for each subject (indicated in different colors); n = 6.

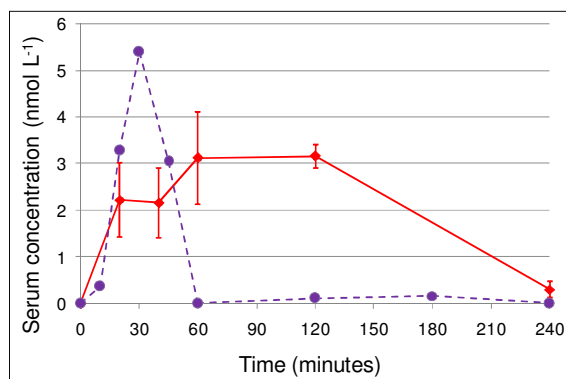
**Comparison of DART TOF-MS and LC-MS quantification of Cyanidin serum levels:**

An example of a DART mass spectrum of a serum sample is provided in Figure 3. The serum sample from the 45-minute post ingestion of an SEBX lozenge is rich in a diversity of chemicals. The internal standard demethoxycurcumin (DMC) is clearly detected at  $m/z$   $[M+H]^+ = 339.1$  as is Cyanidin ( $m/z$   $[M+H]^+ = 287.1$ ), Averionol ( $m/z$   $[M+H]^+ = 359.3$ ), and Istrocyanidin ( $m/z$   $[M+H]^+ = 607.4$ ) (see Table 1). Tristenonol ( $m/z$   $[M+H]^+ = 479.2$ ) and Cyanidin-3-glucoside ( $m/z$   $[M+H]^+ = 449.1$ ) were not detected at this time point, however the masses for these compounds are indicated (see Figure 3).



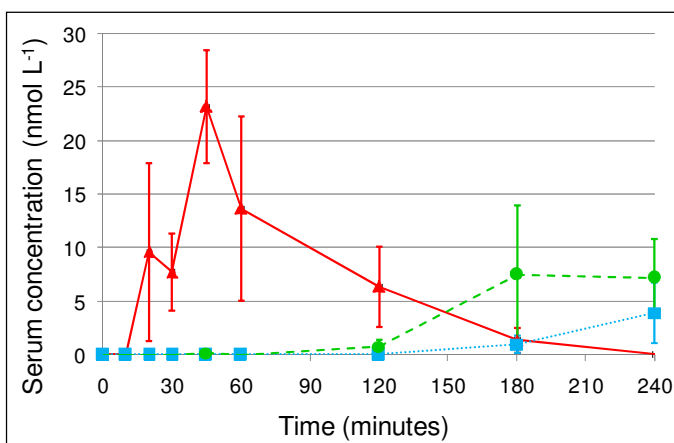
**Figure 3. DART TOF-MS analysis of a 45-minute post-ingestion serum sample when SEBX was taken as a lozenge.** Demethoxycurcumin (DMC;  $m/z [M+H]^+ = 339.1$ , dark blue arrow) was used as an internal standard in the MS analysis. The presence of Cyanidin ( $m/z [M+H]^+ = 287.1$ , green arrow), Averionol ( $m/z [M+H]^+ = 359.3$ , red arrow) and Istrocyanidin ( $m/z [M+H]^+ = 607.4$ , blue arrow) is shown while Tristenonol ( $m/z [M+H]^+ = 479.2$ , violet arrow) and Cyanidin-3-glucoside ( $m/z [M+H]^+ = 449.1$ ; orange arrow) were not detected at this time point.

Serum Cyanidin levels reached  $3.0 \text{ nmol L}^{-1}$  within 60 minutes of consumption of the SEBX lozenge based on the average data for the 6 subjects (Figure 4). Cyanidin was detected within 20 minutes of consumption of the SEBX lozenge and continued to be observed in the serum up to 4 hours post ingestion. Using DART TOF-MS, the concentration of serum Cyanidin reached  $5.4 \text{ nmol L}^{-1}$  within 30 minutes of consumption from the single individual in this part of the study (see Table 1). Cyanidin was detected as early as 10 minutes post-consumption and continued to be detected by DART TOF-MS up to 6 hours post-consumption (data not shown), the last time-point analyzed.



**Figure 4. Serum Cyanidin levels averaged from 6 subjects determined by LC-MS and Cyanidin levels from a single subject determined by DART TOF-MS after delivery of a 175-mg dose SEBX lozenge.** The serum concentrations of Cyanidin ( $\text{nmol L}^{-1}$ ) determined using LC-MS are shown as red diamonds ( $- \blacklozenge -$ ) while those determined using DART TOF-MS are shown as purple circles ( $- \bullet -$ ). Serum concentrations of Cyanidin are plotted as a function of time over 4 hours (240 minutes). Error bars represent  $\pm 1$  S.D.

**Serum levels of anti-influenza flavonoids delivered in the SEBX lozenge form:** The serum concentration of Averionol, Tristenonol, and Istrocyanidin were monitored using DART TOF-MS (Figure 5). Averionol, the most abundant of the three anti-influenza chemicals in the SEBX, was detected in serum within 20 minutes of administering the SEBX lozenge. Averionol reached a maximum serum concentration of 23.2 nmol L<sup>-1</sup> at 45 minutes post-consumption, and was not detected in serum after 4 hours. Tristenonol was detected in serum 3 hours after administering the 175-mg dose and reached a concentration maximum of 3.9 nmol L<sup>-1</sup> at 4 hours post-consumption. Tristenonol was detected in serum up to 6 hours post-administration of the single dose which was the last time-point collected. Istrocyanidin reached detectable levels in serum as early as 45 minutes post-administration of the SEBX, and reached a maximum serum concentration of 7.8 nmol L<sup>-1</sup> at 6 hours post-consumption (see Table 1).



**Figure 5. Serum concentrations (nmol L<sup>-1</sup>) of Averionol (—▲—), Tristenonol (—■—) and Istrocyanidin (—●—) determined by DART TOF-MS from a 175-mg dose SEBX lozenge.** The blood concentration of Averionol, Tristenonol, and Istrocyanidin were determined by DART TOF-MS and plotted as a function of time over 6 hours (240 minutes). Each data point represents the median value of a single subject sampled three times. Error bars represent  $\pm 1$  S.D.

**Table 1. Serum concentrations of Cyanidin as determined by LC-MS and DART TOF-MS, and Averionol, Tristenonol, and Istrocyanidin serum concentrations (nmol L<sup>-1</sup>) determined by DART TOF-MS from a 175-mg dose SEBX lozenge. Data is presented as the average concentration (n = 6 for LC-MS analysis; n = 3 for DART TOF-MS analysis)**

Compound	Serum concentration (nmol L <sup>-1</sup> ) at each timepoint (min)									
	0	10	20	30	45	60	120	180	240	
Cyanidin <sup>1</sup>	0.0	ND	2.2	ND	2.2	3.1	3.1	ND	0.3	
Cyanidin <sup>2</sup>	0.0	0.4	3.3	5.4	3.0	0.0	0.1	0.2	0.0	
Averionol <sup>2</sup>	0.0	0.0	9.6	7.7	23.2	13.7	6.3	1.3	0.0	
Tristenonol <sup>2</sup>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	3.9	
Istrocyanidin <sup>2</sup>	0.0	0.0	0.0	0.0	0.1	0.0	0.7	7.5	7.2	

<sup>1</sup>Determined by LC-MS; <sup>2</sup>Determined by DART TOF-MS

**Pharmacokinetic analysis of the SEBX flavonoids delivered in lozenge form:** The primary pharmacokinetic parameters for the 175-mg dose SEBX lozenge are

summarized in Table 2. Cyanidin reached a maximum urine concentration at 120 minutes post-consumption and reached a maximum serum concentration at 60 (LC-MS) and 30 (DART TOF-MS) minutes post consumption of a single 175-mg SEBX dose. The elimination rate constant  $k$ , defined as the rate at which the compound being analyzed is removed from urine or serum, can be determined. The elimination rate constant was similar for Cyanidin regardless of the analytical method used (Table 2). Based upon the first-order elimination kinetics of Cyanidin (as well as the other flavonoids monitored) the elimination half-life ( $t_{1/2}$ ; the time required for the urine or serum concentration to be reduced by 50%) was determined for Cyanidin, Averionol, Tristenonol, and Istrocyandin (Table 2). Again, the elimination half-life of Cyanidin in serum is similar when the concentration is determined by DART -MS or traditional LC-MS.

**Table 2. The primary pharmacokinetic parameters of Cyanidin and the SEBX anti-influenza compounds after ingestion of a single 175-mg SEBX lozenge.**

Compound	$C_{max}$ (nmol L <sup>-1</sup> )	$T_{max}$ (min)	$k$ (nmol L <sup>-1</sup> min <sup>-1</sup> )	$t_{1/2}$ (min)
LC-MS:				
Cyanidin (urine)	27.5 ± 4.5	120	0.044	16
Cyanidin (serum)	3.1 ± 1.0	60	0.024	29
DART-MS (serum):				
Cyanidin	5.4 ± 2.8	30	0.035	20
Averionol	23.2 ± 5.3	45	0.162	4.3
Tristenonol	3.9 ± 0.8	240	0.026	26
Istroycanidin	7.8 ± 3.6	>360	(0.027)*	(26)*

Averionol reached a  $C_{max}$  of 23.2 nmol L<sup>-1</sup> at 45 minutes post consumption, and was the most rapidly eliminated (0.162 nmol L<sup>-1</sup> min<sup>-1</sup>) from the blood yielding a  $t_{1/2}$  of 4.3 minutes. Tristenonol reached a  $C_{max}$  of 3.9 nmol L<sup>-1</sup> at 240 minutes post-consumption and was eliminated from the blood at a rate of 0.026 nmol L<sup>-1</sup> min<sup>-1</sup>. This is similar to the elimination of Istrocyandin which achieved a  $C_{max}$  of 7.8 nmol L<sup>-1</sup> at >360 minutes after consumption of the SEBX lozenge (Table 2). Because the  $C_{max}$  of Istrocyandin was achieved after the final time point collected, the pharmacokinetic parameters determined in Table 2 for Istrocyandin were based on a single measurement, whereas for Cyanidin, Averionol, and Tristenonol parameters were determined on the average results for all samples. The elimination rate constant ( $k$ ) and half-life ( $t_{1/2}$ ) for Istrocyandin was also determined from a single sample.

**Serum levels of anti-influenza flavonoids delivered as the SEBX drink:** The serum concentration of Averionol, Tristenonol, and Istrocyandin were monitored using DART TOF-MS from one subject who ingested, as a drink, a 350-mg dose of the SEBX (Table 3). The SEBX drink dose was double that of the lozenge because previous studies (Milbury *et al.* 2002; Bitsch *et al.* 2004) showed very low serum uptake of cyanidins when consumed as a drink. Cyanidin was only just detectable in serum after 30 minutes (0.5 nmol L<sup>-1</sup>) and was not detectable after 60 minutes. Averionol, the most abundant of the three anti-influenza compounds in the 350-mg dose, was detected in serum within 30 minutes of administering the drink, increased to a level of 20.3 nmol L<sup>-1</sup> by 60 minutes and was not detectable 180 minutes post-ingestion (Table 3). Tristenonol was

first detected at 40 minutes after drinking the 350-mg SEBX dose, peaked at 60 minutes (17.0 nmol L<sup>-1</sup>) and persisted through at least 180 minutes (4.0 nmol L<sup>-1</sup>) post-administration, which was the last time-point collected. In contrast, Istrocyandin was not detected in serum until 180 minutes (the last time-point collected) at a concentration of 8.2 nmol L<sup>-1</sup> (Table 3).

**Table 3. Average (n = 3) serum concentrations of Cyanidin, Averionol, Tristenonol, and Istrocyandin at each time point (minutes) as determined from pharmacokinetic analysis from a 350-mg SEBX drink determined by DART TOF-MS.**

Compound	Serum concentration (nmol L <sup>-1</sup> ) at each timepoint (min)				
	30	45	60	120	180
Cyanidin	0.5	0.3	0.0	0.0	0.0
Averionol	4.5	13.5	20.3	3.6	0.0
Tristenonol	0.0	2.0	17.0	4.7	5.0
Istrocyandin	0.0	0.0	0.0	0.0	8.2

**Pharmacokinetic analysis of flavonoids delivered as the SEBX drink:** The pharmacokinetic parameters of the 350-mg dose of the SEBX drink are summarized in Table 4. When consumed as a liquid, the  $C_{max}$  of Cyanidin decreased by 10x, the  $C_{max}$  of Averionol remained the same, the  $C_{max}$  of Tristenonol increased 4x, and the  $C_{max}$  of Istrocyandin remained the same when compared to delivery as the SEBX lozenge. The  $C_{max}$  levels for Cyanidin, Averionol, Tristenonol, and Istrocyandin were achieved at 30, 60, 60, and >180 minutes respectively, when the SEBX drink was consumed. The elimination rate-constants,  $k$ , and  $t_{1/2}$  values did not change dramatically when the SEBX lozenge and drink delivery was compared except for Istrocyandin where  $k$  could not be determined because the  $C_{max}$  value was achieved after the final time-point was collected for DART TOF-MS analysis. Tristenonol, an esterified flavonoid, was eliminated much more rapidly from the blood when the SBEX was consumed as a drink (Table 4) than when it was taken as a lozenge (Table 2).

**Table 4. Pharmacokinetic parameters determined from DART TOF-MS analysis of serum after a 350-mg dose of the SEBX drink.**

Compound	$C_{max}$ (nmol L <sup>-1</sup> )	$T_{max}$ (min)	$k$ (nmol L <sup>-1</sup> min <sup>-1</sup> )	$t_{1/2}$ (min)
Cyanidin	0.53	30	0.018	39
Averionol	20.3	60	0.169	4.1
Tristenonol	17.0	60	0.101	6.9
Istrocyandin	8.22	>180	ND	ND

ND = could not determined

**Comparison of the anti-influenza phytonutrients pharmacokinetics from the SEBX lozenge and drink delivery systems:** The bioavailability of the anti-influenza flavonoids Averionol, Tristenonol, and Istrocyandin reached 10.5, 8.6, and 19.7% (wt/wt) respectively, when delivered as an SEBX lozenge (Table 5). When the

bioavailability of the SEBX bioactive phytonutrients was compared between oral delivery (SEBX lozenge), and delivery as a drink (SEBX drink) where the bulk of

**Table 5. Comparative bioavailability of bioactive chemicals in the EB extract when supplied as the SEBX lozenge (oral delivery) and SEBX drink (GI delivery).**

Compound	% Bioavailability	
	Lozenge	Drink
Cyanidin	4.7	0.2
Averionol	10.5	4.6
Tristenonol	8.6	18.8
Istrocyanidin	19.7	10.8

these flavonoids are delivered directly to the stomach, it is clear that two of the bioactives, Averionol and Istrocyanidin, are more bioavailable when delivered in the oral cavity (Table 5). In contrast, Tristenonol showed the opposite pattern with much greater bioavailability when the SEBX was provided as a drink. Cyanidin behaved like Averionol and Istrocyanidin which showed increased bioavailability when delivered as the SEBX lozenge.

## Discussion

The nutritional and health value of many herbal-based dietary supplements and functional foods derives from the bioavailability of the phytonutrients present. Bioavailability varies dramatically with both the chemical form and the site of phytonutrient uptake in the body. For example, phytonutrients like flavonoids are the most abundant polyphenols in the human diet and are readily absorbed in the oral cavity in glycosylated and methylated forms due to the high abundance of sugar transporters in the oral mucosa (Oyama *et al.* 1999), but when introduced into the stomach, bacterial activity can cleave chemical moieties, like sugar residues, that are essential for absorption, thereby rendering the flavonoid unavailable or at best only weakly absorbed (Schneider *et al.* 1999; Crespy *et al.* 2002; Nemeth *et al.* 2003). Gastrointestinal tract microflora also have a major impact on flavonoid uptake, as they actively transform and metabolize these chemicals (Keppler and Humpf 2005).

The precise mechanisms by which flavonoids are taken up by the GI tract are not fully known, though recent studies by Nemeth *et al.* (2003) point to the importance of intestinal epithelial cell trans-membrane and cytosolic  $\beta$ -glycosidases. The authors suggest two mechanisms for uptake. One mechanism involves active transport by a glucose transporter of the hydrophilic glycosides into the cytosol of the epithelial cells of the intestinal lumen where they are deglycosylated to form the hydrophobic aglycones that passively diffuse across the membrane into the portal blood stream. The other, non-mutually exclusive mechanism, involves deglycosylation of glycosides by a trans-membrane  $\beta$ -glycosidase, transport of the aglycones to the cytosol and then into the blood stream.

We sought to evaluate the pharmacokinetics and bioavailability of specific glycosylated, esterified and methylated flavonoids present in a standardized elder berry extract (SEBX) when the SEBX was provided as a slow-dissolve lozenge, targeting oral cavity uptake, and when the extract was provided as a drink, targeting absorption in the small intestine. We also explored the use of a new mass spectrometric method, DART TOF-MS, to address the pharmacokinetics of bioactive chemicals in SEBX as a lozenge and as a drink in order to trace several different flavonoids simultaneously while not requiring chemical reference standards for all the target molecules. The uptake of Cyanidin (delivered as a drink) is typically measured to determine the bioavailability of flavonoids present in berries, grapes and other fruits (Cao and Prior 1999; Frank *et al.* 2002; Mulleder *et al.* 2002), therefore we monitored Cyanidin levels with DART TOF-MS to evaluate its utility for pharmacokinetic studies and compared the analyses with traditional LC-MS techniques.

In the present study, Cyanidin was readily taken up and detected in the blood within 20 to 30 minutes of ingestion, when delivered as both a drink and in lozenge form. Previous studies showed maximum serum levels of Cyanidin were reached between 30 and 75 minutes (Milbury *et al.* 2002; Bitsch *et al.* 2004) when delivered as a drink. The serum concentrations of Cyanidin, determined by LC-MS and DART TOF-MS, were within one standard deviation at  $3.1 (\pm 1.0)$  and  $5.4 (\pm 2.8)$  nmol L<sup>-1</sup>, equivalent to 2.7 and 4.7% bioavailability, respectively. The bioavailability of Cyanidin, when delivered in the SEBX lozenge, increased by almost 100-times (4.7%) when compared to published values on Cyanidin bioavailability when consumed in liquid forms where primary uptake would be in the small intestine (0.07% total Cyanidin, Milbury *et al.* 2002; 0.2% Cyanidin, present study). These findings support the preferred uptake of SEBX Cyanidin, in the glycoside and aglycone forms, in the oral cavity. The urine concentration of Cyanidin spiked at  $27.5$  nmol L<sup>-1</sup> at 2 hours post-consumption which is below the urine concentration previously reported ( $96.1$  nmol L<sup>-1</sup>) by Milbury *et al.* (2002) even though the previous study utilized ca. 360-times the amount of cyanidins used here. This further supports the enhanced uptake of Cyanidin in the oral cavity.

Proanthocyanidins, and methylated, glycosylated and esterified flavonoids are the most bioavailable flavonoid forms (Deprez *et al.* 2000; Matsumoto *et al.* 2001; Ross and Kasum 2002; Mazza 2005). Nutritional supplements that are enriched in esterified, methylated or glycosylated flavonoids will provide enhanced utilization and health benefits (Cook and Samman 1996; Ross and Kasum 2002; Kowalczyk *et al.* 2003). In the studies here, Averionol, a methylated flavonoid, reached a serum concentration of  $23$  nmol L<sup>-1</sup> 45 minutes post-consumption, and was detected in serum as early as 20 minutes after ingestion when delivered in the SEBX lozenge. Averionol followed first-order kinetics and was eliminated in the urine within 4 hours post consumption. The  $C_{max}$  of Averionol represents 10.5% bioavailability of the total amount of Averionol present in a single 175-mg dose SEBX lozenge. This supports the hypothesis that methylation, as well as mode of delivery, increases the bioavailability of flavonoids.

Tristenonol is an esterified flavonoid in elder berry fruits known to contribute to the anti-influenza activity of this botanical (Roschek Jr. *et al.* 2008). Maximum serum

concentration of Tristenonol was achieved 4 hours post-consumption of the SEBX lozenge. Istrocyanidin, an A-type proanthocyanidin in elder berry also known to contribute to anti-viral activity (Fink *et al.* 2008), reached maximum plasma concentrations 6 hours after ingestion of the SEBX lozenge. It was found that B-type proanthocyanidins from cocoa achieved similar plasma levels after being ingested as a beverage (Holt *et al.* 2002). The absolute concentrations of Tristenonol and Istrocyanidin in serum were lower than that of Averionol, but the 175-mg SEBX dose also contained 5- to 6-times lower levels of both Tristenonol and Istrocyanidin. The maximum serum concentrations of Tristenonol and Istrocyanidin represent 8.6 and 19.7% bioavailability, respectively, significantly higher than bioavailability of Cyanidin reported here and in other studies (Milbury *et al.* 2002; Bitsch *et al.* 2004).

The bioavailability of Averionol and Istrocyanidin decreased by ca. 2-fold when ingested as the SEBX drink compared with the lozenge, indicating that Averionol (methylated) and Istrocyanidin (proanthocyanidin) are preferentially absorbed in the oral cavity. Conversely, the bioavailability of Tristenonol increased by ca. 2-fold when the SEBX drink was consumed, indicating that Tristenonol is preferentially absorbed in the small intestine. Further support for the small intestine being the primary site of uptake for Tristenonol comes from the fact that Tristenonol takes nearly 4 hours to reach maximum serum concentration when the SEBX lozenge is consumed, but reaches maximum serum levels in only 60 minutes post-ingestion when the SEBX drink is consumed (i.e. delivered directly to the stomach). Uptake in the small intestine of this esterified flavonoid may require trans-membrane epithelial  $\beta$ -glucosidase activity (Nemeth *et al.* 2003).

When the results obtained by DART TOF-MS are compared with LC-MS analyses of serum, it is clear that DART TOF-MS can be used in a quantitative manner to identify and determine phytonutrient concentrations in biological fluids. This new MS capability allows for rapid pharmacokinetic analyses of multiple phytonutrients at the same time and, as such, should enable an expansion of our understanding of the biological role of food and nutraceutical sources of flavonoids and other phytonutrients in human nutrition and health.

Collectively the findings here demonstrate that Cyanidin and anti-influenza phytonutrients in the SEBX are rapidly absorbed into the bloodstream (within minutes of consumption) when delivered as a slow-dissolve lozenge maximizing oral cavity absorption and persist for up to 4 hours post-ingestion. When the SEBX is taken as a drink, Cyanidin, Averionol and Istrocyanidin show reduced bioavailability, while Tristenonol bioavailability increased most likely because its primary site of absorption was the small intestine. Although bioavailability of the SEBX anti-viral flavonoids was dramatically different between the SEBX lozenge and drink, the pharmacokinetics were similar. These results indicate that mode-of-delivery and, hence, site of uptake is important in the bioavailability of flavonoids and proanthocyanidins. We also demonstrate, for the first time, that DART TOF-MS can be used reliably and in a quantitative manner for pharmacokinetic studies of botanical small molecules in body fluids without significant sample preparation.

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