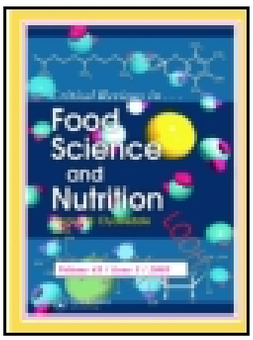

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A THEORETICAL PHYSIOLOGICALLY-BASED PHARMACOKINETIC APPROACH FOR MODELLING THE FATE OF ANTHOCYANINS *IN VIVO*

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Abstract

Recent studies on the pharmacokinetics of anthocyanins (ACNs) and their metabolites have uncovered evidence for hitherto unknown physiological effects affecting the fate of these compounds *in vivo*. In particular, it has been shown that the stomach, in addition to the small intestine, has an important role in absorption. Most studies still use a non-compartmental or one-compartmental approach to determine the pharmacokinetic parameters of ACNs, which does not represent the anatomical and physiological conditions that a compound is subject to in the organism. Thus, the objective of this study was to review the current knowledge of the different processes involved in the metabolism of ACNs once ingested and, based on this information, propose a theoretical physiologically-based, multi-compartmental pharmacokinetic (PBMK) model to describe their fate *in vivo*. This is the first study that reports a PBMK model for ACNs; the model provides a more physiologically representative approach for ANC metabolism, which could be used as a basis for experimental designs and interspecies scale-up.

Keywords

absorption; metabolism; distribution; excretion; bioavailability

ABBREVIATIONS

A_{xi} amount of parent ACN (*i*) in a given compartment (*x*) or in the ingested food (*F*)

$A_{x\_Lu_i}$ amount of parent ACN (*i*) in the lumen of a given compartment (*x*)

AE active renal elimination

AC *N*anthocyanin

ADM *E*absorption, distribution, metabolism, and excretion

AUC area under the curve (amount *x* time/volume)

BE biliary excretion

C_{B_i} concentration of the parent ACN in blood (amount/length^3)

CL_{K_i} renal clearance of the parent ACN (volume/time)

CL'_{Li_i} intrinsic clearance with respect to the parent ACN concentration in the liver (*Li*) (volume/time)

C_{Vx} concentration of the parent anthocyanin (*i*) in the venous blood leaving the compartment (amount/length^3)

C_{xi} concentrations of ACNs in a given compartment (*x*) (amount/length^3)

COMT catechol-*O*-methyltransferase

EHC	enterohepatic circulation
f_{B_i}	fction of free parent ACN in the blood
HS	*A*human serum albumin
I	small intestine
K	kidneys
$K_{a_{x_i}}$	absorption rate constant of the parent ACN in a given compartment (time^{-1})
k_e	renal excretion rate constant (time^{-1})
K_{m,x_mi}	Michaelis-Menten constant (amount/length^3)
L	large intestine
Lg	lungs
LPH	lactase phlorizin hydrolase
Lu	organ lumen
M	mucosa
$P_{B:Air}$	blood:air partition coefficient
$P_{x:B}$	partition coefficient of the ACN between the compartment (*x*) and blood
PBPK	physiologically-based pharmacokinetic

Q_c	cardiac output (volume/time)
Q_v	alveolar ventilation (volume/time)
Q_x	blood flow rates to the compartment (*x*) (volume/time)
RP	rapidly perfused tissues
S	stomach
SP	slowly perfused tissues
SULT	sulfotransferases
UDPGDH	uridine diphosphate-glucose dehydrogenase
UDPGT	uridine diphosphate-glucuronosyltransferases
V_i	volume of distribution of the parent ACN (volume)
V_{max,x_mi}	apparent maximum velocity or the theoretical maximum metabolic (amount/time)
V_x	volume of the compartment (*x*) (% body weight)

INTRODUCTION

The pharmacokinetics (PK) of exogenous compounds refers to their absorption, distribution, metabolism, and excretion (ADME) (Petzinger and Geyer, 2006). For some time, the PK of anthocyanins (ACNs) was unknown and thought to be nearly inexistent as their absorption was considered limited (Nemeth et al., 2003). Although the whole process is not completely

understood, researchers have indicated that existing studies have underestimated the amount of ACNs absorbed (Fernandes et al., 2012) as recent reports have highlighted higher than expected bioavailability rates and longer permanence time of ACN-related metabolites in the circulatory system (Czank et al., 2013; Kalt et al., 2014; Kay et al., 2014).

A limitation of the methods used for the identification and quantification of ACNs and their metabolites in biological fluids is the indirect assessment of the flavylium cation, which is the ACN form that predominates at pH < 2. As a consequence, structures that are not able to regenerate this ion by reacidification of the sample due to *in vivo* metabolism and/or modifications in the chemical structure will not be detected (Fernandes et al., 2012). In addition, some studies have reported a lower than expected recovery of parent ACNs from plasma and urine after ingestion, which could not be representative of the compounds found *in vivo* after metabolism and excretion (He et al., 2005). Another issue is the low concentration of some metabolites that would be present, which would be near the limit of the detection of the methods and equipment used (Czank et al., 2013; Kay et al., 2004), potential degradation of these compounds during sample preparation (Felgines et al., 2003; He et al., 2005), and binding of the compounds and metabolites to proteins in the bloodstream (Al Bittar et al., 2014; Tang et al., 2014).

Current studies often use a non-compartmental or one-compartmental approach to assess bioavailability of ACNs. Although simple, these methods do not appropriately represent the anatomical, physiological (e.g. tissue volumes and blood flow), and biochemical conditions (e.g. metabolic rate constants) that a particular compound encounters in the organism, in association

with their physicochemical characteristics (e.g. partition coefficient) (Pastino and Conolly, 2000). For this reason, a theoretical physiologically-based pharmacokinetic (PBPK) model is proposed to better describe the bioavailability of ACNs. Here, a review of the current literature related to the PK of ACNs is presented. This information is then used to develop a PBPK model that accounts for their passage through different compartments in the body, taking into consideration the different variables that impact their PK. This model will ultimately provide insights on the absorption, distribution, metabolism, and excretion of ACNs and their metabolites, and could contribute to the experimental design of *in vitro* and *in vivo* studies taking into account the physiologically relevant concentration of circulating compounds. To the best of our knowledge, this is the first time a PBPK model is used to describe the fate of ACNs *in vivo*.

1 PHARMACOKINETICS OF ANTHOCYANINS

After ingestion, exogenous compounds are generally absorbed in the gastrointestinal (GI) tract, distributed by the systemic circulation, metabolized, and finally excreted. In the case of ACNs, in addition to absorption in the small intestine, a considerable body of evidence has demonstrated that the stomach could also have a role (Passamonti et al., 2003). It is also worth noting that ACNs can be found in different structures, which results in varied PK parameters and pharmacological effects (Pojer et al., 2013). Tables 1 and 2 summarize some of the PK parameters for ACNs described in the literature for animal models and in humans, respectively. The following sections review the literature available for the PK of ACNs, where the absorption, distribution, metabolism, and excretion are described.

1.1 Absorption

Oral absorption rates differ greatly among polyphenols and can be influenced by chemical (e.g. pH), biological (e.g. intestinal microbiota and enzymes), and physiological conditions (e.g. barriers, presence of other nutrients, disorders and/or pathologies) along the GI tract (Ugalde et al., 2009), in addition to the chemical structure and the matrix where the compounds are found (Charron et al., 2009). For example, Charron et al. (2009) showed that nonacylated ACNs have higher bioavailability when compared to acylated ACNs. When comparing different food matrices, the total amount of ACNs absorbed was similar when ingested as carrot juice and whole carrots; however, the absorption of these compounds from the juice sample was faster since it was not subject to the same digestive time required for the whole carrot.

Research has suggested that the stomach could have a role in the absorption of ACNs (Cai et al., 2011; Felgines et al., 2007), which could justify the rapid detection of these compounds in plasma and where the pH conditions favor their chemical stability (Woodward et al., 2011). At physiological gastric pH, ACNs are likely to be found in the flavylium cation form (depending on the *pKa* of the compound) (Fernandes et al., 2012), where absorption by passive diffusion is likely to be limited (Lipinski et al., 1997). However, it has been suggested that ACN absorption in the stomach could occur with bilitranslocase, an organic anion carrier (Passamonti et al., 2003; Passamonti et al., 2005a), where increasing the concentration of ACNs would ultimately result in the saturation of this carrier and reduction of their absorption (Fernandes et al., 2012; Kurilich et al., 2005; Talavera et al., 2003).

According to Vanzo et al. (2011), the involvement of bilitranslocase in the absorption process

could justify the rapid uptake of cyanidin 3-glucoside after intravenous administration in Winstar rats. In their study, they detected peonidin 3-glucoside (a methylated derivative of cyanidin 3-glucoside) within 15 s after the administration of cyanidin 3-glucoside and attributed this to the rapid cellular uptake of the parent ACNs, methylation, and transport of the metabolite to the plasma. These results are supported by the concomitant (and rapid) reduction of the cyanidin 3-glucoside concentration in plasma. It is believed that bilitranslocase could mediate the influx of cyanidin 3-glucoside and efflux of the metabolites to the circulatory system (Vanzo et al., 2011).

ACNs are also absorbed in the small intestine. It has been suggested that polyphenol glycosides are first hydrolyzed to their aglycone forms prior to absorption, and the exception of cyanidin and delphinidin glucosides (Nemeth et al., 2003), ACNs and other flavonoids can be hydrolyzed at the mucosal brush-border membrane by β-hydroxylase LPH (Day et al., 2000). To account for the presence of parent ACN in blood and urine, a mechanism of absorption was proposed involving the sodium-glucose co-transporter (SGLT) carrier, similarly to other flavonoids (Cao et al., 2001; McGhie et al., 2003; Tsuda et al., 1999). However, Felgines et al. (2008) demonstrated that the intestinal absorption of ACNs in rats was not affected by concomitant administration of glucose, which could indicate that their transport is not mediated by SGLT.

In situ perfusion of ACNs in the jejunum and ileum of Wistar rats for 45 min resulted in the absorption of high amounts of ACN glycosides. The exact amount varied depending on the compound structure: between 10.7 (malvidin 3-glucoside) to 22.4% (cyanidin 3-glucoside) (Talavera et al., 2004). Absorption was also affected by the sugar moiety as the uptake of cyanidin 3-glucoside was significantly higher than its 3-galactoside and 3-rutinoside derivatives.

However, He et al. (2009) attributed the reduction of cyanidin 3-glucoside concentration in intestinal fluid to degradation, and not higher absorption.

1.2 Distribution

Andres-Lacueva et al. (2005) and Passamonti et al. (2005b) demonstrated that ACNs can cross the blood-brain barrier in blueberry-fed rats. These compounds were identified in regions of the brain related to learning and memory after an 8-week exposure period (Andres-Lacueva et al., 2005). Intact blackcurrant ACNs also crossed the blood-aqueous and blood-retinal barriers after oral administration in rats (Matsumoto et al., 2006). Using healthy weanling pigs as a model for human nutrition, Kalt et al. (2008) investigated the effects of long-term exposure (four weeks) of these animals to blueberries (1, 2, and 4%, w/w, added to the basal diet). Pigs were used in this study due to similarities with humans in relation to digestive absorption and cardiovascular physiology. Eleven intact ACNs were reported in brain cortex, liver, eye, and cerebellum tissues, whereas none were detected in plasma and urine (Kalt et al., 2008).

ACNs and their metabolites can bind to human serum albumin (HSA) under physiological pH conditions (Wiese et al., 2009), similarly to other polyphenols, which would affect their half-life in plasma and tissue distribution. Indeed, HSA is known to mediate the transport of a large variety of ligands (Varshney et al., 2010) and it has been suggested that the polyphenols would bind to sites characterized by the presence of hydrophobic pockets containing positive charges due to arginine and lysine amino acid residues (Al Bittar et al., 2014). In *in vitro* conditions, Al Bittar et al. (2014) demonstrated that chalcones, an ACN metabolite, have higher affinity for HSA than their parent compounds, possibly due to their linear structure. It has also

been demonstrated that increasing the number of hydroxyl substituents in ring B of the ACN backbone structure contributes to a higher binding affinity to HSA (e.g. delphidin 3-glucoside has higher affinity than cyanidin 3-glucoside) (Tang et al., 2014). Since albumin can escape reabsorption in the body through the kidneys, it could contribute to the elimination of ACNs via urine.

1.3 Metabolism

In general, ACNs are subjected to extensive metabolism after absorption, as recently demonstrated for cyanidin 3-glucoside (Ferrar et al., 2014). It has been suggested that their metabolites persist for longer periods of time in the circulation, exerting their activities as long as their main molecular structure is maintained (Felgines et al., 2003; Pojer et al., 2013).

The mouth is often referred to as a benign environment for exogenous compounds that are ingested orally due to the short residence time in this cavity. However, researchers have showed that the hydrolysis of ACNs can occur in saliva and through oral microbial activity. Using a rinse solution containing ACNs, Mallery et al. (2011) reported the presence of aglycones in the mouth due to the β-glucosidase activity of enzymes produced by the host and oral microbiota. They also identified other key phase II enzymes, including uridine diphosphate-glucuronosyltransferases (UDPGT), catechol-*O*-methyltransferase (COMT), UDP-glucose dehydrogenase (UDPGDH), breast cancer resistance protein (BCRP), and lactase phlorizin hydrolase (LPH), in addition to sulfatase and β-glucoronidase reported by Chauncey et al. (1954). Comparing different rinse solutions, it was interesting to note that those containing flavoring agents resulted in lower salivary ACN levels, suggesting that the liquid was perceived as food by the sensory system,

leading to release of saliva (confirmed by an increase in volume) and, consequently, β-glucosidase (Mallery et al., 2011). These results are in agreement with those by Kamonpatana et al. (2012), who showed that the ACN degradation promoted by saliva and buccal microbiota could be reduced by the use of the antibacterial chlorhexidine.

In the liver, phenolic aglycones are methylated by COMT (Galati et al., 2006) and/or conjugated with glucuronic acid (El Mohsen et al., 2006) or sulphate by UDPGT (Woodward et al., 2011) or phenol sulfotransferases (SULT), respectively (Felgines et al., 2003). According to Fleschhut et al. (2006), ACNs and their aglycone forms are poor substrates for UDPGT, which would result in low glucuronidation rates and could explain the presence of intact ACNs in the circulation. These authors also indicated that ACNs and anthocyanidins are not substrates for enzymes of the cytochrome P450 family as they were not able to identify phase I metabolites *in vitro*. It has been suggested that sulfation predominates only when low doses of phenolic-derived drugs are administered, possibly because this pathway is easily saturated (Kay, 2006). Ferrars et al. (2014) recently identified several metabolites from cyanidin 3-glucoside in different body fluids.

Kay et al. (2005) presented the pharmacokinetics of ACNs after oral administration of a 721 mg dose of cyanidin 3-glycosides (galactoside, arabinoside, xyloside, and glucoside) from chokeberry extract in a small human trial consisting of healthy male volunteers (n = 3). This amount would be equivalent to 120-230 g of fresh whole berries. Unlike previous reports, approximately 32% of the total amount of ACNs identified in the blood and urine consisted of the parent compounds while 68% accounted for their respective metabolites (glucoronidated and methylated forms). Glucoronidation represented the main metabolic transformation, accounting

for more than 50% of the total ACNs detected in blood and urine, followed by methylation (approximately 48%) (Kay et al., 2005).

Passamonti et al. (2009) noted that when ACNs and other flavonoids that undergo hepatic glucuronidation are ingested in high doses, this could lead to jaundice. Also that the antioxidant capacity of plasma which is commonly reported after their consumption would exhibit a transient and reversible increase, depending on the concentration of circulating bilirubin, an endogenous antioxidant. This would be due to the ACNs and bilirubin competing for bilitranslocase in the liver, which may explain the slight increase observed in bilirubin after the administration of ACN-rich extract from elderberry in postmenopausal women in the study by Curtis et al. (2009).

1.4 Excretion

The type of metabolite (or conjugate) formed will determine the route of excretion of ACNs. For instance, glucuronides formed in the liver are readily available for biliary excretion unlike those formed in the intestine, which can reach and remain in the systemic circulation for a longer duration (higher $t_{1/2}$). Fecal elimination can be reduced by enterohepatic circulation (EHC), which also extends the residence time of exogenous substances and their metabolites in the body (Kalt et al., 2014). The EHC can lead to the sulfation of compounds, diverting them from biliary excretion towards urinary excretion as highly polar compounds are not significantly reabsorbed by the intestine (Kay, 2006). The compounds that are not absorbed are hydrolyzed by colonic bacteria and eliminated in the feces (Felgines et al., 2008; Fleschhut et al., 2006; Sánchez-Patán et al., 2012).

Values for fecal elimination are underestimated for most classes of polyphenols. In the case of

ACNs, He et al. (2005) demonstrated that feces are one of the main routes for their excretion in rats. These results were confirmed by Felgines et al. (2010) and Czank et al. (2013), who assessed the fecal elimination of ACNs and their metabolites through the quantification of ^{14}C and ^{13}C isotope content in mice and humans, respectively. Czank et al. (2013) also showed that the content of ^{13}C isotope in the breath was approximately 6.9±1.6% of the initial dose, indicating that the lungs are also a route of excretion for ACN metabolites (Czank et al., 2013).

In relation to excretion via urine, He et al. (2009) showed that after stomach intubation of black raspberry extract, ACNs were detected in urine within 30 min. This observation may be explained by the presence of bilitranslocase in renal tissue at the basolateral membrane domain (Elias et al., 1990), which could indicate its involvement in the excretion of ACNs. Vanzo et al. (2008) demonstrated that the concentration of ACNs in the kidneys is three times higher than in the systemic plasma and liver, which corroborates the hypothesis of the transport being mediated by bilitranslocase. These results also indicated that the kidneys are more efficient with the uptake of ACNs than liver and, consequently, the excretion of these compounds would occur preferentially through the urine rather than bile (Passamonti, 2011; Vanzo et al., 2008). The localization of bilitranslocase in the basolateral and not in the apical domain suggests that the ACNs that eventually escape the glomerular filtration could be taken up into tubular cells and then excreted into the urine.

1.5 Bioavailability

When discussing PK parameters, the bioavailability is also an important aspect that should be explored. It refers to the rate and extent to which a drug reaches its site of action, i.e., the amount

of compound ingested that will reach the systemic circulation and the specific organs or tissues, which will later result in a biological effect. Until recently, the bioavailability of ACNs was also considered very low, although recent reports have indicated that the data available from earlier studies in the literature could have underestimated ACN bioavailability since they did not monitor or considered the role of ACN metabolites (Kay, 2006). For example, Kalt et al. (2014) demonstrated that the bioavailability is higher than previously assumed and the elimination of metabolites continues for days after the consumption of ACNs has been discontinued, confirming the results obtained by Czank et al. (2013).

According to Ferrars et al. (2014), the conversion of cyanidin 3-glucoside into its metabolites, peonidin 3-glucoside and methoxy-cyanidin 3-glucoside-glucuronide, is reversible. Hwang et al. (1981) indicated that in cases where a drug has a reversible metabolism (or biotransformation), the conventional concept of bioavailability and experimental strategies may not be adequate. This idea is relevant for the analysis of ACNs as both parent compounds and their metabolites can exhibit activity *in vivo*, and metabolites can be considered latent sources of active moiety, with prolonged effects (Hwang et al., 1981). EHC should also be considered a reversible metabolism in the study of ACN PK. When analyzing the results of absorption studies, proper consideration must be given to the role of different absorption site, as multiple peaks in the blood concentration do not necessarily indicate EHC. These multiple peaks could result from factors such as differences in carrier density and reduced gastric motility (Gabrielsson and Weiner, 2000).

In addition, blood AUC is commonly used to determine the bioavailability of exogenous

compounds. However, according to Pastino and Conolly (2000) this strategy may not be appropriate for accurately estimating the bioavailability of compounds where their metabolism can be saturated. For example, an increase in the amount ingested (or dosed) by 2-fold is not accompanied by the same increase in the AUC. As discussed previously, the sulfation route is known to be easily saturated (Kay, 2006). It is also possible that technological constraints have limited the identification of sulfated metabolites, as reported by Walle et al. (2004) for resveratrol metabolites. In addition, comparing the consumption of 250 and 500 g servings of microwave cooked purple carrot, researchers observed that the absorption of individual ACNs decreased with the larger dose (Novotny et al., 2012), supporting the idea that the carrier molecules can also be saturated.

2 PHYSIOLOGICALLY-BASED PHARMACOKINETIC (PBPK) MODELLING OF ANTHOCYANINS

2.1. Motivation

The fate of ACNs *in vivo* can be described by using one- or multi-compartment model models. In both situations, it is assumed that the compounds and their metabolites are linearly transported between a central compartment (e.g. blood and rapidly perfused tissues) and peripheral tissue compartments (e.g. slowly perfused tissues) (Chen and Gross, 1979). Determining the PK parameters of exogenous compounds and establishing an appropriate PBPK model is important for the following reasons: (a) it enables interspecies scale-up (e.g. data obtained in mice can be used to predict outcomes in humans) (Bradshaw-Pierce et al., 2007); (b) it provides an association between the concentration of the compound, effects, and responses, which can help

in determining the dose regimen; and (c) it can help in elucidating the effects of disease states on the PK of the compound (e.g. different blood flow rates can be used to better mimic a pathological condition *in vivo*).

As discussed previously, one-compartment models offer limited information about the PK of ACNs and their metabolites after ingestion. In addition, it takes some time for the compounds to distribute into the organs and tissues and reach equilibrium, which makes the one-compartment model unsuitable for exogenous compounds where concentration is measured frequently (Gabrielsson and Weiner, 2000). On the other hand, the PBPK multi-compartment model is much more comprehensive and accounts for the anatomic site of absorption, metabolism, distribution, and excretion of the exogenous compounds, as well as factors such as the blood flow rates, physiologic organ or tissue volumes, and tissue binding (Chen and Gross, 1979). This model consists of differential equations for each compartment, where a mass balance and the following assumptions are observed: (1) the model is flow-limited (i.e., it is assumed that the concentration of the exogenous compound in the arterial blood reaches rapid equilibrium with each compartment and concentrations in each compartment would be proportional to that in the venous blood leaving the organ/tissue); (2) the plasma protein and tissue binding are linear (i.e., the coefficient of distribution ratio or partition coefficient of the compound between the tissue and plasma is independent of the concentration of the compound); and (3) concentration of the exogenous compound is homogeneous in a given compartment (Chen and Gross, 1979).

Boonpawa et al. (2014) recently described a PBPK model for quercetin and its metabolites to predict the time-dependent concentrations of these compounds in plasma and

other compartments. These authors reported that the model was adequate in predicting biliary and urinary excretion and intestine efflux of quercetin metabolites through the quantification of these compounds (Boonpawa et al., 2014). Although their proposed model could be used for any flavonoid, it is lacking a separate compartment for the stomach, which may be a significant omission given the role of the stomach in the absorption of ACNs.

In the following sections, a comprehensive theoretical PBPK model is first developed for the parent ACNs and then a separate PBPK model developed for their metabolites. The equations for each compartment are presented separately to better explain the model. For a comprehensive review and summary of appropriate values to use for some of the non-specific variables common to other models, the reader is referred to the work by Brown et al. (1997).

2.2 Multi-compartmental PBPK model for parent anthocyanins

The PBPK model for ACNs that is proposed in this study consists of nine separate compartments, representing the organs or tissues that are involved in the ADME of the parent compounds (Figure 2). These compartments are the stomach, small intestine, large intestine, liver, kidney, slowly perfused tissues, rapidly perfused tissues, lungs, and blood, where arrows are used in Figure 2 to indicate the movement of the digestive bolus through the digestive tract, connecting the compartments for the stomach and intestines. In this model, it is also assumed that blood from the hepatic artery and the portal vein mix in the sinusoids before entering the liver (Kan and Madoff, 2008), and that the concentration of the compound in the tissue is proportional to the outgoing venous concentration of the parent anthocyanin (Wagner, 1993). It

is worth noting that the aglycone anthocyanidins are not included in the discussion of the model as their occurrence in nature is still not completely characterized (Macz-Pop et al., 2006).

The dotted arrows to the right of large intestine, liver, and kidneys indicate loss of the parent ACN as a result of elimination or excretion, whereas those to the left of the small and large intestines and liver indicate loss of the parent ACNs by metabolism (from microbiota or host). A fraction of these metabolites is excreted and the remainder is absorbed. In the case of the kidneys, there are two arrows leaving this compartment indicating passive and active urinary excretion. Further details regarding absorption of the metabolites are given in the sub-compartment model for metabolites described in section 2.3. The model equations for the different compartments shown in Figure 2 are presented below. These equations were developed from other studies in the literature, such as those described by Boonpawa et al (2014) for quercetin.

Stomach. The first site for ACN absorption in the body is the stomach (Cai et al., 2011; Felgines et al., 2007). The equations for this compartment consider the ACNs that would be bioacessible, accounting for food that is ingested and/or digested over time, along with the bolus that is transferred to the small intestine. In addition, the quantity of ANCs being absorbed and circulated through the gastric tissue via blood is included. Even though it has been suggested that passive diffusion and absorption involving the bilitranslocase carrier could occur simultaneously in the stomach depending on the ACN structure and gastric conditions (Fernandes et al., 2012), in this model they were combined into one term in the equation.

$$A_{S\_Lu_i} = A_{F_i} - dA_{I\_Lu_i} \quad (1)$$

$$\left(dA_{S_i} / dt\right) = K_{a_{S_i}} A_{S\_Lu_i} + Q_S \left(C_{B_i} - {C_{S_i}}/{P_{S:B_i}} \right) \quad (2)$$

$$C_{S_i} = \left({A_{S_i}}/{V_S} \right) \quad (3)$$

Small intestine

In addition to stomach, the small intestine constitutes an important organ for absorption of ACNs (Talavera et al., 2004), even though its pH could lead to the chemical degradation of these compounds (He et al., 2009). Similar to stomach, the equations that are described take into account the amount of bolus that enters the small intestine and is transferred to the large intestine over time, as well as the quantity that is absorbed or eliminated through metabolism, either by the host or microbiota. It is possible that different isoenzymes are used to metabolize the ACNs, in which case, the model would need to account for this with different values of K_m.

$$A_{I\_Lu_i} = \left(A_{F_i} - A_{S_i} \right) - A_{L\_Lu_i} \quad (4)$$

$$\left(dA_{I_i} / dt\right) = K_{a_{I_i}} A_{I\_Lu_i} + Q_I \left(C_{B_i} - {C_{I_i}}/{P_{I:B_i}} \right) - \left[\left(\frac{V_{max,I\_mi} \, {C_{I_i}}/{P_{I:B_i}}}{K_{m,I\_mi} + {C_{I_i}}/{P_{I:B_i}}} \right)_{metab} + \left(\frac{V_{max,I\_mi} \, {C_{I_i}}/{P_{I:B_i}}}{K_{m,I\_mi} + {C_{I_i}}/{P_{I:B_i}}} \right)_{microb} \right]$$

(5)

$$C_{I_i} = \left({A_{I_i}}/{V_I} \right) \quad (6)$$

where *mi* is the metabolite; *metab* and *microb* indicate the metabolism of the compound by the host and microbiota, respectively.

Another important consideration is the EHC that could contribute to the concentration of ACNs in the intestine. In this post-absorption situation, the model would have a term to account for the amount of ACNs from the liver via biliary excretionhen, equation 5 would be expressed as $C_{I_i} = \left(A_{I_i} + A_{I_{i\_EHC}} / V_i \right)$ where $A_{I_{i\_EHC}}$ represents the amount of parent ACN that is being circulated in the EHC.

Large intestine

The equations for the large intestine are very similar to those for the small intestine compartment, however the amount that will be eliminated through the feces is also considered here. Feces comprise of approximately 25% solids, from which 30% are undigested material associated with bile salts (Barbosa, 2013). It is possible that ACNs (and their metabolites) could interact with these compounds. For example, Aura et al. (2005) showed that the recovery of ACNs from spiked feces was incomplete, which could indicate that these compounds are able to bind to the fecal matrix. Feces seem to be the main vehicle of elimination of ACNs (He et al., 2005), even though most results have been underestimated.

$$A_{L\_Lu_i} = A_{F_i} - (A_{S_i} + A_{I_i}) - A_{Feces_i} \quad (7)$$

$$\left(dA_{L_i} / dt \right) = Q_L \left(C_{B_i} - C_{L_i} / P_{L:B_i} \right) - \left[\left(\frac{V_{max,L\_mi} \; C_{L_i} / P_{L:B_i}}{K_{m,L\_mi} + C_{L_i} / P_{L:B_i}} \right)_{metab} + \left(\frac{V_{max,L\_mi} \; C_{L_i} / P_{L:B_i}}{K_{m,L\_mi} + C_{L_i} / P_{L:B_i}} \right)_{microb} \right] \quad (8)$$

$$C_{L_i} = \left(A_{L_i} / V_L \right) \quad (9)$$

Liver

Parent ACNs are subjected to extensive metabolism in the liver. In this model, the clearance of parent ACNs through biliary excretion is included. In addition, the model accounts for the concentration of ACNs in the liver from the ingested food along with the amount circulated in the EHC, as already shown for the small intestine.

$$\left(dA_{Li_i}/dt\right) = Q_S\left(C_{S_i}/P_{S:B_i}\right) + Q_I\left(C_{I_i}/P_{I:B_i}\right) + Q_L\left(C_{L_i}/P_{L:B_i}\right) + Q_{Li}\left(C_{B_i} - C_{Li_i}/P_{Li:B_i}\right) - \left[\left(\frac{V_{max,BE\_mi}\, C_{Li_i}/P_{Li:B_i}}{K_{m,BE\_mi} + C_{Li_i}/P_{Li:B_i}}\right)_{\text{metab}} + \left(CL'_{Li_i}\, f_{B_i} C_{Li_i}/P_{Li:B_i}\right)\right] \quad (10)$$

$$C_{Li_i} = \left(A_{Li_i}/V_{Li}\right) \quad (11)$$

Kidneys

ACNs and their metabolites have been identified in the urine, which is the biological fluid commonly assessed to determine the bioavailability of these compounds. Using a similar approach to that used for the liver compartment, a term is included for the renal clearance of parent ACNs through urine. In addition, a term is also included to account for active excretion (AE) of the compounds that eventually escape the glomerular filtration, since bilitranslocase was also identified in the basolateral membrane domain in the renal tissue (Elias et al., 1990).

$$\left(dA_{K_i}/dt\right) = Q_K\left(C_{B_i} - {C_{K_i}}/{P_{K:B_i}}\right) - \left[\left(CL_{K_i} f_{B_i} {C_{K_i}}/{P_{K:B_i}}\right) + \left(\frac{V_{max,AE} \; {f_{B_i} C_{Ki_i}}/{P_{K:B_i}}}{K_{m,AE} + {f_{B_i} C_{Ki_i}}/{P_{K:B_i}}}\right)\right] \quad (12)$$

$$C_{K_i} = \left({A_{K_i}}/{V_K}\right) \quad (13)$$

$$CL_{K_i} = k_e V_i \quad (14)$$

Slowly and rapidly perfused tissues

Examples of slowly perfused tissues include the skin and muscles, whereas the brain and heart would be categorized as rapidly perfused organs. They were included in this model since research has shown that they are sites for distribution and accumulation of parent ACNs and their metabolites (Andres-Lacueva et al., 2005; Kalt et al., 2008). Equations 15 and 16 refer to slowly perfused tissues, whereas equations 17 and 18 are for the rapidly perfused compartment.

$$\left(dA_{SP_i}/dt\right) = Q_{SP}\left(C_{B_i} - {C_{SP_i}}/{P_{SP:B_i}}\right) \quad (15)$$

$$C_{SP_i} = \left({A_{SP_i}}/{V_{SP}}\right) \quad (16)$$

$$\left(dA_{RP_i}/dt\right) = Q_{RP}\left(C_{B_i} - {C_{RP_i}}/{P_{RP:B_i}}\right) \quad (17)$$

$$C_{RP_i} = \left({A_{RP_i}}/{V_{RP}}\right) \quad (18)$$

Lungs

Although the lungs are not involved in the elimination of the parent ACNs, a separate compartment was included in this model as a site for converting venous blood into arterial blood, which will ultimately be distributed to other compartments.

$$\left(dA_{Lg_i} / dt\right) = Q_{Lg}\left(C_{Lg_i} \Big/ P_{Lg:B_i} - C_{B_i}\right) \quad (19)$$

$$C_{Lg_i} = \left(A_{Lg_i} \Big/ V_{Lg}\right) \quad (20)$$

Blood

The concentration of parent ACNs in the blood is the sum of the concentration of the various compartments in the model, taking into account the blood flow rate in these organs/tissues and the partition coefficient of the compounds, in addition to the cardiac output (although the heart is not considered a separate compartment). As discussed previously, parent ACNs can be bound to different components in the blood, such as HAS (Wiese et al., 2009), and there is an equilibrium of the free and bound form of these compounds.

$$\left(dA_{B_i} / dt\right) = \left[\left(Q_S + Q_I + Q_L\right) C_{Li_i} \Big/ P_{Li:B_i} + Q_K C_{K_i} \Big/ P_{K:B_i} + Q_{SP} C_{SP_i} \Big/ P_{SP:B_i} + Q_{RP} C_{RP_i} \Big/ P_{RP:B_i} + Q_{Lg} C_{Lg_i} \Big/ P_{Lg:B_i}\right] - \left(Q_C C_{B_i}\right) \quad (21)$$

$$C_{B_i} = \left(A_{B_i} \Big/ V_B\right) \quad (22)$$

2.3 Sub-compartmental model for metabolites[n]:

This model, shown schematically in Figure 3, is very similar to the one proposed for the parental ACNs in section 2.2, where the notation [n] indicates that the compartmental model can be

used for *n* generations of metabolites. The differences are: (a) the absence of a compartment for stomach, since it is considered that the generation of the metabolites occurs after ingestion due to the limited residence time in the mouth and stability in the gastric environment, (b) and inclusion of a compartment for the lungs as some metabolites can be eliminated by this route (through breath) (Czank et al., 2013). As all other compartments have been previously described in the model for the parent ACNs, only the equations for the lung compartment are given in this section for the metabolite model.

Lungs

ACN metabolites have been shown to be eliminated through the breath (Czank et al., 2013), in a similar way to other flavonoids. In the case of quercetin, approximately 52% of an isotope-labeled compound administered to healthy human volunteers was recovered in respiratory carbon dioxide (Petrakis et al., 1959; Walle et al., 2001).

$$\left(dA_{Lg_{mi}} / dt\right) = Q_{Lg}\left(C_{Lg_{mi}} \Big/ P_{Lg:B_{mi}} - C_{B_{mi}}\right) - Q_v\left[C_{Lg_{mi}} \Big/ \left(P_{Lg:B_{mi}} P_{B:Air_{mi}}\right)\right] \quad (23)$$

$$C_{Lg_{mi}} = \left(A_{Lg_{mi}} \Big/ V_{Lg}\right) \quad (24)$$

where Q_v is the alveolar ventilation; $P_{B:Air}$ is the blood:air partition coefficient.

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Table 1 -- PK parameters for ACNs from studies performed in animal models

Animal	ACN source	Dose[a]	C_{max}[b]	t_{max} (h)[c]	AUC[d]	Urinary excretion (%)[e]	Reference
Rat	Bilberry (extract)	400 mg	2-3 μg/mL	0.25			Morazzoni et al., 1991
	Black currant (extract)	489 mg delphinidin 3-rutinoside 476 mg cyanidin 3-rutinoside 359 mg cyanidin 3-glucoside	0.36 mg/L 0.51 mg/L 0.38 mg/L	2 0.5 0.5	0.81 mg h/L 1.51 mg h/L 0.68 mg h/L		Matsumoto et al., 2001
	Black currant (extract)	100 mg delphinidin 3-glucopyranoside	0.19 mg/L	0.25			Ichiyanagi et al., 2004
	Elderberry/black currant	320 mg cyanidin 3-glucoside	1.56 mg/L	0.25	1.62 mg		Miyazawa et al., 1999

	(extract)	40 mg cyanidin 3,5-diglucoside	0.19 mg/L		h/L		
	Purple corn (extract)	404 mg cyanidin 3-glucoside	0.14 mg/L	0.5			Tsuda et al., 1999
Rabbit	Black currant (extract)	117 mg 164 mg 53 mg (delphinidin glycosides)	780 µg/L 100 µg/L 450 µg/L	0.5	6.6 µg h/L 0.76 µg h/L 1.8 µg h/L	0.035 (4 h) 0.009 (4 h) 0.023 (4 h)	Nielsen et al., 2003
Pig	Marionberry (powder)	74.2 mg	16 µg/L (C3G) 9.2 µg/L (C3Rut)	1	29.1 µg h/L 24.7 µg h/L	0.088 (24 h)	Wu et al., 2004

[a] amount per kg of body weight (BW). Total amount of ACNs (if not specified)

[b] maximal plasma concentration

[c] time to reach maximum plasma concentration (C_{max})

[d] area under the curve

[e] % of initial dose. The numbers in parenthesis indicate the time after ingestion when the assessment was made

Table 2 -- PK parameters for ACNs estimated in humans

ACN source	**Dose**[a]	C_{max}[b]	t_{max} **(h)**[c]	**AUC**[d]	**Urinary excretion (%)**[e]	**Reference**
Bilberry/lingonberry (puree)	650 mg	62 µg/L	1.5			Nurmi et al., 2009
Blackberry (fruit)	431 mg				0.16 (24 h)	Felgines et al., 2005
Blackcurrant (extract)	111 mg delphinidin 3-rutinoside 82 mg cyanidin 3-rutinoside 32 mg delphinidin 3-glucoside 11 mg cyanidin 3-glucoside	45 µg/L 27 µg/L 11 µg/L 2.3 µg/L	1.75 1.50 1.50 1.25	176 µg h/L 99.8 µg h/L 32 µg h/L 4.1 µg h/L	0.11 (8 h)	Matsumoto et al., 2001
Blackcurrant juice (200 mL)	153 mg				0.02-0.05 (5 h)	Netzel et al., 2001

Blackcurrant (juice)	1095 mg (undiluted juice) 672 mg (juice) 672 mg (juice+rice cake)	53 µg/L 16 µg/L 32 µg/L	0.75 0.75 1.5	19 µg h/L 19 µg h/L 16 µg h/L	0.072 (4 h) 0.048 (4 h) 0.045 (4 h)	Nielsen et al., 2003
Blackcurrant (concentrate)	188.5 mg				0.064 (7 h)	McGhie et al., 2003
Blood orange (juice)	71 mg cyanidin 3-glucoside	0.85 µg/L	0.5	3.1 µg h/L	1.2 (24 h)	Vitaglione et al., 2007
Blueberry (powder)	1200 mg	13.1 µg/L	4			Mazza et al., 2002
Blueberry (extract)	439.1 mg				0.02 (7 h)	McGhie et al., 2003
Boysenberry (concentrate)	344.5 mg				0.029 (7 h)	McGhie et al., 2003
Chokeberry (extract)	1300 mg cyanidin 3-	265 µg/L	0.5-2			Kay et al., 2004

	glycosides					
Chokeberry (extract)	721 mg cyanidin-3-glycosides	43.2 µg/L	2.8	169 µg h/L	0.15 (24 h)	Kay et al., 2005
Cranberry (juice)	94.47 mg	2.15 µg/L	~3	8.7 µg h/L	00.078-3.2 (4 h)	Milbury et al., 2010
Dealcoholized red wine	58 mg malvidin 3-glucoside	0.7 µg/L	1.5	106 µg h/L	<0.03 (6 h)	Bub et al., 2001
Elderberry (extract)	1500 mg	100 µg/L	0.5			Cao & Prior, 1999
Elderberry (juice)	180 mg	35 µg/L	1			Murkovic et al., 2000
Elderberry (juice)	500 mg				0.05 (6 h)	Murkovic et al., 2001
Elderberry (extract)	720 mg	43.8 µg/L	1.2		0.05 (24 h)	Cao et al., 2001
Elderberry (concentrate)	1900 mg				0.03-0.012 (6 h)	Mulleder et al., 2002

Elderberry (extract)	720 mg	43.8 µg/L	1.2		0.05 (24 h)	Milbury et al., 2002
Elderberry (juice)	3570 mg				0.053 (5 h)	Bitsch et al., 2004b
Elderberry/black currant (extract)	148 mg cyanidin 3-glucoside 13.7 mg cyanidin 3,5-diglucoside	13 µg/L	0.5			Miyazawa et al., 1999
Hibiscus (extract)	147.4 mg	3.4 µg/L	1.5	7.37 µg h/L	0.018 (7 h)	Frank et al., 2005
Red grape (juice)	117 mg malvidin 3-glucoside	1.5 µg/L	2	327 µg h/L	<0.03 (6 h)	Bub et al., 2001
Red grape (juice)	283.5 mg	100 µg/L	0.5	168.4 µg h/L	0.23 (7 h)	Frank et al., 2003
Red grape (juice)	283.5 mg	100	0.5	168.4	0.23 (7 h)	Bitsch et al., 2004a

		µg/L		µg h/L		
Red wine	218 mg				1.5-5.1 (12 h)	Lapidot et al., 1998
Red wine	68 mg malvidin 3-glucoside	0.7 µg/L	0.8	142 µg h/L	<0.03 (6 h)	Bub et al., 2001
Red wine	279.6 mg	42.9 µg/L	1.5	100 µg h/L	0.18 (7 h)	Frank et al., 2003
Red wine	279.6 mg	42.9 µg/L	1.5	100 µg h/L	0.18 (7 h)	Bitsch et al., 2004a
Strawberry (fruit)	77 mg pelargonidin 3-glucoside				1.8 (24 h)	Felgines et al., 2003

[a] amount per kg of body weight (BW). Total amount of ACNs (if not specified)

[b] maximal plasma concentration

[c] time to reach maximum plasma concentration (C_{max})

[d] area under the curve

[e] % of initial dose. The numbers in parenthesis indicate the time after ingestion when the assessment was made

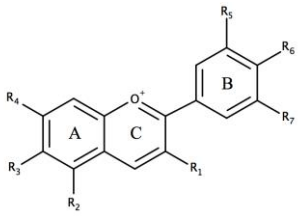

Figure 1 -- Backbone structure of ACNs

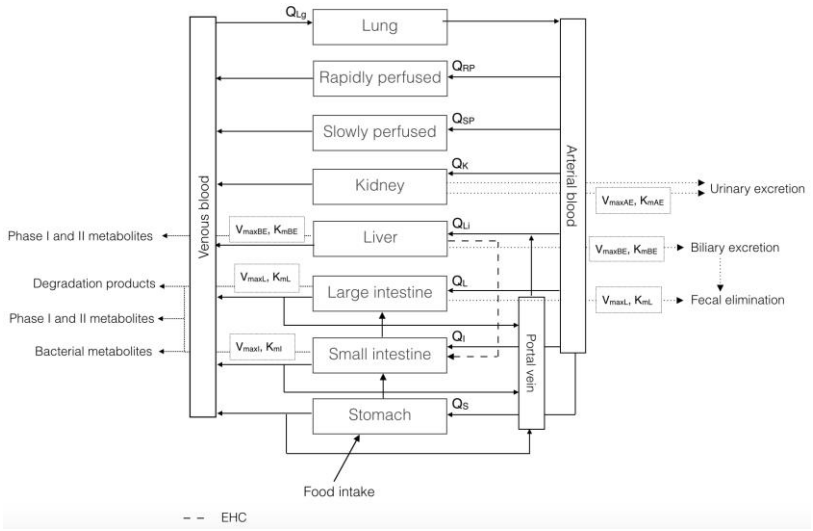

Figure 2 -- Multi-compartmental PBPK model for parent CAN

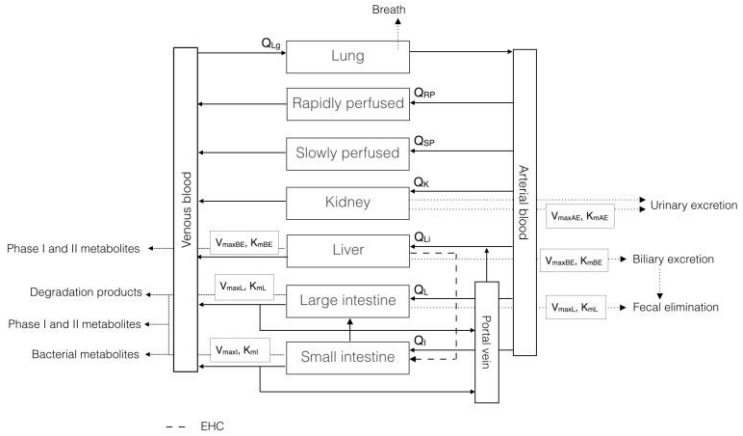

Figure 3 -- PBPK compartment-model for metabolites

