UNIVERSITY OF SÃO PAULO
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Area of Bromatology

RAFAELA ROSSI E SILVA

BIOAVAILABILITY OF ELLAGITANNINS FROM CAMBUCI (*CAMPOMANESIA PHAEA* BERG) IN HEALTHY AND IN OBESE SUBJECTS

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Advisor: Prof. Dr. Maria Inés Genovese Rodriguez

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Judging committee of the dissertation to obtain a degree of Master of Science

_____

Prof. Dr. Maria Inés Genovese
Advisor

_____

1º. Examiner

_____

2º. Examiner

_____

3º. Examiner

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RESUMO

SILVA, R.R. **Avaliação da biodisponibilidade dos elagitaninos de cambuci (*Campomanesia phaea* Berg) em indivíduos saudáveis e com sobrepeso/obesidade.** Faculdade de Ciências Farmacêuticas – Universidade de São Paulo, São Paulo, 2022.

O cambuci (*Campomanesia phaea* Berg), é um fruto nativo da mata Atlântica, pertencente à família das mirtáceas, rico em elagitaninos (ET), proantocianidinas e outros compostos bioativos fenólicos (CBF) que estão associados a vários efeitos biológicos benéficos à saúde humana, tais como atenuação inflamação sistêmica e da resistência à insulina. Evidências apontam que os efeitos benéficos de alguns CBF, como os elagitaninos, estão associados com sua ingestão crônica e à ação dos metabólitos produzidos. As urolitinas são os principais metabólitos produzidos após o consumo de uma fonte rica em elagitaninos. De acordo com o metabólito produzido, os indivíduos podem ser classificados em metabotipos (A, B e 0). No entanto, nada se sabe sobre a absorção e a metabolização dos CBF do cambuci. Desta forma, os objetivos deste trabalho foram a caracterização físico-química da polpa de cambuci, a identificação dos CBF e a avaliação da biodisponibilidade dos polifenóis presentes no suco deste fruto, em seres humanos saudáveis e com sobrepeso/obesidade. Para tanto, os voluntários (n = 28, sendo 15 saudáveis e 13 obesos) consumiram suco de cambuci, e suas respectivas urinas 24 horas, após a ingestão da bebida, foram coletadas para identificação dos metabólitos de elagitaninos. O cambuci apresentou uma alta acidez, com valores de pH de ~2,3 e acidez total titulável de ~1,9 g equivalentes de ácido cítrico/100 g em base úmida (b.u.), e teor de sólidos solúveis de ~7,5 ºBrix, não sendo, portanto, muito ácido. O teor de fenólicos totais encontrado foi ~ 780 mg equivalentes de ácido gálico/100 mL de suco. A quantidade total de flavan-3-ois encontrada na polpa de cambuci foi de 45,45 g/kg em base seca (b.s.) e os principais monômeros identificados foram a galocatequina (22,25 g/kg b.s.) e a epigalocatequina galato (16,48 g/kg b.s.). O grau de polimerização do flavan-3-ol foi de 32,78 indicando uma alta intensidade de adstringência e baixa biodisponibilidade. Através de LC-MS foi feita a identificação de 26 CBF, sendo sua grande maioria derivados de elagitaninos, e dentre os identificados podemos destacar telemagrandina II e pedunculagina. O teor de elagitaninos encontrado foi de ~6,2 mg/g (b.s.), demonstrando que o cambuci é um fruto rico em ácido elágico e seus derivados. Os 28 voluntários que consumiram o suco de cambuci para ensaio de biodisponibilidade foram classificados, pela primeira vez, em metabotipos de acordo com o tipo de urolitina produzida. O metabotipo A foi o mais prevalente (64,3%), seguido pelo metabotipo B (17,9%) e 0 (17,9%). Quando analisados de acordo com o estado nutricional, o metabotipo A foi prevalente em ambos os grupos. Conclui-se, portanto, que o perfil de CBF do cambuci se destaca pela presença de ET, tais como telimagrandina II e pedunculagina. Devido ao alto grau de polimerização não foram observados metabólitos de proantocianidinas. O metabotipo A foi o mais prevalente na população deste estudo, e o estado nutricional pode não ser um fator determinante no tipo de urolitina produzida.

Palavras-Chaves: compostos fenólicos, cambuci, biodisponibilidade, urolitinas.

ABSTRACT

SILVA, R.R. **Bioavailability of ellagitannins from cambuci (*Campomanesia Phaea* Berg) in healthy and obese subjects.** Faculty of Pharmaceutical Sciences – University of São Paulo, São Paulo, 2022.

Cambuci (*Campomanesia phaea* Berg) is a native fruit of the Atlantic Coastal Forest, belonging to the Myrtaceae family, rich in ellagitannins (ET), proanthocyanidins and other bioactive phenolic compounds (BPCs) related to beneficial effects to human health, such as systemic inflammation and attenuation of insulin resistance. Evidence indicates that the beneficial effects of some BPCs, such as ellagitannins, are associated to their chronic intake and the action of the metabolites produced. Urolithins are the main metabolites produced after consumption from a rich source of ellagitannins. According to the metabolite produced, subjects can be classified into metabotypes (A, B e 0). However, nothing is known about the uptake and metabolism of BPC from cambuci. Thus, the objectives of this study were the physical-chemical characterization of cambuci pulp, identification of BPC profile and the determination of their bioavailability in healthy and overweight/obese subjects. Therefore, subjects (n = 28, being 15 healthy and 13 overweight/obese) consumed cambuci juice, and their respective urines 24 hours after drinking were collected to identify the metabolites of ellagitannins. Cambuci presented high acidity, with pH values of ~ 2.3 and titratable acidity of ~ 1.9 g citric acid equivalents/100 g fresh weight (FW), and solids content of ~7.5 ° Brix, not being characterized as a very sweet fruit. The total phenolic content was ~ 780 mg gallic acid/100 mL juice. The total amount of flavan-3-ols found in the fruit was 45.45 g/kg dry weight (DW) and the main monomers identified were gallocatechin (22.25 g/kg DW) and epigallocatechin gallate (16.48 g/kg DW). The degree of polymerization of flavan-3-ol was 32.78, indicating a high intensity of astringency and low bioavailability. Through LC-MS, 26 BPCs were identified, most of them being derived from ellagitannins, and among those identified, telemagrandin II and, pedunculagin. The total ellagic acid content found was ~6.2 mg/g DW, demonstrating that cambuci is a fruit rich in ellagic acid and its derivatives. The 28 volunteers who consumed cambuci juice for the bioavailability assessment were classified, for the first time, into metabotypes according to the type of urolithin produced. Metabotype A was the most prevalent (64.3%), followed by metabotype B (17.9%) and 0 (17.9%). When analyzed according to nutritional status, metabotype A was prevalent in both groups. In conclusion, the BCP profile of cambuci stands out for the presence of ET, such as telemagrandin II and pedunculagin. Due to the high degree of polymerization, no proanthocyanidin metabolites were observed. Metabotype A was the most prevalent in this study population, and nutritional status may not be a determining factor in the type of urolithin produced.

Keywords: phenolics compounds, cambuci, bioavailability, urolithins.

FIGURE LIST

TABLE LIST

SYMBOLS AND ABBREVIATIONS

BMI – body index mass

BPCs – bioactive phenolic compounds

C18 – Octadecylsilane resin

CAE – citric acid equivalents

COMT – catechol-O-methyltransferase

DAD – Diode Array Detector

DP – degree polymerization

DW – dry weight

ESI – Electrospray ionization

ET– ellagitanninns

FW – fresh weight

GAE – Gallic acid equivalent

GE – glucose equivalent

HCl – Chloride acid

HHDP – hexahydroxydiphenic

HIV – Human Immunodeficiency Virus

H2O – Water

HPLC – High-performance liquid chromatography

Iso Uro A – isourolithin A

IsoUro A 3-glur – isourolithin A 3-glucuronide

IsoUro A 9-glur – isourolithin A 9-glucuronide

Kcal – Kilocalories

LADME – liberation, absorption, distribution, metabolism, and elimination

LC-MS – Liquid chromatography/mass spectrometry

M – mol/L

m/z – mass-to-charge

mDP – mean degree polymerization

MS – Mass spectometry

N2 – Nitrogen

NaOH – Sodium hydroxide

OS – oxidative stress

PA – Proanthocyanidin

PTFE – Teflon

PVDF – polyvinylidene difluoride membrane

RONS – reactive oxygen and nitrogen species

ROS – reactive oxygen species

RT – Retention time

SPE – Solid phase extraction

SULT – sulfotransferase

TA – Total titratable acidity

T1DM – Type 1 diabetes mellitus

T2DM – Type 2 diabetes mellitus

TSS – Total soluble solids

UGT – uridine 5'-diphospho glucuronosyltransferase

Uro A – urolithin A

Uro A-glur – urolithin A-glucuronide

Uro B – urolithin B

Uro B-glur – urolithin B-glucuronide

UV – Ultraviolet

v/v – volume/ volume

WHO – World health organization

SUMMARY

1. INTRODUCTION

1.1. BIOACTIVE PHENOLIC COMPOUNDS IN FOOD

Cardiometabolic diseases which include cardiovascular disease, type 2 diabetes mellitus (T2DM), obesity, and their risk factors are the main causes of morbidity and mortality worldwide (LANDBERG *et al*., 2019). It is estimated that the annual Brazilian economic burden of cardiometabolic diseases represent approximately R$3.45 billion for the health care system (NILSON *et al*., 2020). Population studies have shown that adherence to a healthy lifestyle is associated with a longer life expectancy free of major chronic diseases (LI *et al*., 2020). An adequate and regular intake of fruits and vegetables has been reported as a relevant factor in promoting human health by reducing the risk of cardiometabolic diseases and other chronic non-communicable diseases (LAPUENTE *et al*., 2019).

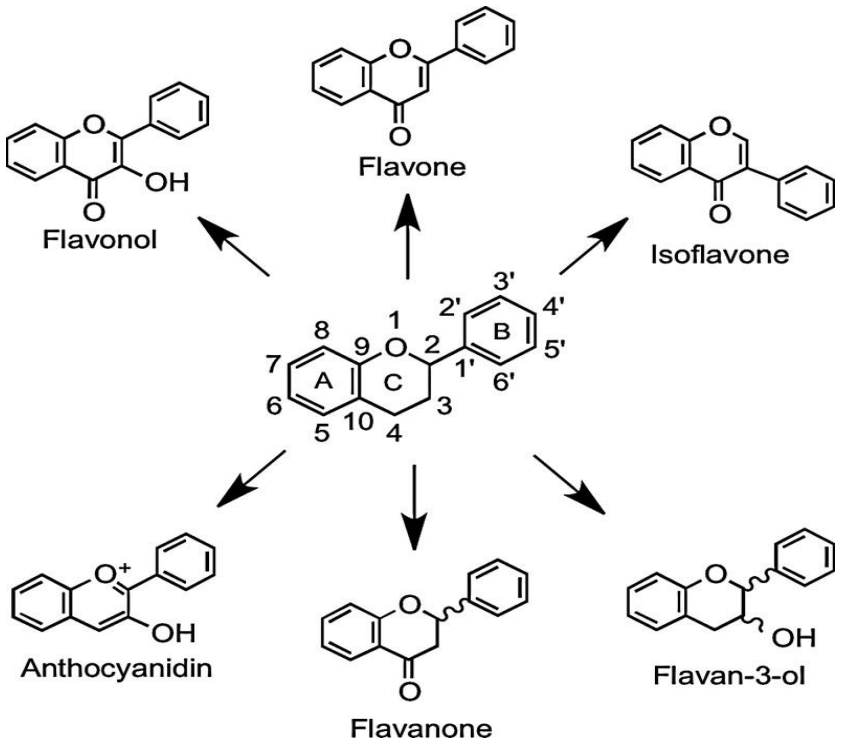
The nutritional relevance of fruits and vegetables is attributed to the content of macronutrients such as fibers, as well as micronutrients including vitamins and minerals. Besides these macros and micronutrients, foods of plant origin are recognized as sources of bioactive compounds, also known as phytochemicals, and among these polyphenols (phenolic compounds) play a crucial role (BRESCIANI *et al*., 2017).

Polyphenols are secondary metabolites produced by plants and are related to defense responses against external aggressions or interactions with the environment. They may act as attractants or repellents to insects, influence the color, oxidative stability, and flavor of the plant or fruit (LIN *et al*., 2016). The concentration of polyphenols depends on the type of plant, stage of maturation, type of soil, post-cultivation storage, among others (KLEPACKA; GUSJKA; MICHALAK, 2011).

Phenolic compounds are characterized by the presence of at least one phenolic skeleton with one or more hydroxyl groups attached to their chemical structures. Several different molecules have already been identified being grouped into classes according to their chemical structure (BASTOS; ROGERO;

ARÊAS, 2009). They often are divided into two different classes: flavonoids and non-flavonoids. The chemical structure of flavonoids is composed of two aromatic rings connected by a three-carbon bond (C6-C3-C6). Flavonoids are found in high abundance in the plant kingdom and are divided into subclasses depending on the oxidation state of the central ring (**Figure 1**). The main subclasses are: flavones, flavonols, flavanones, flavanols, isoflavones, and anthocyanidins. Non-flavonoids such as phenolic acids include benzoic acid and derivatives (i.e. hydroxybenzoic, vanillic, gallic) and cinnamic acid and derivatives (i.e. cumaric, caffeic, ferulic, chlorogenic) (CROZIER; JAGANATH; CLIFFORD, 2009).

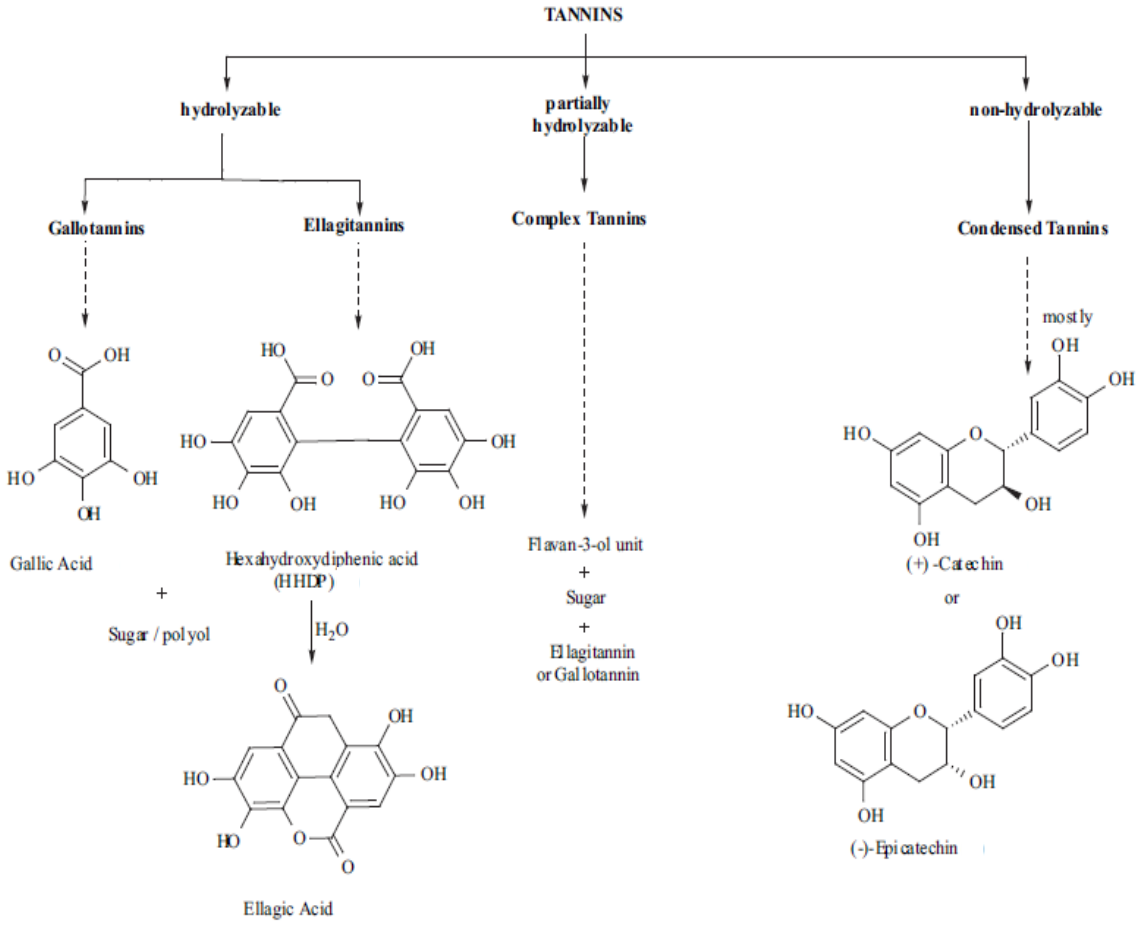
Figure 1. Chemical structure of flavonoids



Source: Adapted from (DEL RIO *et al*., 2013).

Tannins are phenolic compounds of high molecular weight, soluble in water, and can precipitate with protein and alkaloids. Although they have these properties in common, their structural characteristics are different and are divided regularly into three main groups: condensed tannins (known as proanthocyanidins), hydrolyzable tannins (gallotannins and ellagitannins), and complex tannins, with less distribution in nature (**Figure 2**) (COS *et al*., 2004).

Figure 2. Classification of tannins.

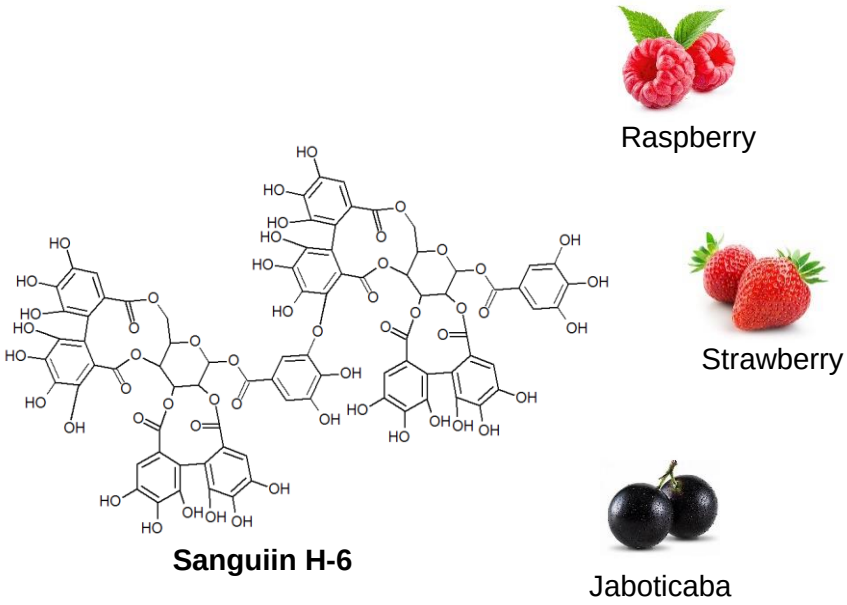


Source: Adapted from (COS *et al*., 2004).

The main ellagitannins identified in plant foods, especially in fruit and nuts, are punicalagin, lambertianin C, pedunculagin, casuarictin, and sanguiin H-6 (**Figure 3**). The occurrence of ellagitannins in plant foods is restricted to few fruits such as berries of the genus *Rubus* (cherry, blackberry, blueberry, cranberry), *Fragaria* (strawberry), and *Punica* (pomegranate) (VILLALBA *et al*., 2019).

However, other plant foods have been identified as sources of ellagitannins including fruits of the Myrtaceae family such as cambuci (*Campomanesia phaea*), jaboticaba (*Myrciaria jaboticaba*), grumixama (*Eugenia brasiliensis*), and camu-camu *(Myrciaria dubia* Mc) (ABE; LAJOLO; GENOVESE, 2012).

Figure 3. Chemical structure of the main ellagitannins and their sources in the diet.

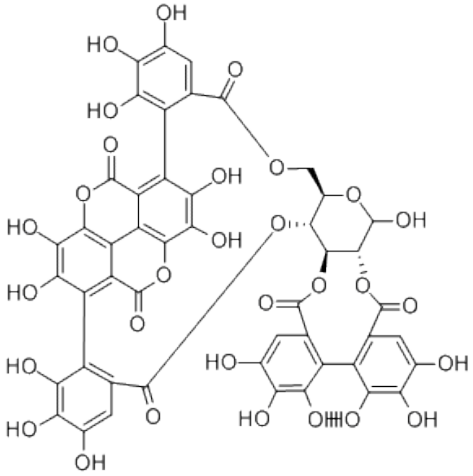


Sanguiin H-6

Raspberry

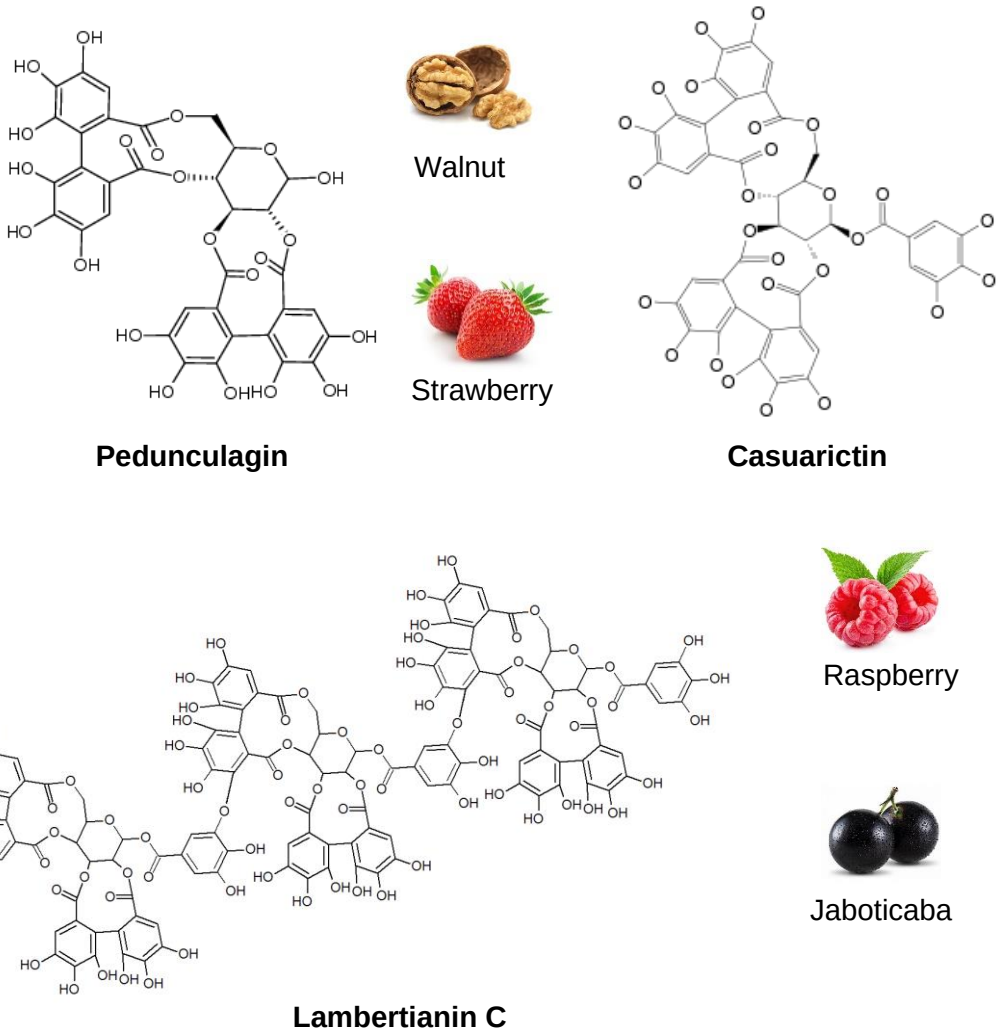
Strawberry

Jaboticaba



Punicalagin

Pomegranate



Pedunculagin **Casuarictin**

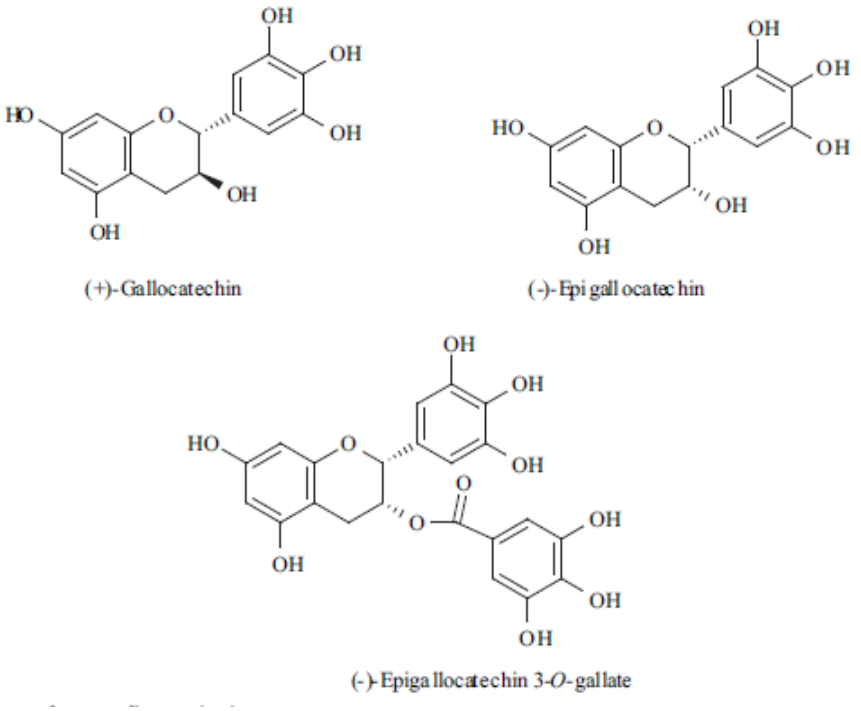
Lambertianin C

Source: Adapted from (VILLALBA *et al*., 2019)

Proanthocyanidins (PAs, condensed tannins) are oligomer or polymers formed by monomeric units of flavan-3-ol derivatives. In contrast to hydrolyzable tannins, condensed tannins do not present a polyol group attached to the molecule in the central core and are not readily hydrolyzed (COS *et al*., 2004). The most studied condensed tannins are based on molecules with monomeric units of flavan-3-ols, mainly (-) - epicatechin and (+) - catechin. Other important flavan-3-ols are (-) - epigallocatechin gallate, (-) - epigallocatechin and (+) – gallocatechin (**Figure 4**). The structures of PAs differ in the position and configuration of their monomeric linkages. According to the structure of their

monomer, PAs may be classified as procyanidins, prodelphinidins, and propelargonidins (MENA *et al*., 2014).

Figure 4. Chemical structure of some flavan-3-ols.



Source: Adapted from (COS *et al*., 2004).

PAs are present in nuts, fruits, flowers, bark, and seeds of several plants as complex mixtures of polymers with a degree of polymerization varying from 3 to 11. The main dietary sources of PAs are red wine, teas, chocolate, and fruits such as grape, apple, persimmon, and cranberry (RAUF *et al*., 2019). Some fruits from the Myrtaceae family, such as grumixama, jaboticaba, camu-camu are also proanthocyanidin sources in a plant-based diet (ABE; LAJOLO; GENOVESE, 2012).

Phenolic compounds have been widely studied due to their biological effects, which can be beneficial to human health. These benefits are mainly related to their direct and indirect antioxidant actions (COSME *et al*., 2020). Phenolic compounds can neutralize reactive oxygen and nitrogen species (RONS), which are byproducts of physiological aerobic metabolism. Under

normal conditions, the generation of RONS is kept under control by cellular antioxidant defenses and repair systems and is involved in the production of energy from organic molecules, in the immune defense, and signaling process. When there is excessive production, cell defense systems fail and RONS accumulate, playing an important role in the onset of oxidative stress (OS) and inflammation, causing irreversible damage to DNA, lipids, and proteins, promoting aging, age-related diseases, and various degenerative diseases. OS induces a cascade of events that triggers and accompanies molecular/cellular pathogenic events, responsible for several human disorders, including carcinogenesis, atherosclerosis, cardiovascular and neurodegenerative diseases (ALFEI; MARENGO; ZUCCARI, 2020).

Polyphenols are able to donate electrons to oxidizing species, eliminate free radicals and chelate metal ions, or indirectly attenuate the production of reactive oxygen species (ROS), either by improving the activity of antioxidant enzymes or by inhibiting enzymes that induce pro-oxidant effects. Furthermore, polyphenols also have other biological effects related to their antioxidant capacity, such as antimicrobial, anti-inflammatory, anticancer, and cardioprotective activities (COSME *et al*., 2020).

1.2. BIOAVAILABILITY OF PHENOLIC COMPOUNDS

The term bioavailability has been widely used and widespread in pharmacology and refers to the amount of a drug, administered in a pharmaceutical form, that reaches the systemic circulation, and the speed necessary for this process to occur (ANVISA, 2017). For the bioavailability of food, many definitions have been suggested over the years of studies of this concept, and the most appropriate defines bioavailability of food as being the fraction of a nutrient or an ingested compound that reaches the systemic circulation and subsequently specific targets to exert its biological action (D'ARCHIVIO *et al*., 2010).

Thus, to exert a health benefit, the phenolic compound needs to reach the tissue where it will perform its function. For this, after ingestion, the phenolic

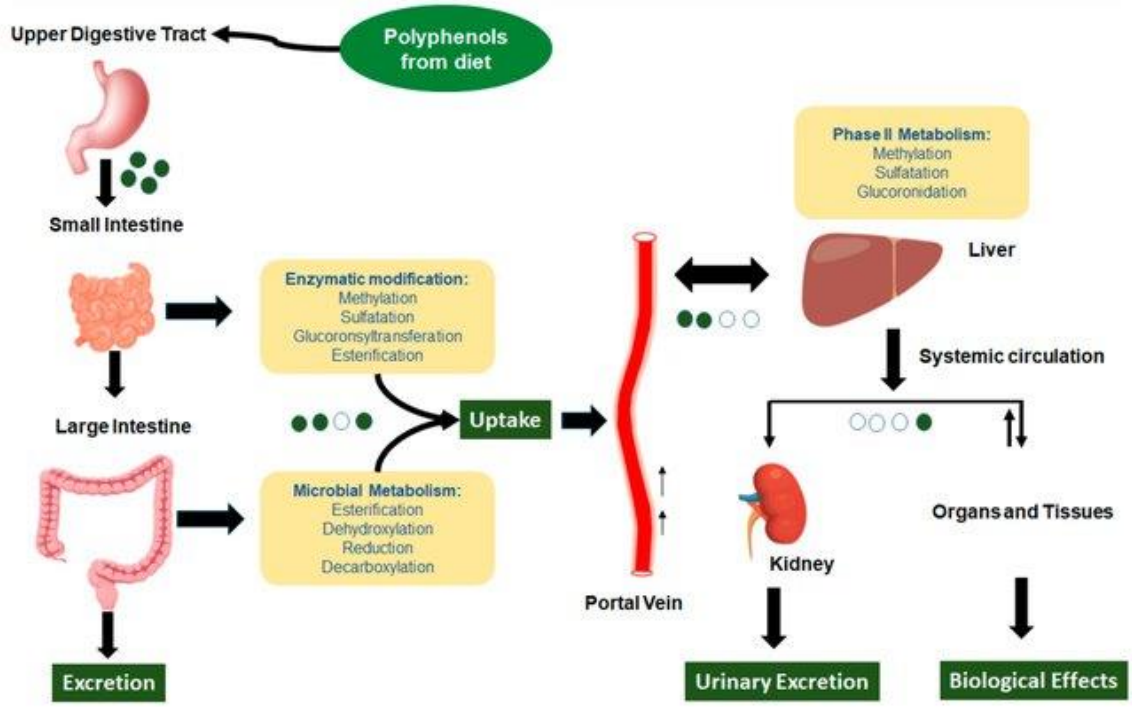
compound must be released from the matrix and be bioaccessible in the gastrointestinal tract, to be metabolized. Several factors can influence bioavailability such as the composition of the food matrix, chemical structure of the compound, and intake associated with other foods (PARADA; AGUILERA, 2007).–This suggests that the daily consumption of foods that contain high concentrations of polyphenol does not guarantee that these compounds will have biological properties. Bioavailability covers several processes such as the liberation from a food matrix and phases of absorption, distribution, metabolism, and elimination (LADME) (REIN *et al*., 2013).

Polyphenols can be absorbed in the gastrointestinal tract after being released from the food matrix and being bioaccessible. Interaction with other foods in the diet, solubility, different cell transporters, molecular transformation, metabolism, and interaction with the intestinal microbiota, can influence the absorption of these compounds-(REIN *et al*., 2013).

Polyphenols are found in foods as polymers, glycosides, or esters and are unabsorbed as such (**Figure 5**). This reaction releases the aglycone form of the polyphenol, which can then be absorbed by the enterocyte. Aglycone forms can also be released into the enterocyte by hydrolysis mediated by the cytosolic β-glycosidase enzyme. In enterocytes, aglycone forms can be resulting in glucuronidated and/or methylated forms through being conjugated by phase II enzymes. As a large part of the polyphenols are unabsorbed in the small intestine, these compounds are metabolized by the microbiota into smaller molecules when they reach the large intestine (CIPOLLETTI *et al*., 2018). The molecules that have been absorbed are metabolized in the liver to form sulfated, glucuronidated and/or methylated metabolites through the action of phase II sulfotransferase (SULT), uridine 5'-diphospho glucuronosyltransferase (UGT), and catechol-O-methyltransferase (COMT). These biotransformations represent a metabolic detoxification process, which facilitates biliary and urinary elimination due to the increased solubility of these compounds (CROZIER; DEL RIO; CLIFFORD, 2010). From the liver, bioactive metabolites may be excreted in the bile or systemic circulation. The metabolites present in the systemic circulation

are lastly distributed to the target tissues or excreted in the urine (REIN *et al*., 2013).

Figure 5. Schematic representation of the absorption and metabolism of dietary polyphenols.



Source: Adapted from (CIPOLLETTI *et al*., 2018).

One of the main objectives of the assessment of polyphenols bioavailability is the identification of biomarkers. Through this approach, biological activities and their respective mechanisms of action can be elucidated, and even the ingestion of food sources, as well as an association between diet and health, can be assessed. Thus, in order to establish a biomarker from a specific polyphenol, the exploration of its pharmacokinetic parameters is essential (ZAMORA-ROS *et al*., 2012).

Current scientific knowledge suggests that substances such as PAs, which have a high molecular weight and a high degree of polymerization, are very unlikely to be absorbed intact and reach target tissues. The PAs ingested by the diet do not undergo metabolism in the oral cavity, and interact with the proteins

present in the saliva, causing astringency. They reach the stomach and resist acidic conditions. In the small intestine, PAs form complexes with proteins and digestive enzymes, limiting their absorption. The remaining PAs reach the caecum without suffering extensive depolymerization. In the colon, intact or hydrolyzed PAs undergo intense biotransformation by resident colonic microflora, forming phenolic acids, hypuric acids, simple phenols, and valerolactones. Some metabolites produced are efficiently absorbed and travel to the liver to undergo metabolization, producing glucuronidated, methylated or sulfated metabolites. Unabsorbed metabolites are excreted in feces and urine. PAs with a degree of polymerization above 3 are almost unabsorbed in the upper gastrointestinal tract with more than 90% of the ingested PAs reaching the colon, where their active metabolization is closely related to the host microbiota (MENA *et al*., 2014).

Ellagitannins are metabolized in a similar way to PAs during their passage through the gastrointestinal tract. In the small intestine, ellagitannins are hydrolyzed to ellagic acid and gallic acid due to the physiological alkaline pH and/or the action of microbial enzymes. A small part of these phenolic acids is absorbed by the enterocyte and undergoes phase II metabolism (for reactions of glucuronidation, sulphation, methylation) in the enterocyte and/or in the liver. The remaining ellagic acid that has not been absorbed reaches the colon and is biotransformed into urolithins by the action of the intestinal microbiota. Thus, urolithins are the main biomarkers of ellagitannin consumption (MENA *et al*., 2014).

As the intestinal microbiota is responsible for the bioconversion of ellagitannins into urolithins, an imbalance of the intestinal microbiome (dysbiosis), which is associated with some chronic diseases, inflammatory bowel diseases, colon cancer, cirrhosis, metabolic syndrome, and obesity, may be behind of the difference in the metabolism of ellagitannins (TOMÁS-BARBERÁN *et al*., 2014). Although the mechanisms are not yet fully understood, it is known that different bacterial species regulate this bioconversion process (TOMÁS-BARBERÁN *et al*., 2016).

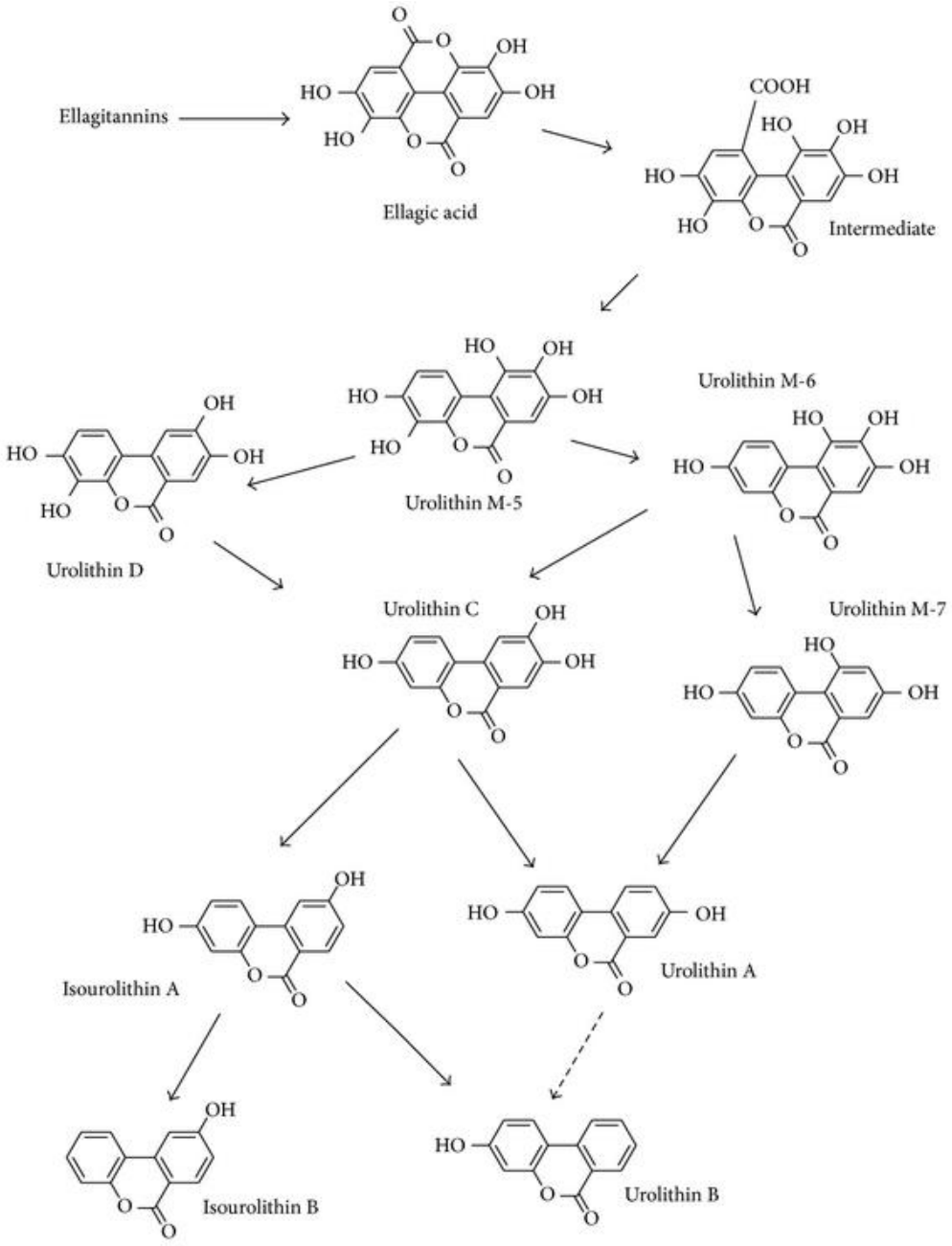
The first metabolite produced from ellagic acid is called urolithin M-5 (pentahydroxy-urolithin), formed from the opening of the lactone ring and a decarboxylation (**Figure 6**). Uro-M5 is considered a determinant intermediate

since successive dehydroxylation reactions occur and other derivatives are produced from it. The first isomers formed are Uro-D and Uro-M6 (tetrahydroxy-urolithins), which are converted to Uro-C and Uro M-7 (trihydroxy-urolithins), and finally, Uro-A and IsoUro-A (dihydroxy-urolithins), or even Uro-B and IsoUro-B (monohydroxy-urolithins), which are the main metabolites detected in human plasma and urine (ESPÍN; GONZÁLEZ-SARRÍAS; TOMÁS-BARBERÁN, 2017; TOMÁS-BARBERÁN *et al*., 2016).

The aglycone urolithins produced by the action of the intestinal microbiota are absorbed, reach the liver, and undergo phase II reactions. These metabolites, now more soluble, reach the systemic circulation and can reach their respective target tissues to exert biological actions. Finally, conjugated urolithins are excreted in the urine (CROZIER; DEL RIO; CLIFFORD, 2010; MENA *et al*., 2014).

The differences in the production of urolithins could explain, at least in part, the large variability in health effects observed *in vivo*. Urolithin A is the main metabolite detected in human plasma and urine, mainly in the conjugated form with glucuronic acid. However, conjugates of isourolithin A and urolithin B were also observed in some volunteers, while in others there was no production of these urolithins. Therefore, recently, stratification of human beings has been proposed according to their capacity to produce specific microbial metabolites derived from the consumption of ellagitannins independent of demographic characteristics, health status, food source of ellagitannin. Three metabotypes are suggested as follow: metabotype A (producers of free or conjugated urolithin A), metabotype B (producers of urolithin A, isourolithin A, and free or conjugated urolithin B) or metabotype 0 (non-producers of these forms of urolithins) (TOMÁS-BARBERÁN *et al*., 2014).

Figure 6. Bioconversion of ellagitannins to urolithins.



Source: Adapted from (ESPÍN *et al*., 2013).

1.3. CAMBUCI

Cambuci (*Campomanesia phaea* Berg) is a native Brazilian fruit belonging to the Myrtaceae family, originating from the Atlantic Forest biome. The Myrtaceae are aromatic trees or shrubs, which often produce edible fruits, and several members of this family are used in folk medicine as antidiarrheals, antimicrobials, antioxidants, antirheumatics, anti-inflammatories and/or lipid-lowering agents (STEFANELLO; PASCOAL; SALVADOR, 2011). Myrtaceae fruits are also known to be a rich source of ellagitannins. Until recently, berries were believed to be exclusive sources of ellagitannins; however, a study conducted by Abe *et al*. (2012) found that fruits belonging to the Myrtaceae family are also rich in dietary sources of ellagitannins (LANDETE, 2011).

The cambuzeiro occurs in the states of Minas Gerais and São Paulo, mainly in the Serra do Mar region. Formerly, it was abundant in the city of São Paulo, having even given rise to the name of the Cambuci neighborhood. Nowadays, it is one of the species of the Atlantic Forest in danger of extinction. The height of the trunk varies from 3 to 5 m, and the diameter from 20 to 30 cm. According to Vallilo *et al*. (2005), during the months from August to November, cambuci shows a white flowering and its fruiting occurs in the months of January and February. The similarity of the edible fruit with pots formerly made by the Indians probably gave rise to the name "cambuci", which in Tupi-Guarani language means clay pot (LANDRUM; KAWASAKI, 1997).

The leaves of this species are rich in linalool (11.1%), caryophyllene oxide (11.8%), beta-caryophyllene (6.3%), beta-selinene (6.3%), and alfa-cadinol (1.9%), constituents of high commercial value for the pharmaceutical and cosmetics industry (ADATI, 2001).

The ripe fruits are rhomboid, green, fleshy, juicy, extremely aromatic, and widely used in the preparation of juices, ice creams, and alcoholic beverages ("pinga de cambuci") by the local population (**Figure 7**). They usually measure 5 to 6 cm in diameter in the median region, by 3 to 4.5 cm in height. They have an intense pleasant aroma, giving them great potential as flavoring agents in food and beverages. The cambuci is considered a fruit with high acidity (pH 2.4 - pH 3.0) and a high fiber content when compared to other species of the same

botanical family, as has considerable sodium, potassium, and magnesium contents (AZEVEDO *et al*., 2017).

Figure 7. Cambuci

Source: available in http://cunhacomerciodefrutas.com.br/product/cambuci/

In the case of bioactive phenolic compounds, the fruits of this plant have high levels of ellagic acid, mainly in the form of ellagitannins (ABE; LAJOLO; GENOVESE, 2012). Flavonoids such as quercetin and kaempferol derivatives, although in small amounts, are also present in cambuci (GONÇALVES; LAJOLO; GENOVESE, 2010).

Due to the high content of ellagitannins present in cambuci, studies have been conducted to assess the possible beneficial health effects. Donado-Pestana *et al*. (2015) demonstrated through an experimental model using C57BL/6J mice with induced obesity by a diet rich in sucrose and fats, that cambuci phenolic extracts, rich in ellagitannins, reduced fasting glucose and glucose intolerance. In another study, following a similar experimental model, cambuci reduced body weight gain, inflammation, fatty liver, hyperglycemia, glucose intolerance, and insulin resistance in the liver and skeletal muscle, and these effects were associated with the activation of AKT and AMPK pathways in these tissues (DONADO-PESTANA *et al*., 2021).

Human health properties have been associated to polyphenols consumption. A study conducted by Balisteiro *et al*. (2017) evaluated the effect of some fruits of the Myrtaceae family on postprandial glycemia in healthy

individuals during a meal test (30 g of carbohydrates contained in one unit of white bread). It was observed that after 2 h of consumption of the clarified cambuci juice, the amount of glucose absorbed was 36% lower in comparison to the control water, demonstrating that cambuci may be a promising fruit as a co-adjuvant in the treatment for hyperglycemia (BALISTEIRO *et al*., 2017).

Although cambuci is a source of ellagitannins, it is unknown which classes of ellagitannins are present nor if they are susceptible to metabolism/absorption after human consumption. In this context, the present work aimed to chemically characterize the phenolic compounds present in cambuci, especially ellagitannins, and to evaluate the ellagitannins metabolites formed after the consumption of the commercial fruit pulp by both, healthy and obese subjects.

2. OBJECTIVES

2.1. GENERAL OBJECTIVE

The present study aimed to evaluate the metabolization of ellagitannins present in cambuci fruit juice, both in normal and obese subjects

2.2. SPECIFIC OBJECTIVES

- To chemically characterize cambuci phenolic compounds, including ellagitannins.
- To determine whether cambuci ellagitannins could be metabolized to urolithins.
- To verify eventual differences in cambuci ellagitannins metabolization among normal and obese individuals.

3. MATERIALS AND METHODS

3.1 MATERIAL

Cambuci frozen pulps, ready for human consumption, were purchased from the Institute AUÁ of Socioenvironmental Enterprise (São Paulo, Brazil). The cambuci juice were prepared defrosting 300 g of the pulp by adding 200 mL of water.

3.2. METHODS

3.2.1. Total soluble solids (TSS)

A digital refractometer determined TSS content (ATAGO N1, Atago, Tokyo, Japan) with automatic temperature compensation of 20 °C. Results were expressed as °Brix (AOAC, 1995).

3.2.2. Total sugars

Total sugars were determined using a spectrophotometer (HITACHI mod. U-1100) (DUBOIS *et al*., 1956). An aqueous glucose solution (1%) was used to obtain a standard curve. Samples were analyzed at 490 nm wavelength, and results were expressed in grams of glucose equivalents per 100 g of cambuci pulp (GE/100 g).

3.2.3. Total titratable acidity (TA)

TA was determined by titration, using NaOH (0.1 mol/L) as titrant solution and phenolphthalein as indicator (AOAC, 1995). Results were expressed as citric acid equivalents per 100 g of cambuci pulp (CAE/100 g).

3.2.4. Determination of hydrogenionic potential (pH)

Hydrogenionic potential (pH) was determined using a digital potentiometer (model pH20-pH21, Hanna Instruments, Woonsocket, USA) calibrated with pH 4.0 and 7.0 buffer solutions (Synth, Diadema, BR).

3.2.5. Ash

Cambuci pulp was incinerated in a muffle furnace for 4 h (Robertshaw 318-24, Quimis, Diademas, BR) at 550 °C to determine ash content (AOAC, 1995).

3.2.6. Moisture

Cambuci pulp was dried for six hours at 70 ºC under reduced pressure in vacuum oven (Orion 515, Fanem, São Paulo, BR) to determine moisture content (AOAC, 1995).

3.2.7. Lipids

Lipids from cambuci pulp were extracted with ethyl ether using Soxhlet extractor (Sebelin TE-188, Tecnal, Piracicaba, Brazil) for eight hours. Then, solvent was evaporated, and vial containing lipids were weighed to determine fat content (AOAC, 1995).

3.2.8. Proteins

Proteins were quantified by the micro-Kjeldahl procedure (TE-0363, Tecnal, Piracicaba, Brazil), using a general factor of 5.75 (AOAC, 1995).

3.2.9. Carbohydrates

Carbohydrates were calculated by subtracting moisture, lipids, proteins, fibers, and ash percentages from 100%.

3.2.10. Determination of total fibers

Total fibers were determined in cambuci pulp according to AOAC (1995), which is applicable for foods with less than 2% starch contents (dry basis). Vials containing 5 mL of pulp and 20 mL of water (n = 4) were covered with foil and incubated in a 37 °C water for 90 minutes. Then, 100 mL of absolute ethanol was added, forming a suspension that was kept for 60 min in a 25 °C water. After this period, the suspension was vacuum filtered using glass wool in a pre-tared filter crucible n°. 2 (coarse ASTM 40-60 mm). Residues were washed sequentially with aqueous ethanol solution (78%, two times), ethanol (95%, two times), and acetone (one time). Then, they were oven-dried at 105 ºC for 120 min, cooled in a desiccator, and weighed. Protein and ash contents were analyzed in residues, and subsequently were discounted for the calculation of total fibers. The results were expressed in grams of fibers per 100 g of cambuci pulp FW.

3.2.11. Mineral composition

Mineral composition was assessed by determining boron, calcium, copper, iron, magnesium, manganese, nitrogen, phosphorus, potassium, selenium, sodium, sulfur, and zinc. Sodium and potassium were quantified by flame-emission spectrophotometry and other minerals by atomic absorption spectrophotometry (MALAVOLTA, 1989).

3.2.12. Total phenolic content

Total phenolic content was determined according to Singleton with modifications Magalhães *et al*. (2010). Gallic acid standard solution or diluted samples and Folin-Ciocalteu reagent (50 µL) were pipetted in a 96-well microplate and stirred for 5 min at room temperature. Then, NaOH (100 µL, 0.35 mol/L) was added, and the blend was stirred for another 5 min. Finally, absorbance was read at 760 nm using a spectrophotometer (BioTek Instruments,

Winooski, VT). Results were expressed as gallic acid equivalents per 100 g of cambuci pulp FW.

3.2.13. Identification and characterization of the phenolic compounds of cambuci

3.2.13.1. Identification and characterization of cambuci phenolic compounds by High-Performance Liquid Chromatography coupled to mass detector (HPLC-DAD MS/MS)

To identify phenolic compounds, 500 mg of cambuci was extracted from pulp with 70% methanol (10 mL) for 1 min (n=3) and centrifuged at 4,696 *g* for 10 min (20ºC). Supernatant was collected, filtered using a 0.22 µm PVDF filter (Millipore Ltd., Bedford, MA, USA), analyzed in chromatographic system Agilent 1200 series (Agilent Technologies, Waldbronn, Germany) equipped with diode array detector, and coupled to an electrospray ionization ion trap mass spectrometer (Bruker, Daltonics, Bremem, Germany). Phenolic compounds were separated in a Pursuit XRs C18 reverse-phase column (250 x 4 mm, 5 µm, Agilent Technologies, Waldbronn, Germany) at 25 ºC. Gradient mobile phase consisted of 1% aqueous formic acid (eluent A) and acetonitrile (eluent B) at a flow rate of 800 µL/min. Before sample injection, column was equilibrated with 5% of eluent B. Then, sample was injected (8 µL), and eluent B concentration was increased to 9% B at 5 min, 16% at 15 min, 50% at 45 min, and 90% from 47 to 49 min. Initial conditions were re-established at 52 min and kept isocratic until 57 min. Optimal ESI-MS parameters using nitrogen as nebulizer gas were capillary voltage of 3500 V, drying gas flow of 10 L/min, nebulizer pressure of 40 psi, and drying temperature of 300 ºC. MS spectra were acquired in negative ionization mode and measured in selective ion monitoring mode. Phenolic compounds were identified by comparing fragmentation patterns, characteristic of each compound, from their respective molecular ions (GARCÍA-VILLALBA *et al*., 2015).

3.2.13.2. Ellagitannins by acid hydrolysis

Quantification of ellagitannins was adapted from García-Villalba *et al*. (2015). Initially, lyophilized cambuci pulp (100 mg) was dissolved in 4 N HCl solution (5 mL), homogenized in a vortex, and placed in a digester block at 90 ºC for 12h. After cooling to room temperature, pH was adjusted to 2.5 with 5 N NaOH solution, and samples were centrifuged at 3,000 *g* for 10 min at 4 ºC. Subsequently, supernatant volume was adjusted to 10 mL with ultra-pure water and filtered using a 0.45 µm filter. Pellet was re-extracted with 10 mL of methanol:dimethyl sulfoxide (50:50 v/v), followed by vortex homogenization. Supernatant from re-extraction of the pellet was filtered with 0.22 µm filter PTFE membrane (Millipore, MA, USA). Samples were injected into Hewlett-Packard 1100 equipped with an automatic sample injector, quaternary pump, and diode array detector. Chromatographic separation of urolithins was performed using LiChromCART C-18 reverse phase column (250 mm X 4 mm, 4.5 µm, Merck, Darmstadt, Germany) at 25ºC. Ellagitannins were quantified at 280 nm with an ellagic acid standard curve (1-2000 mg/L) purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2.13.3. Proanthocyanidins by phloroglucinolysis

Proanthocyanidins analysis was performed according to Karonen *et al*. (2007) (n=3). Initially, lyophilized cambuci pulp (50 mg) was dissolved in 0.8 mL of phloroglucinol solution and methanol acidified with 0.1 M HCl containing 50 mg/mL phloroglucinol and 10 mg/mL ascorbic acid. This blend was vortexed and placed in a water at 50 ºC for 20 min. Then, it was cooled in an ice bath, and 1 mL of 40 mM sodium acetate was added to stop the reaction. Finally, the blend was centrifuged at 3,000 *g* for 10 min at 4 ºC, filtered using a 0.45 µm filter, and analyzed by HPLC-DAD-IT MS/MS. Chromatographic conditions were the same described in section **3.2.13.1**. Commercial standards were used for quantification of total flavan-3-ols (flavan-3-ol monomers and phloroglucinol adducts). Apparent mean degree of polymerization (mDP) was calculated by adding all subunits

(flavan-3-ol monomer and phloroglucinol adducts) and dividing by all flavan-3-ol monomers.

3.3. BIOAVAILABILITY OF CAMBUCI (*Campomanesia phaea* Berg) PHENOLIC COMPOUNDS - STUDY PROTOCOL

3.3.1. Individuals and experimental design

Thirty-two volunteers self-reported healthy were recruited to assess bioavailability of phenolic compounds. Exclusion criteria were presence of type 1 diabetes mellitus (T1DM), T2DM or pre-diabetes, dyslipidemia, untreated hypothyroidism, HIV, heart disease, tuberculosis, hormone replacement treatment for menopause, gastrointestinal disorders, bleeding or hemophilia, and pregnancy. After evaluating anthropometric data, volunteers were divided according to body mass index (BMI) into two groups, normoweight (between 18.5 and 25 kg/m²) and overweight/obese (≥ 25 kg/m²) (WHO, 2000). Volunteers were instructed to fast overnight (10-12 h before intervention). The first urine at intervention day and after 30 min of consuming 500 mL of cambuci juice (300 mL of cambuci pulp + 200 mL of water) were discarded. Urine during the next 24 h was collected in an appropriate bottle and stored at -80 ºC (**Figure 8**). Volunteers were instructed to maintain physical activities and eating routines and avoid phenolic-rich foods (e.g., wine, cocoa, chocolate, grapes, berries, teas, and nuts) 24 hours before and after juice ingestion. Project and informed consent form were approved by the research ethics committee of Faculty of Pharmaceutical Sciences, University of São Paulo (n° 1.637.867, CAEE: 53727916.0.0000.0067). The study was explained to all volunteers, and they agreed to participate by signing the informed consent form.

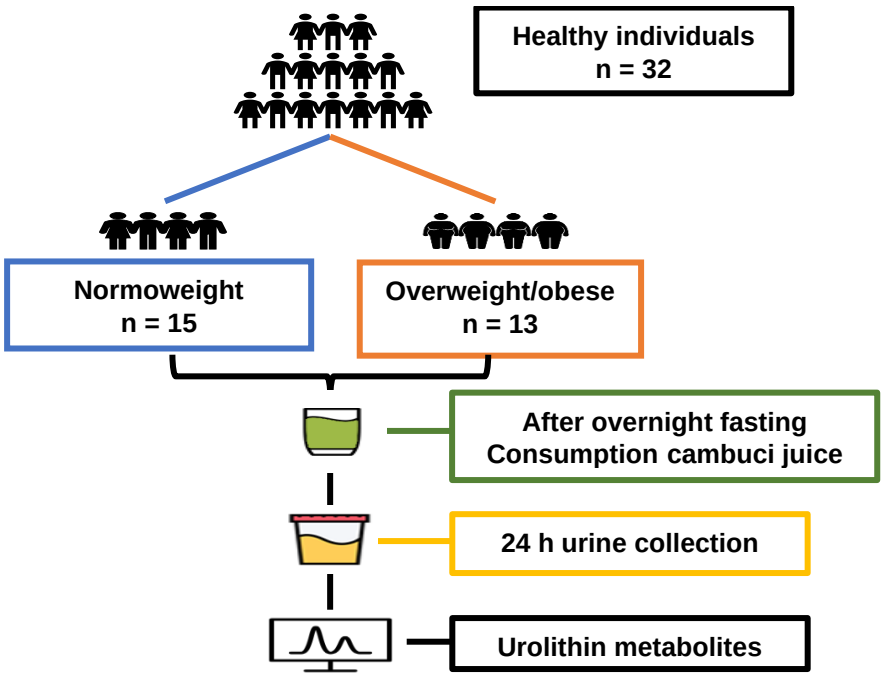
3.3.2. Urolithin metabolites analysis by HPLC-DAD

Urine samples were thawed, vortexed, and centrifuged at 14,000 *g* for 15 min at 5 ºC (n=3). A Hewlett-Packard 1100 chromatography system equipped with automatic sample injector, quaternary pump, and diode array detector was

used. Chromatographic separation of urolithins was obtained using LiChromCART C-18 reverse phase column (250 mm X 4 mm, 4.5 µm, Merck, Darmstadt, Germany) at 25ºC. Gradient mobile phase consisted of water:formic acid (99:1 v/v) (eluent A) and acetonitrile (eluent B). Before sample injection, column was balanced with 5% eluent B. Sample was injected (5 µL), and eluent B concentration was increased to 17% in 2 min, 25% in 7 min, 35% in 15 min, and 50% from 20 to 28 min. Then, initial conditions were restored in ten minutes. Urolithins were identified (diode array detected urolithins at 305 nm) by comparing retention time and spectral characteristics.

For unidentified urolithins, urine samples were purified through solid-phase extraction on octadecylsilane (C18) columns (Supelclean™ LC-18, Supelco) manually prepared. Previously, columns for solid-phase extraction were preconditioned with 20 mL of methanol and 60 mL of distilled water per gram of adsorbent. After eluting 25 mL of urine samples, columns were washed with water, and urolithins were eluted with methanol (50 mL). Finally, the extract was concentrated by methanol evaporation, re-suspended in 80% methanol, and re-injected in the chromatographic system described above.

Figure 8. Experimental design to evaluate the metabolism of ellagitannins in cambuci juice.



Source: the author (2021)

4. RESULTS AND DISCUSSION

4.1. CHEMICAL CHARACTERIZATION OF CAMBUCI PULP

Table 1 shows proximate composition of cambuci pulps. Cambuci pulp presented a high moisture content (~84%), which is characteristic of the Myrtaceae family. Indeed, other fruits from this family have high moisture contents, for instance jaboticaba with 87.85%, pitanga with 90.47%, and red guava with 85.81%. High moisture content indicates fruits are fleshy and juicy when ripe (VALLILO *et al*., 2005). Cambuci pulp had lipid content (1.44%) similar to kiwi (1.37%) and higher than fruits of the same family, such as jaboticaba (0.89%), pitanga (0.23%), and red guava (0.34%) (VALLILO *et al*., 2005).

Cambuci pulp fiber content was different from those found by Vallilo *et al*. (2005), although the value obtained was similar to other fruits of Myrtaceae family. This disparity may be due to different analytical techniques, intraspecific variations of species, and edaphoclimatic variations.

Table 1. Proximate composition of cambuci pulp

	Cambuci Pulp (FW)
Moisture (g/100 g)	84.41 ± 0.78
Ash (g/100 g)	0.23 ± 0.01
Lipids (g/100 g)	1.44 ± 0.06
Proteins (g/100 g)	0.30 ± 0.01
Total fiber (g/100 g)	2.00 ± 0.13
Carbohydrates (g/100 g) *	11.62
Calories (kcal/100 g) **	59.74
Total soluble solids (ºBrix)	7.5 ± 0.1
pH	2.32 ± 0.01
Total titratable acidity (g CAE/100 g)	1.93 ± 0.02
Total sugars (g GE/ 100 g)	9.31 ± 0.40
Total phenolics (mg GAE/100 g)	780.0 ± 0.1

* Calculated as the difference (100% – %moisture - %ash – %lipid – %protein – %fiber = %carbohydrates).

** Sum of proteins plus carbohydrates multiplied by factor 4 (Kcal/g) added to the total lipid content multiplied by factor 9 (Kcal/g).

The results are expressed as media ± SD (n=3); CAE = citric acid equivalents; GE = glucose equivalents; GAE = gallic acid equivalents.

TSS is an indirect method to quantify sugar content in a sample and varies according to species, growing conditions, maturation stage, and climate. Cambuci pulp TSS was 7.5 ºBrix (**Table 1**). Compared with three of the main fruits of Brazilian diet, cambuci has TSS content similar to 'Pera' orange (8-12º Brix) and 'Tahiti' acid lime (9º Brix) (LADANIYA, 2008). Therefore, cambuci is not very sweet since it presents approximately half TSS content of banana 'Prata' (22-23 ºBrix) (VIVIANI; LEAL, 2007). Besides low sugar content, cambuci presented high acidity, according to pH and titratable acidity (**Table 1**). Therefore, cambuci can be considered an acidic fruit since it has titratable acidity similar to 'Tahiti' acid lime (5-6 g equivalents citric acid/100 mL juice) (LADANIYA, 2008). Regarding reducing sugars, cambuci has 9.31 g of glucose equivalent /100 mL of pulp.

Chemical composition of fruits is highly dependent on growing and processing conditions. Frozen fruit pulps are one of the most convenient food products obtained from fresh fruit processing since they can be stored for long periods, preserving most nutritional characteristics, allowing higher availability and broader distribution of local products, overcoming seasonality problems, and avoiding losses due to over maturity. Moreover, fruit pulps are often used to manufacture fruit jams, jellies, nectars, puddings, and baby food. Chemical composition varies according to geographic region and time period. Furthermore, during pulp processing, fruit parts (seeds and peel mainly) are discharged, altering the concentration of final products.

Regarding mineral composition of cambuci pulp (**Table 2**), potassium was predominant, followed by magnesium, sulfur, and phosphorus. Potassium is widely distributed in nature, unlike phosphorus, magnesium, and sulfur, which are often present in their poorly soluble chemical structure in the soil, leading to poor absorption by plants. However, levels found may be associated with harvest site location. Vallilo *et al*. (2005) found a high sodium concentration in cambuci harvested from Núcleo Caraguatatuba in the Serra do Mar State Park. This

ecosystem is near to the ocean, exposing the soil to direct and frequent influence of fog, wind, and rain, which carry chemical elements; therefore, justifying higher sodium concentrations than fruits from other regions (VALLILO *et al*., 2005).

Table 2. Mineral composition of cambuci pulp

Minerals (g/Kg DW)	**Cambuci pulp**
Nitrogen (N)	16.88
Phosphorus (P)	0.49
Potassium (K)	8.73
Calcium (Ca)	<0.2
Magnesium (Mg)	0.85
Sulfur (S)	0.52
Boron (B)	0.003
Zinc (Zn)	0.015
Iron (Fe)	0.029
Manganese (Mn)	0.003
Copper (Cu)	0.005
Sodium (Na)	0.059

DW = dry weight

Characterization of cambuci pulp phenolic profile was performed using HPLC-MS. Compounds were identified through selective molecular weight and fragmentation profile of molecular ion characteristics. Due to absence of calibration standards, identification of cambuci polyphenols was performed by comparing fragments of molecular ions generated by mass spectrometry in MassBank®, Metlin®, and ReSpect® databases. Twenty-six compounds were identified (**Figure 9** and **Table 3**).

Figure 9. Chromatographic profile of cambuci pulp at 280 nm.

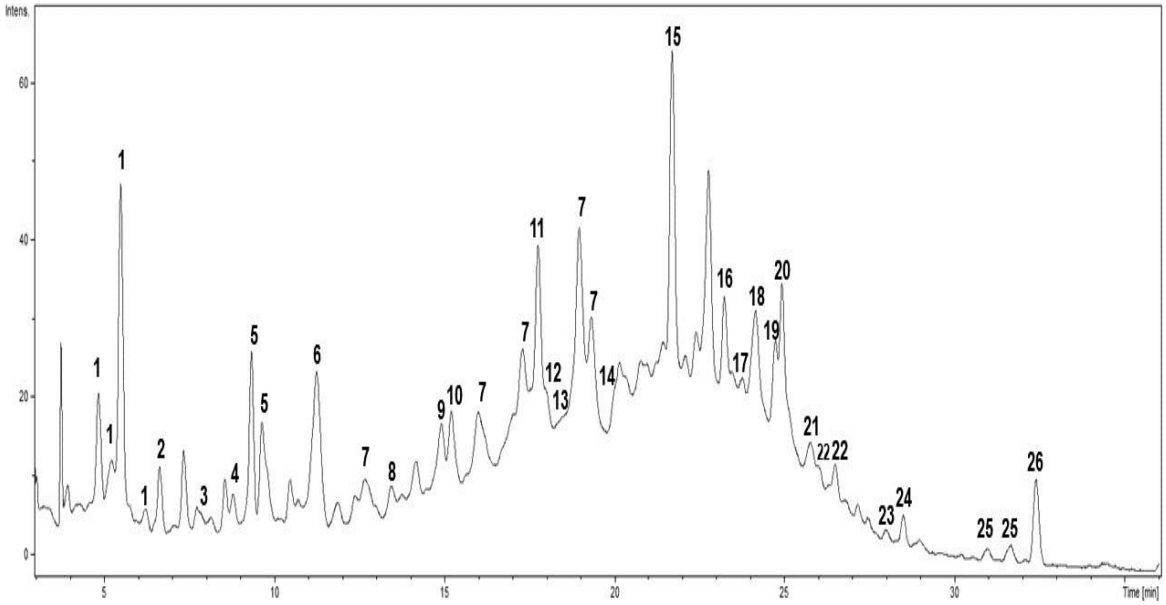


Table 3. Identification of phenolic compounds present in the cambuci pulp by high-performance liquid chromatography/mass spectrometry (LC-MS).

(continue)

No	Compounds	RT (min)	Fragments (*m/z*)
1	Galloyl-hexoside	4.87 5.25 5.52 6.24	**331,** 169**,** 271
2	Gallic acid	6.66	**169,** 125
3	Galloyl-quinic acid	7.75	**343,** 169, 125
4	Bis-HHDP-hexoside (Pedunculagin)	8.80	**783,** 301, 481
5	Galloyl-shikimic acid	9.35 9.66	**325**,169,125
6	Bis-HHDP-glucose	11.27	**783,** 391,375
7	Digalloyl-HHDP-glucose (Tellimagrandin II)	12.60 16.01 17.30 18.97 19.32	**785**, 301,483
8	Digalloyl-hexoside	13.46 25.76	**483,** 169, 313

Table 3. Identification of phenolic compounds present in the cambuci pulp by high-performance liquid chromatography/mass spectrometry (LC-MS).

(conclusion)

9	Galloyl-bis-HHDP-Glucose	14.92	**935,** 633, 917
10	n-Coumaric acid hexoside	15.21	**325,** 162
11	Trigalloyl-HHDP-glucose	17.76	**1087,** 850, 917
12	Methyljasmonate	18sh	**385,** 153, 223
13	(Epi)Gallocatechin gallate	18.20	**457,** 169, 305
14	Ellagic acid-hexoside	20.16	**463,** 301, 257
15	Tris-Galloyl-HHDP-Glucose	21.70	**937,** 301, 767
16	Tetragalloyl hexoside	23.24	**787,** 617, 721
17	(Epi) Catechin gallate	23.76	**441,** 289, 331
18	Ellagic acid desoxyhexoside	24.15	**447,** 300, 227
19	Ellagic acid	24.74	**301,** 257, 229
20	Pentagalloyl hexoside	24.93	**939,** 769, 616
21	Quercetin pentoside	25.76 26.49	**433,** 301, 371
22	Quercetin rhamnose	27.44	**447**¸ 301, 271
23	Quercetin-O-(O-galloyl)-pentoside	27.97	**585,** 301, 151
24	Methylellagic acid rhamnoside	28.48	**461,** 315, 300
25	Methylellagic acid sulphate	30.95 31.64	**395,** 315, 327
26	Abscisic acid	32.37	**263,** 153, 219

RT: retention time; min: minutes; Molecular ion in bold; m/z: mass/charge.

Among compounds found, 15 hydrolyzable tannins were identified and separated into two classes. Nine compounds were gallotannis (galloyl esters of glucose) (1, 3, 5, 8, 10, 14, 16, 18, and 20), and six were ellagitannins (galloyl

and hexahydroxydiphenoyl esters of glucose) (4, 6, 7, 9, 11, and 15). Free ellagic acid (19) and gallic acid (2) were also found.

Besides hydrolyzable tannins, few flavonols were identified by retention time and fragmentation patterns. Glycosides and acylated (galloyl) glycosides of flavonol quercetin (m/z 301) were detected (21, 22, and 23). Other compounds were also identified, including two flavanol compounds (13 and 17) and ellagic acid derivatives (24 and 25).

Biazotto *et al*. (2019) characterized phenolic profile of several species of Brazilian native fruits, including cambuci. Phenolic compounds found in both studies were tris-galloyl-HHDP-glucose (m/z 937), galloyl-bis-HHDP-glucose (m/z 935), digalloyl-HHDP-glucose (m/z 785), bis-HHDP-glucose (m/z 783), digalloyl-hexoside (m/z 483), methylellagic acid rhamnoside (m/z 461), and gallic acid (m/z 169). Moreover, they found ellagitannins derivatives with m/z different from those found in our study probably because we performed a more accurate identification and did not use chromatographic separation methods (BIAZOTTO *et al*., 2019). Despite this, phenolic profiles of cambuci were similar between studies.

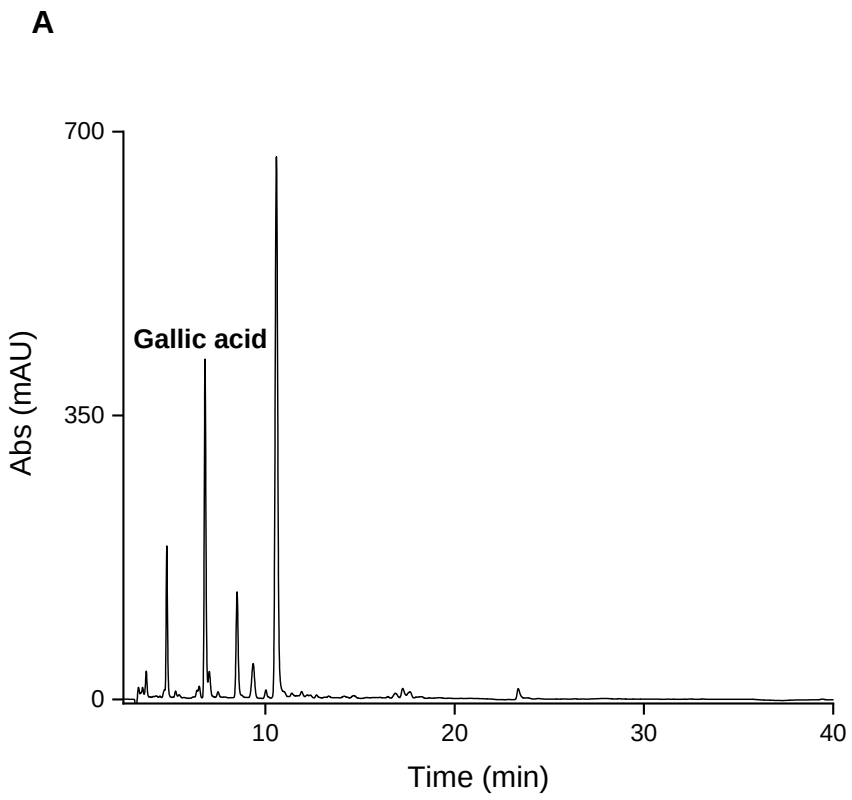
Donado-Pestana *et al*. (2021) also characterized phenolic profile of cambuci pulp. Their study used a chromatographic separation method and observed the presence of ellagic acid (m/z 301), pedunculagin (m/z 783), tellimagrandin II (m/z 785), pentoside and rhamnoside quercetins (m/z 433 and 447), and gallotannins derivatives (m/z 483) (DONADO-PESTANA *et al*., 2021).

Figure 10 shows the chromatographic profile of the cambuci after acid hydrolysis at 280 nm (A) and 360 nm (B). Main products identified and quantified were ellagic acid, gallic acid, valoneic acid dilactone, and sanguisorbic acid dilactone. Due to solubility, gallic acid was identified in supernatant and other compounds in the pellet.

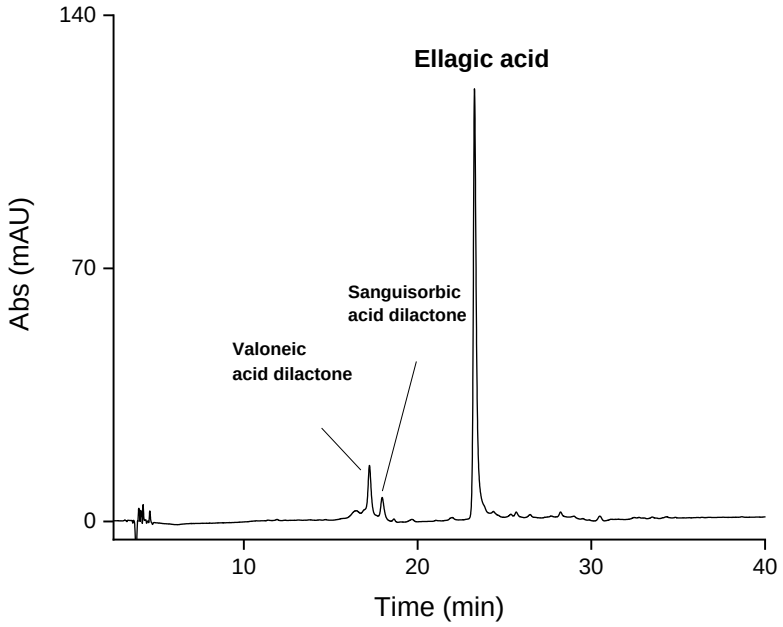
This is the first time dilactone sanguisorbic acid and dilactone valoneico acid were identified in cambuci (**Figure 10B).** Hydrolysis products were identified by comparing retention time and ultraviolet spectrum (GARCÍA-VILLALBA *et al*., 2015). These two compounds are isomers, with 469 mass/charge ratio, consistent with ellagic acid derivatives in which an additional gallic acid is attached to ellagic acid molecule via ether bond. Structural difference between these two isomers is found in hydroxyl position that connects

hexahydroxydiphenyl group (HHDP) to galloyl group and may belong to either HHDP or galloyl group (GARCÍA-VILLALBA *et al*., 2015). Fischer, Carle & Kammerer (2011) identified valoneic acid dilactone in pomegranate peel for the first time. In another study, valoneic acid dilactone and sanguisorbic were also identified in different parts of pomegranate fruit (husk and peel) and juice (GARCÍA-VILLALBA *et al*., 2015). The presence of dilactone sanguisorbic acid and dilone valoneic acid after hydrolysis indicates the occurrence of ellagitannins with bonds involving an additional galloyl group linked to HHDP ester (GARCÍA-VILLALBA *et al*., 2015).

Figure 10. Chromatographic profile of cambuci after acid hydrolysis at 280 nm (A) and 360 nm (B).



B



Concentration of ellagitannins (6.16 mg/g pulp DW) in cambuci corresponds to the sum of hydrolysis products (**Table 4**). Ellagic acid quantified (4.80 mg/g) corresponds to total ellagic acid content (i.e., those in free form and in ellagitannins structure). Goncalves *et al*. (2010) evaluated chemical composition of frozen commercial pulps of Brazilian native fruits and found 5.12 mg/g of ellagic acid in cambuci commercial pulps. Azevedo *et al*. (2017) evaluated total ellagic acid content of cambuci fruits from different regions in São Paulo and observed differences between regions (1.31-7.86 mg/g FW of fruits), concluding that cambuci maturation and total ellagic acid content are inversely correlated. Therefore, total ellagic acid and ellagitannin contents may vary according to harvest location, level of maturation, and production process.

Table 4. Quantification of ellagitannins present in cambuci.

Gallotannins (mg/g DW)	
Gallic acid	5.25 ± 0.12
Ellagitannins and derivates (mg/g DW)	
Sanguisorbic acid dilactone	0.46 ± 0.03
Valoneic acid dilactone	0.90 ± 0.02
Ellagic acid	4.80 ± 0.10

DW: dry weight.
Results expressed as mean ± standard deviation (n = 6)

Proanthocyanidins were quantified after acid catalysis in the presence of phloroglucinol as nucleophilic reagent. In these reactions, proanthocyanidins were converted into constitutive subunits. Subunits are monomeric units of flavan-3-ols and can be divided into two groups: terminal and extension subunits. The latter have an electrophilic flavan-3-ol intermediate that can be captured by nucleophilic reagent to generate analyzable adducts (KENNEDY, 2001). In a phloroglucinolysis reaction, content of total flavan-3-ol in cambuci pulp is the sum of terminal and extension subunits.

From a qualitative viewpoint, cambuci pulp presented a rich source of PA with various monomeric units of flavan-3-ol in its molecules. Epicatechin (monomer), gallocatechin (adduct), epicatechin gallate (monomer and adduct), and epigallocatechin gallate (monomer and adduct) were formed after acid-catalyzed cleavage of proanthocyanidins from cambuci pulp (**Figure 11** and **Table 5**). Similar monomeric flavan-3-ol units, associated with prevention of neurological disorders, cancer, and cardiovascular disease, have been widely described in green tea. (ZAVERI, 2006).

We applied phloroglucinolysis to characterize proanthocyanidin profile of cambuci samples for the first time. Proanthocyanidin content was estimated by subtracting flavan-3-ol monomers (separately quantified by HPLC–UV–Vis–IT without phloroglucinolysis) from phloroglucinolysis results (sum of flavan-3-ol monomers and phloroglucinol adducts) (JAKOBEK; GARCÍA-VILLALBA; TOMÁS-BARBERÁN, 2013). Total amount of proanthocyanidins found was 45.45 g/kg of catechin equivalents. Concentration of different varieties of strawberry ranged from 539 to 1632 μg/g when phloroglucinolysis was used for

quantification (BUENDIA, 2010). Some varieties of apples showed PAs between 1,703 and 5,329 µg/g also applying phloroglucinolysis method (JAKOBEK; GARCÍA-VILLALBA; TOMÁS-BARBERÁN, 2013). Similar results were found in cambuci fruits from Paraibuna region (PA content of 46.45 g equivalents of quebracho tannin/kg DW) (AZEVEDO *et al*., 2017). Although quantification methods were different in both studies, final PA contents were very similar (AZEVEDO *et al*., 2017).

Table 5. Quantification of the flavan-3-ol present in the cambuci pulp by phloroglucinolysis.

Compound	g/kg DW	RT (min)
Epicatechin	3.32 ± 0.07	
Epicatechin monomer	-	
Epicatechin adduct	3.32 ± 0.07	9.30
Gallocatechin	22.25 ± 0.90	
Gallocatechin monomer	-	
Gallocatechin adduct	22.25 ± 0.90	5.90
Epicatechin gallate	3.40 ± 0.13	
Epicatechin gallate monomer	0.08 ± 0.01	22.24
Epicatechin gallate adduct	3.32 ± 0.13	14.2
Epigallocatechin gallate	16.48 ± 0.58	
Epigallocatechin gallate monomer	1.70 ± 0.06	16.15
Epigallocatechin gallate adduct	14.78 ± 0.52	8.90
Total Flavan-3-ols (proanthocyanidins)	**45.45 ± 1.68**	
mDP	**32.78**	

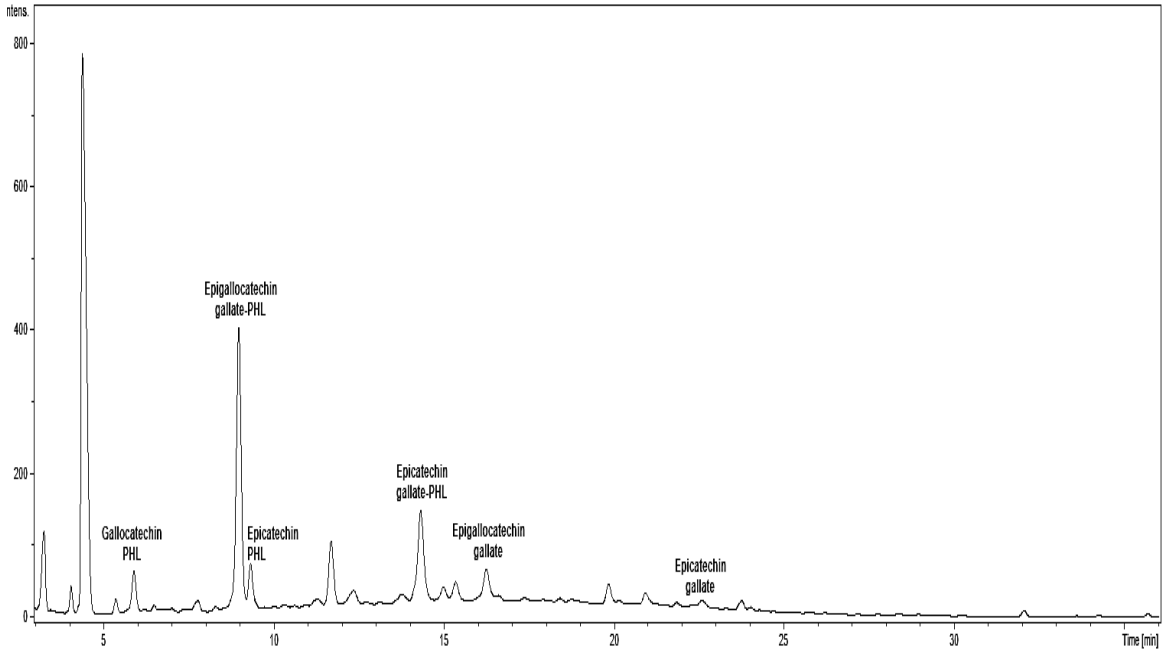
mDP, mean degree polymerization.
DW: dry weight.
Results expressed as mean ± standard deviation (n = 6).

Proanthocyanidins mDP was measured by calculating molar ratio of total monomers and total adducts by total monomers. Cambuci pulp mDP was high (32.78). We are reporting proanthocyanidins DP from cambuci for the first time. Considering DP is the frequency a monomeric unit is repeated in proanthocyanidin macromolecule, our results indicate proanthocyanidins from cambuci pulp have very long chains. Cambuci mDP is higher than apple (3.8 to 9.4) (JAKOBEK; GARCÍA-VILLALBA; TOMÁS-BARBERÁN, 2013) and

strawberry varieties (3.4 to 5.8) (BUENDIA, 2010). mDP may be an important parameter for some quality characteristics features of Southeastern European apples. Oligomeric (mDP 3–5) and more polymerized proanthocyanidins (mDP 6–10) contribute to bitterness and astringency, respectively (SANONER *et al*., 1999). Therefore, cambuci is an acid and highly astringent fruit.

PAs with low DP are more probable to be absorbed through intestinal barrier, whereas PAs with high DP reach the colon and are metabolized by intestinal microbiota (DIAZ-MULA., 2019). A high DP of proanthocyanidins was observed in cambuci pulp, indicating few changes in polymeric polyphenols during digestion and minimal absorption in the small intestine (HILARY *et al*. 2020).

Figure 11. HPLC-chromatogram (360 nm) of cambuci pulp after acid-catalysis in presence of phloroglucinol.



4.2. EVALUATION OF METABOLIZATION OF ELLAGITANNINS FROM CAMBUCI

The metabolization of ellagitannins from cambuci was evaluated after the administration of 500 mL of cambuci juice. The juice was composed by 300 g cambuci pulp defrosted and 200 mL of water, and without sweetener.addition The content of ellagitannins consumed by subjects was 545 mg, as presented in **Table 6.**

Table 6. Quantification of ellagitannins present in cambuci juice.

Gallotannins (mg/ 300 g)	
Gallic acid	251 ± 5
Ellagitannins and derivates (mg/300 g)	
Sanguisorbic acid dilactone	22 ± 1
Valoneic acid dilactone	43 ± 1
Ellagic acid	229 ± 5

DW: dry weight.
Results expressed as mean ± standard deviation (n = 6)

Food sources rich in ellagitannins, such as pomegranate, walnuts, strawberry, and jaboticaba, have been widely used in studies to assess the bioavailability of ellagitannins. However, nothing is known about the absorption and metabolism of ellagitannins present in cambuci. Therefore, this study aimed to administer cambuci pulp, which is a rich source of ellagitannins, in subjects of both genders, different age groups, and different BMIs, and to evaluate the metabolites present in the 24-hour urine of these volunteers.

Thirty-two volunteers were eligible to participate in the study. Of those, 28 completed the study, and 4 were excluded. Of excluded volunteers, everybody felt nauseated and had episodes of emesis after its consumption. Of concluded participants, fifteen were classified as normoweight (BMI 21 ± 2 kg/m^2) and 13 were classified overweight/obese (BMI 31 ± 4 kg/m^2). Thirteen were male and fifteen were female and with a mean age of 33 ± 9 years (**Table 7**).

Table 7. Characteristics of the subjects participating in the study (n=28).

Parameters	Mean ± SD
Subjects (F/M)	15/13
Age (Years)	33 ± 9
BMI (kg/m^2)	
Normoweight	21 ± 2
Overweight/obese	31 ± 4

Data were expressed as mean ± standard deviation, BMI = body index mass

After the consumption of cambuci juice, 6 metabolites of ellagitannins were detected in urine, being them, urolithin A-glucuronide (Uro A-glur), isourolithin A 3-glucuronide (IsoUro A 3-glur), isourolithin A 9-glucuronide (IsoUro A 9-glur), urolithin B-glucuronide (Uro B-glur), urolithin A (Uro A), urolithin B (Uro B), and isourolithin A (Iso Uro A) (**Table 8**). This is the first time that the metabolization of ellagitannins from cambuci was reported. After consumption of other ellagitannins-rich food sources all metabolites identified here have already been described in urine (TOMÁS-BARBERÁN *et al*., 2014). The identification of urolithins was performed comparing the retention time and UV/Vis absorption spectra with the literature (GARCIA-VILLABA, 2015) (**Figure 12**).

Table 8. Identification of urolithin metabolites in the 24h urine of normoweight, overweight and obese individuals after the consumption of cambuci juice.

(continue)

Volunteers	Metabolites	RT	Metabotype
1	Uro A-glur Uro A	16.143 20.857	A
2	Uro A-glur Uro A	16.143 20.876	A
3	Uro A-glur	16.186	A
4	-	-	0
5	Uro A-glur	16.273	A
6	Uro A-glur	16.764	A

Table 8. Identification of urolithin metabolites in the 24h urine of normoweight, overweight and obese individuals after the consumption of cambuci juice.

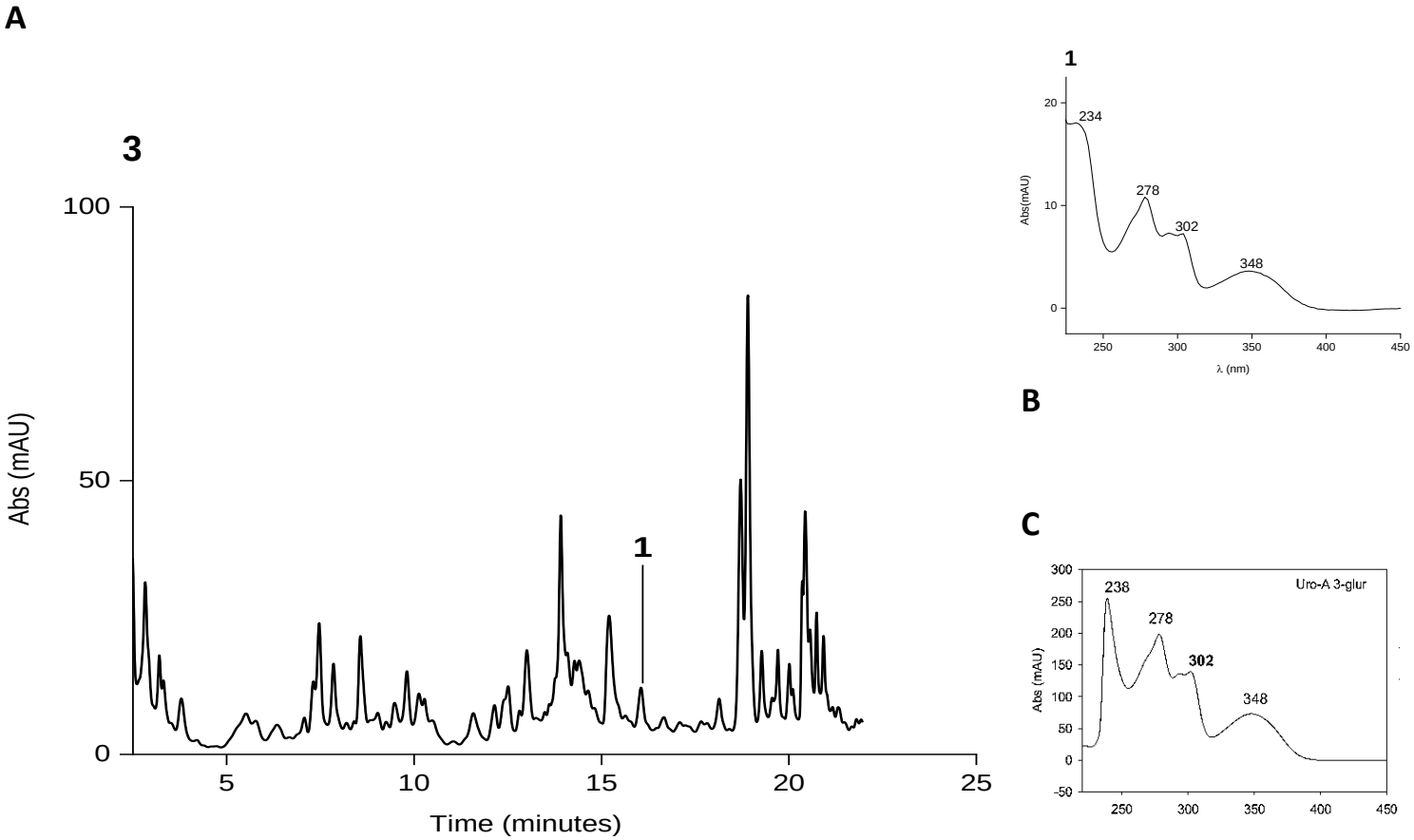
(continue)

7	Uro A-glur Uro A	16.167 20.847	A
8	Uro A-glur	16.103	A
9	IsoUro A 9-glur IsoUro A 3-glur Uro B-glur	16.195 16.848 20.188	B
10	Uro A-glur IsoUro A-glur	16.201 16.908	B
11	Uro A-glur Uro A	16.207 20.853	A
12	-	-	0
13	-	-	0
14	IsoUro A 9-glur IsoUro A 3-glur Uro B-glur IsoUro A	15.663 16.225 19.943 20.562	B
15	Uro A-glur Uro A	15.661 20.687	A
16	-	-	0
17	Uro A-glur	15.737	A
18	Uro A-glur	15.709	A
19	Uro A-glur	15.772	A
20	Uro A-glur	15.781	A
21	-	-	0
22	IsoUro A 9-glur IsoUro A 3-glur IsoUro A	16.22 16.88 20.72	B
23	Uro A	20.84	A
24	Uro A-glur Uro A	16.19 20.86	A

25	IsoUro A 9-glur	16.24	B
	IsoUro A 3-glur	16.89	
	Uro B-glur	20.21	
	IsoUro A	20.73	
26	Uro A-glur	15.595	A
	Uro A	20.628	
27	Uro A	20.85	A
28	Uro A	20.842	A

RT = retention time
Uro A-glur = urolithin A-glucuronide; IsoUro A 3-glur = isourolithin A 3-glucuronide; IsoUro A 9-glur = isourolithin A 9-glucuronide; Uro B-glur = urolithin B-glucuronide; Uro A = urolithin A; Uro B = urolithin B; Iso Uro A = isourolithin A

Figure 12. HPLC-UV Chromatograms at 305 nm of urine of the healthy volunteers after cambuci juice consumption.



Chromatographic profile at 305 nm (A); UV spectra of urolithin metabolite in urine (B); Typical UV spectra of the urolithin-A 3 glucuronide (C). Referring to volunteer L.L., the chromatographic profiles of the remaining volunteers are in appendix A.

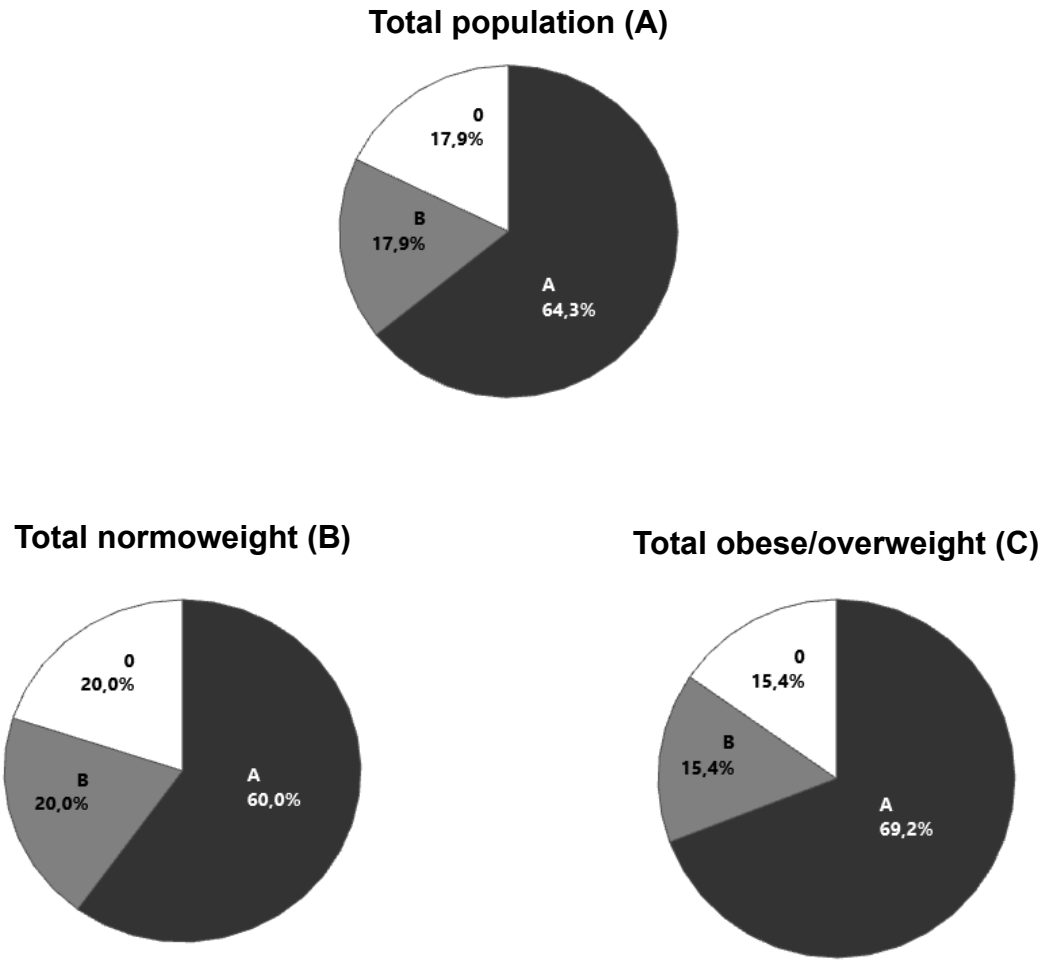
After 24h of cambuci juice consumption, Uro A-glur was the most prevalent metabolite (57%), being excreted by 16 individuals (**Table 8**) and their aglycone form was the second prevalent metabolite (36%), being excreted by 10 individuals. IsoUro A-glur was excreted by 6 individuals, being the third prevalent metabolite (21%). As expected, there was a prevalence of urolithin glucuronides, when compared to their aglycones forms (GONZÁLEZ-SARRÍAS *et al*., 2015), since these compounds are extensively metabolized and undergo conjugation reactions both in the small intestine and in the liver, to increase the hydrophilicity of the molecule and be excreted (CROZIER; DEL RIO; CLIFFORD, 2010). Thus, our results agree with other literature reports (ARAUJO, 2021; INADA, 2020; ESPÍN *et al*., 2007; NUÑEZ-SÁNCHEZ *et al*., 2014), which suggest that, regardless of the food source and the dose of ellagitannins ingested, urolithins are preferably conjugated with glucuronic acid.

Recently, three metabotypes have been described relative to ellagic acid and ellagitannin metabolism. Producers of Uro A and Uro A-glur (metabotype A), producers of Uro A, IsoUro A and Uro B (metabotype B) and non-producers of urolithins (metabotype 0). The present study reports here for the first time the metabolization of ellagitannins from cambuci and investigate urolithin metabotypes in Brazilian subjects from the city of São Paulo, Brazil. These metabotypes have already been described in European populations (TOMÁS-BARBERÁN *et al*., 2014). As expected, regardless of amount or source of ellagic acid or ellagitannins consumed, health status and demographic characteristics (gender, BMI and age), the three metabotypes forementioned were observed in this study. However, some of the factors mentioned above may have influenced the relative distribution of the metabotypes (SELMA *et al*., 2016; TOMÁS-BARBERÁN *et al*., 2014).

Considering all individuals in the present study (n = 28), 64.3% were classified as metabotype A (n=18), 17.9% as metabotype B (n=5) and 17.9% as metabotype 0 (n=5) (**Figure 13A**). A clinical trial conducted in the Brazilian population identified the profile of metabotypes after the consumption of gelatine with jaboticaba. This intervention presented 54.3% metabotype A, 28.6% metabotype B and 17.1% metabotype 0. As already seen, jaboticaba is a fruit that belongs to the Myrtaceae family as well as cambuci. The main ellagitannins found in jaboticaba were castalagin and vescalagin Despite this ellagitannins

found in both fruits were different, the metabolism presented similarity in a healthy population, being agrees with literature reports. In another intervention trial, 20 healthy subjects were stratified according to metabotype, 65% as metabotype A, 20% as metabotype B, and 15% as metabotype 0, after the consumption of ellagitannin-rich pomegranate extract (TOMÁS-BARBERÁN *et al*., 2014).

Figure 12. Distribution of urolithins metabotypes.



Normoweight volunteers showed a higher prevalence of metabotype B (20%) and 0 (20%) (**Figure 13B**) in comparison to obese/overweight (15.4% and 15.4%, respectively), when volunteers were stratified according to their nutritional status (**Figure 13C**). Metabotype A distribution was higher in obese/overweight volunteers than in normoweight. This distribution was different when compared to other studies. Selma *et al*. (2016) analyzed the differences between the profile

of metabolism of ellagitannins in overweight-obese patients and individuals of normoweight. The results showed that the intestinal disorder associated with being overweight and obese can alter the metabolism of ellagic acid compared to healthy individuals. Although all three metabotypes were found in both groups, metabotype B prevailed in obese individuals (31%) compared to eutrophic (20%), whereas metabotype A was more prevalent in eutrophic patients (70%) than in obese individuals (57%). Recently, a study conducted in Metabolic Syndrome patients observed the majority of patients were metabotype A (72%) compared to metabotype B (26%), and these results were associated to alterations in gut microbiota by pharmacological treatments (CORTÉS-MARTÍN *et al*., 2021). Nevertheless, in the clinical trial conducted with the Brazilian population that consumed ellagitannin-rich jaboticaba extracts, the distribution of urolithin metabotypes according to nutricional status was 56.3% to metabotype A, 37.5% to metabotype B, and 6.3% to metabotype 0 for obese/overweight. Normoweight distribution was 52.6% to metabotype A, 21.1% to metabotype B, and 26.3% to metabotype 0 (INADA *et al*., 2019). Thus, the prevalence of metabotype A was even higher in obese/overweight volunteers. The main difference in distribution in both groups was a decrease prevalence of metabotype 0 for overweigth/obese.

Aging of individuals is another described situation that can influence the distribution of metabotypes (CORTÉS-MARTÍN *et al*., 2018). Recently, a clinical study with a large cohort, with volunteers from 5 to 90 years of age and mainly Caucasians, observed that the proportion of metabotype 0 remained constant from 5 to 90 years of age. However, there was an increase progressive for metabotype B in the 40 to 90 years of age (CORTÉS-MARTÍN *et al*., 2018). This can partly explain the high human variability of the present study, since the majority individuals were aged between 20 and 40 years and just two were older than 50 years.

Because not all metabolites from polyphenols have the same biological activity, stratification of individuals according to their metabotype opens a field of exploration necessary to elucidate the effects of phenolic compounds on health, particularly ellagitannins. González-Sarrías *et al*. (2015) evaluated whether the effect of the polyphenols on the lipid profile could suffer an interindividual difference. For this, overweight-obese patients were distributed according to their metabotype A, B or 0. All groups consumed capsules containing pomegranate

extract rich in ellagitannins for 3 weeks. The first results showed that before the interventions with pomegranate extracts, the individuals belonging to metabotype B presented clinical characteristics above the average of the individuals belonging to metabotypes A and 0, in relation to the levels of total cholesterol, LDL fraction, oxidized LDL and apoliprotein B. These results indicated that overweight/obese patients who are metabotype B may have a higher cardiovascular risk than patients belonging to metabotype A and 0. However, the focus of the study was aimed at improving the risks associated with cardiovascular health, and only individuals with metabotype B achieved a significant reduction in total cholesterol, LDL fraction and non-HDL after consumption of the capsules containing pomegranate extract, suggesting that the type of urolithin produced by the individual could reduce the risk of cardiovascular complications. The authors concluded that these effects could be attributed to the individual levels of *Gordonibacter spp*, since after consumption of the pomegranate capsules, this bacterial genus increased significantly in the feces of the metabotypes B. The identification of the bacterial genus that produce the final forms of urolithins can lead to an understanding of interindividual differences in the metabolism of polyphenols, allowing the study of new therapeutic approaches that contribute to the promotion of health.

Clinical trials, meta-analyses and epidemiological studies have showed the possible actions of acid ellagic and ellagitannins in human health (CROZIER; JAGANATH; CLIFFORD, 2009; HANHINEVA *et al*., 2010). However, the most studies are *in vitro*, followed by animal model. Although the potential biological effects of urolithins on human health are still poorly explored, recent studies have demonstrated that urolithins present anti-inflammatory, anticancer, antioxidant, anti and hypolipidemic properties (CORTÉS-MARTÍN *et al*., 2020).

The present study demonstrated that ellagitannins present in cambuci juice undergo bioconversion by the gut microbiota of eutrophic and overweight/obese subjects. It was observed a high interindividual variability in terms of ellagitannin metabolism, which may be associated to differences in gut microbiota by aging and not necessarily associated to nutritional state. Some limitations of the present study must be addressed. Regarding the number of participants, no formula was used for determining sample size as it was very difficult to recruit volunteers. During the development of this study, the world was

affected by the COVID-19 pandemic, which became the main limiting factor of this study. Considering that the main measure to contain the spread of the virus was social isolation, this directly interfered with the recruitment of more volunteers. These results concerning both the distribution of urolithin metabotypes and the metabolism of ellagitannins from cambuci in Brazilian subjects are critical for future studies on the bioactivity of cambuci or of other ellagitannin food sources in the Brazilian population.

5. CONCLUSIONS

The profile of phenolic compounds in cambuci pulp is characterized by the presence of ellagitannins, such as telemagrandin, pedunculagin, in addition to gallotannins, proanthocyanidins, and flavonols. For the first time, the constitutive units and the degree of polymerization of proanthocyanidins were reported.

Regarding bioavailability, it has been shown, in an unprecedented way, that cambuci ellagitannins are susceptible to bioconversion to urolithins by the intestinal microbiota of healthy and overweight/obese individuals.

The main metabolite identified was the urolithin-A glucuronide, corroborating the prevalence of the metabotype A. Furthermore, it was also demonstrated that the nutritional status may not be a determining factor in the production of metabolites.

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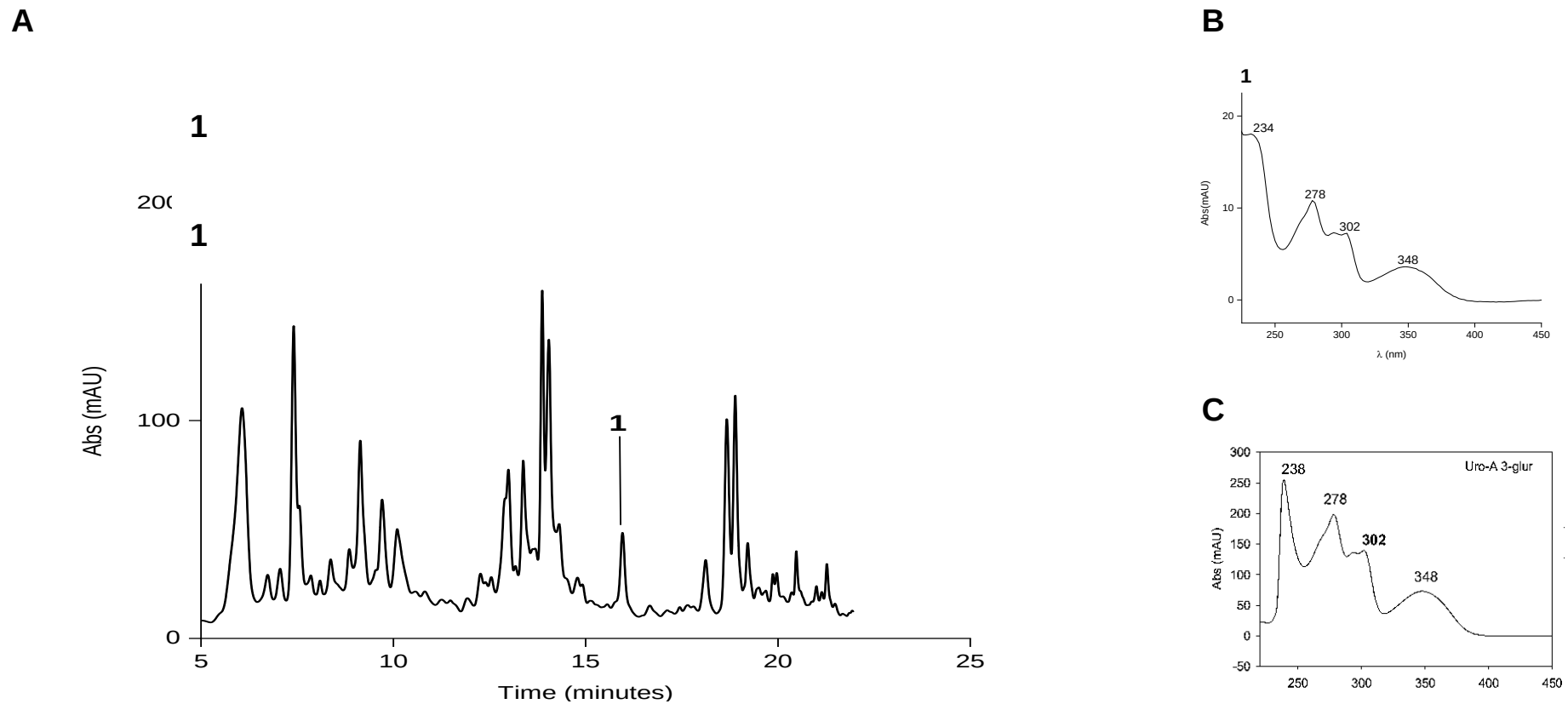
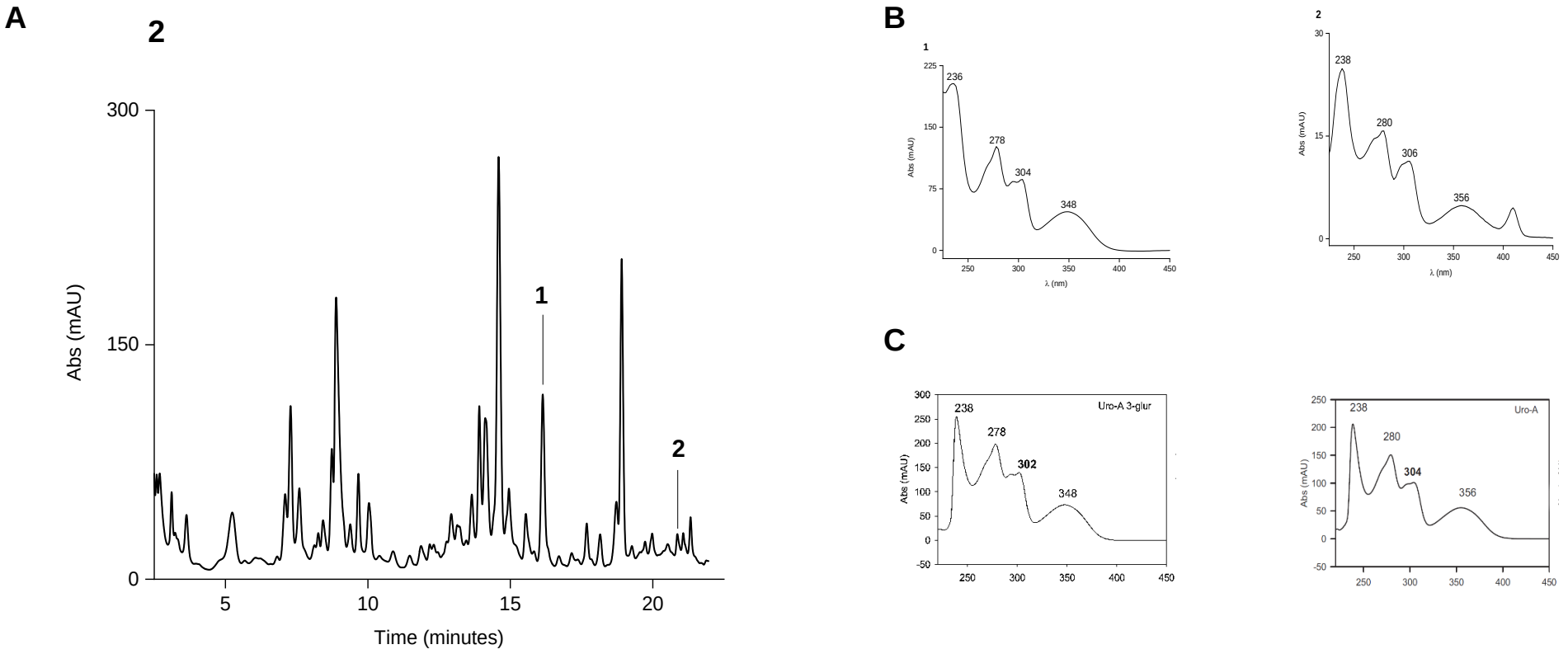
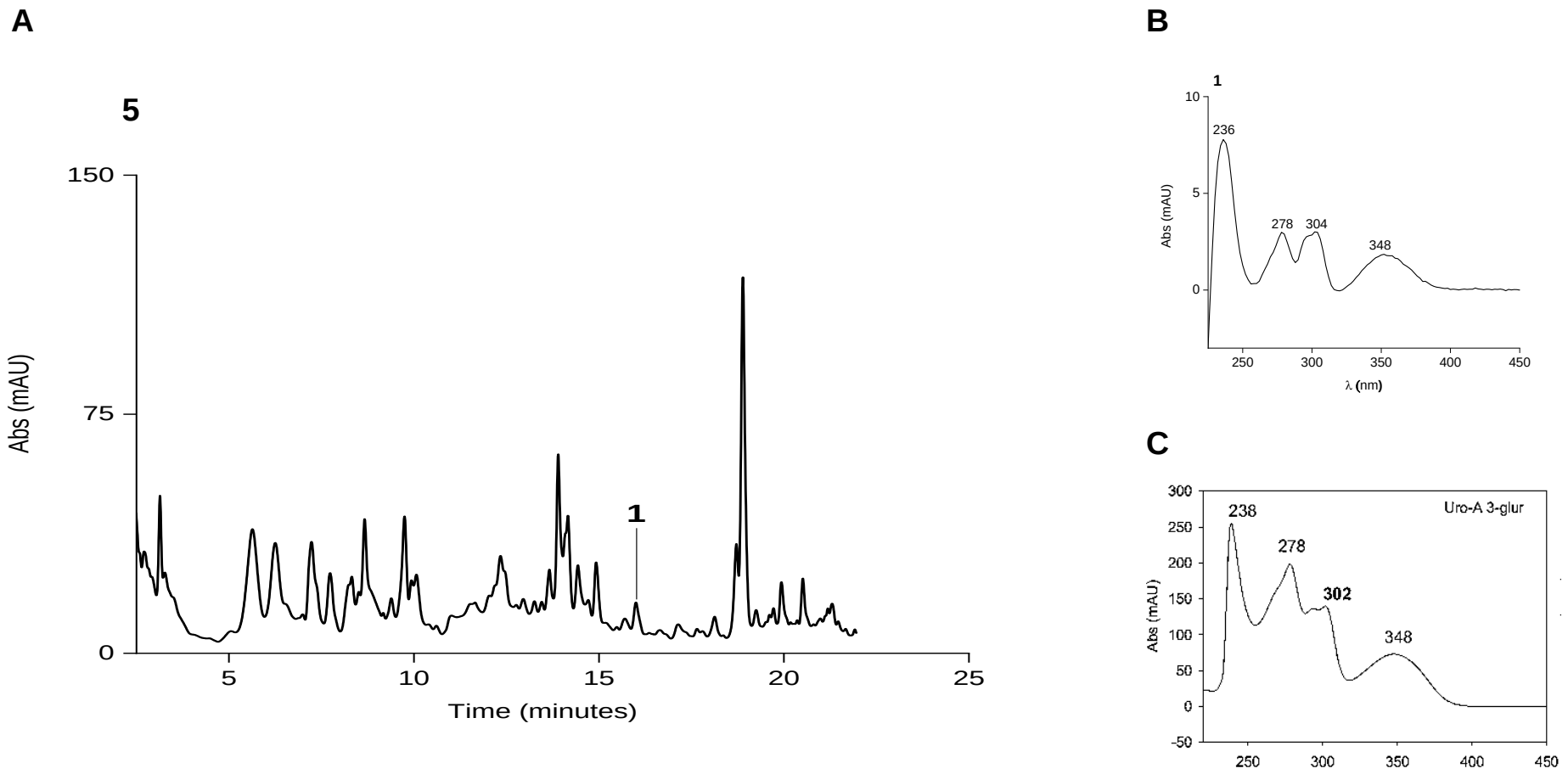
APPENDIX A - HPLC-UV CHROMATOGRAMS AT 305 NM OF URINE OF THE VOLUNTEERS AFTER CAMBUCI JUICE CONSUMPTION



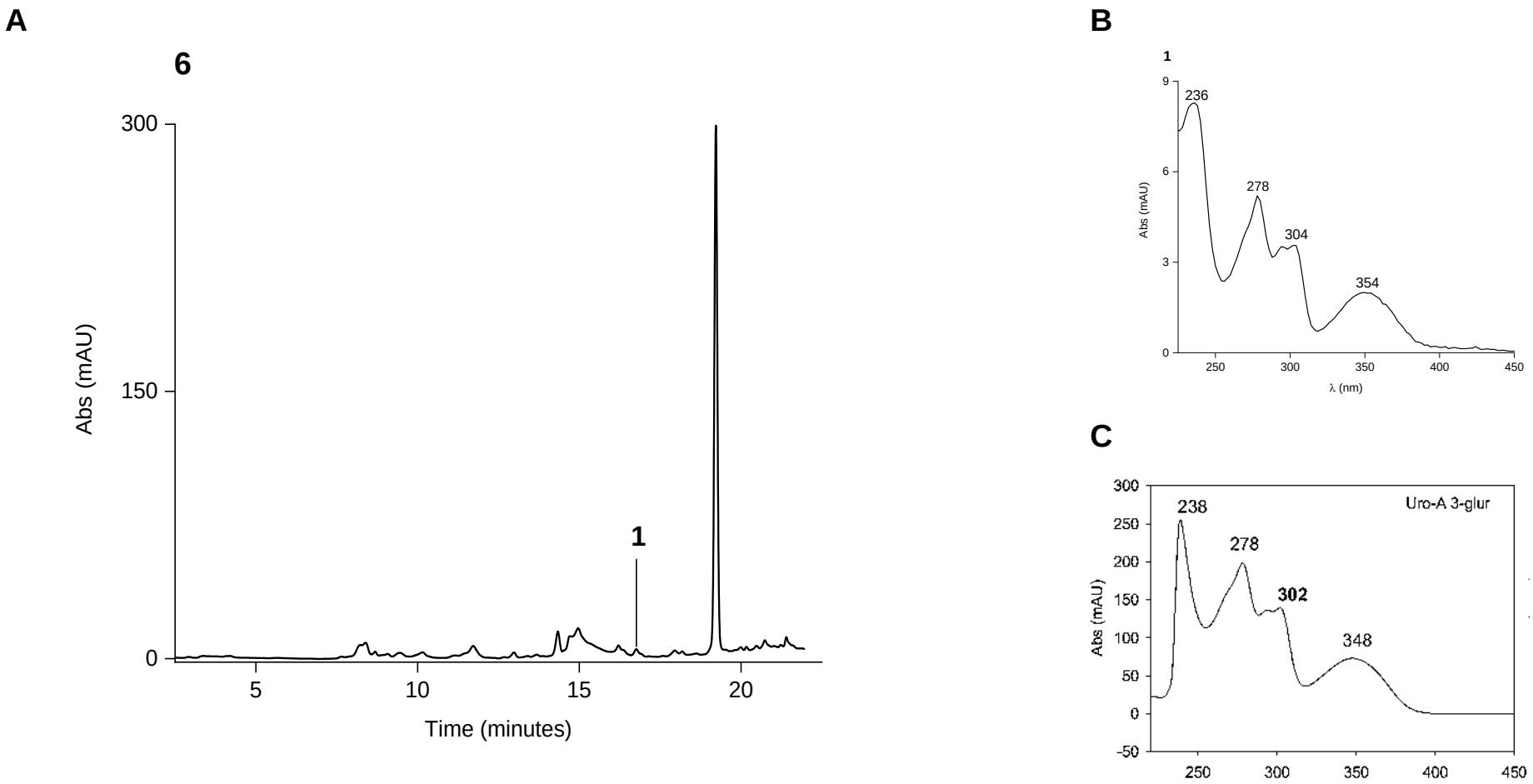
Chromatographic profile at 305 nm (A); UV spectra of urolithin metabolite in urine (B); Typical UV spectra of the urolithin-A 3 glucuronide (C).



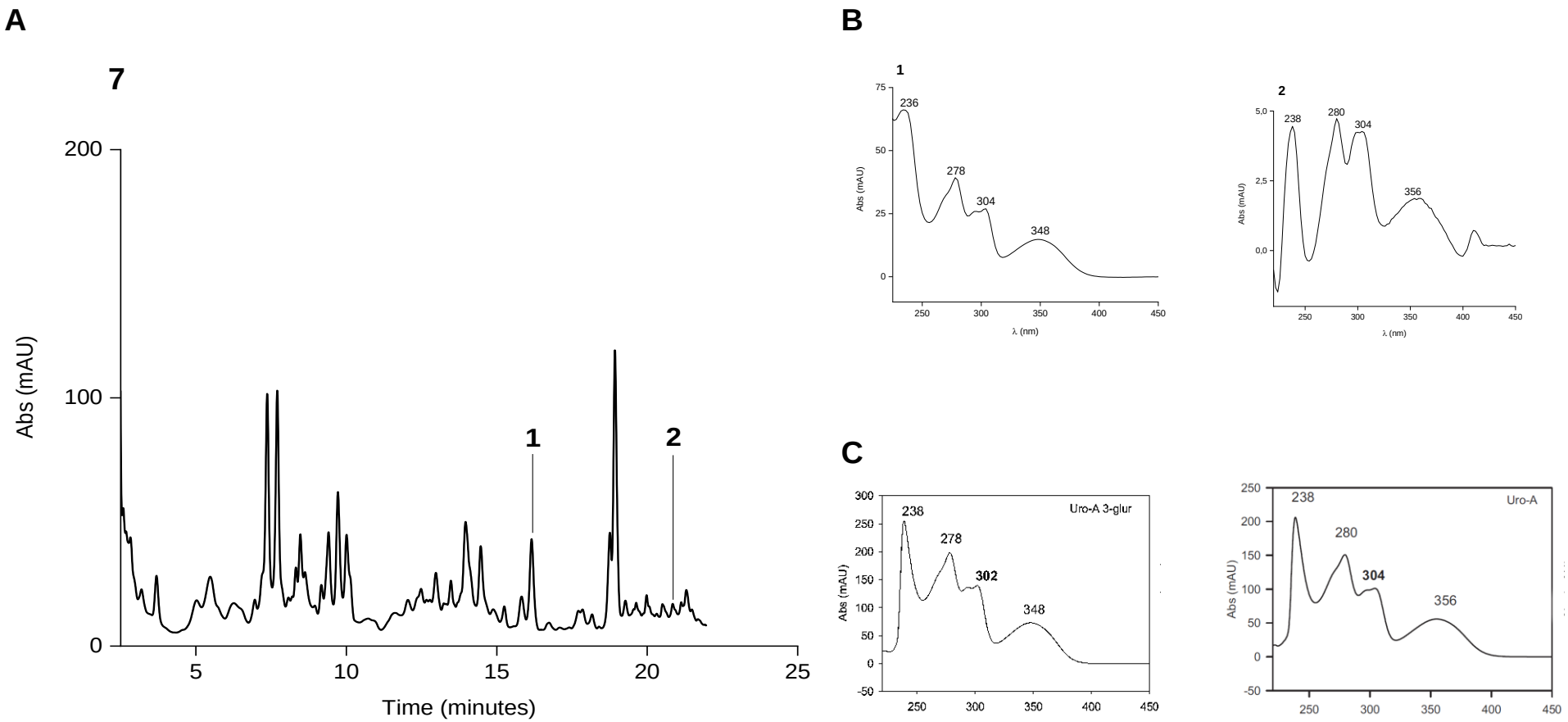
Chromatographic profile at 305 nm (A); UV spectras of urolithins metabolites in urine (B); Typical UV spectra of the urolithin-A 3 glucuronide and urolithin-A(C)



Chromatographic profile at 305 nm (A); UV spectra of urolithin metabolite in urine (B); Typical UV spectra of the urolithin-A 3 glucuronide (C).



Chromatographic profile at 305 nm (A); UV spectra of urolithin metabolite in urine (B); Typical UV spectra of the urolithin-A 3 glucuronide (C).



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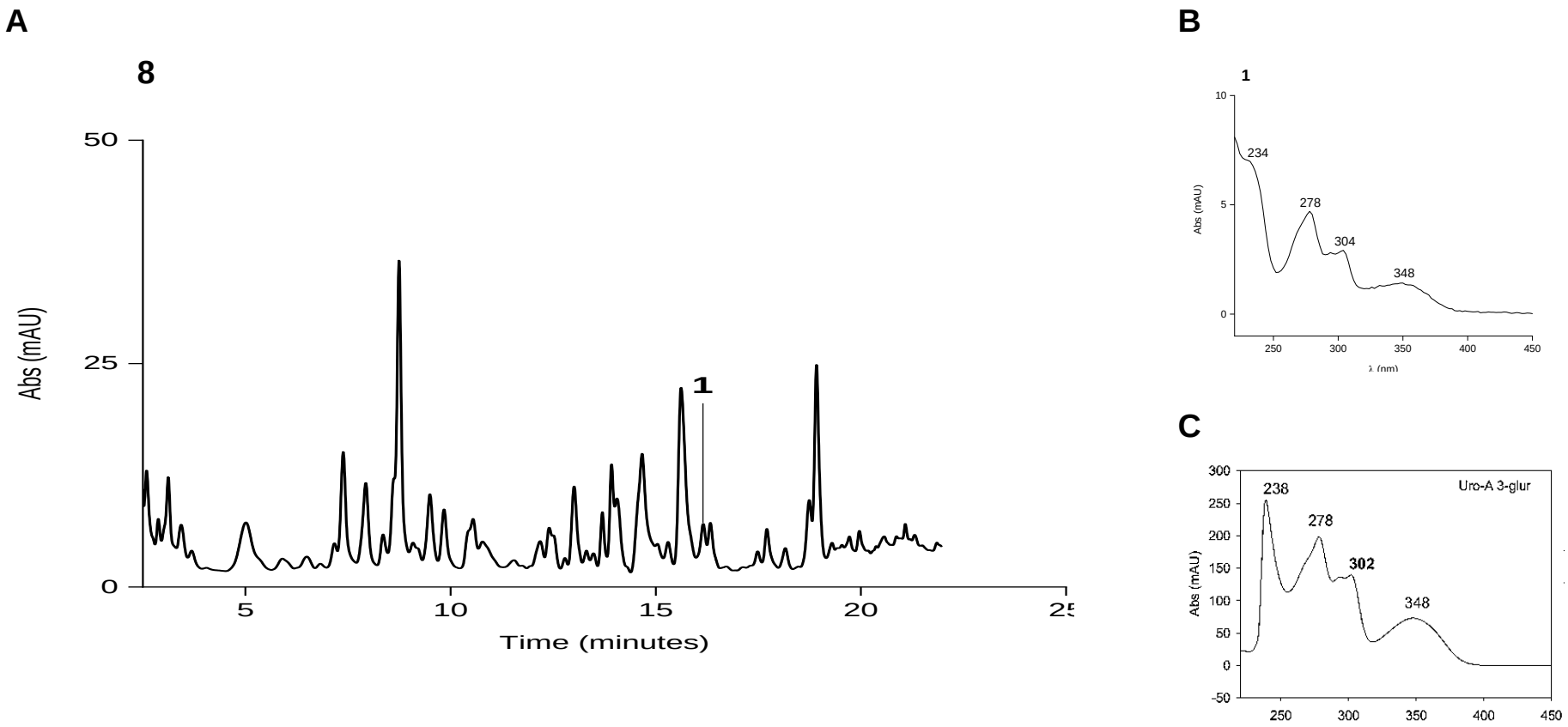
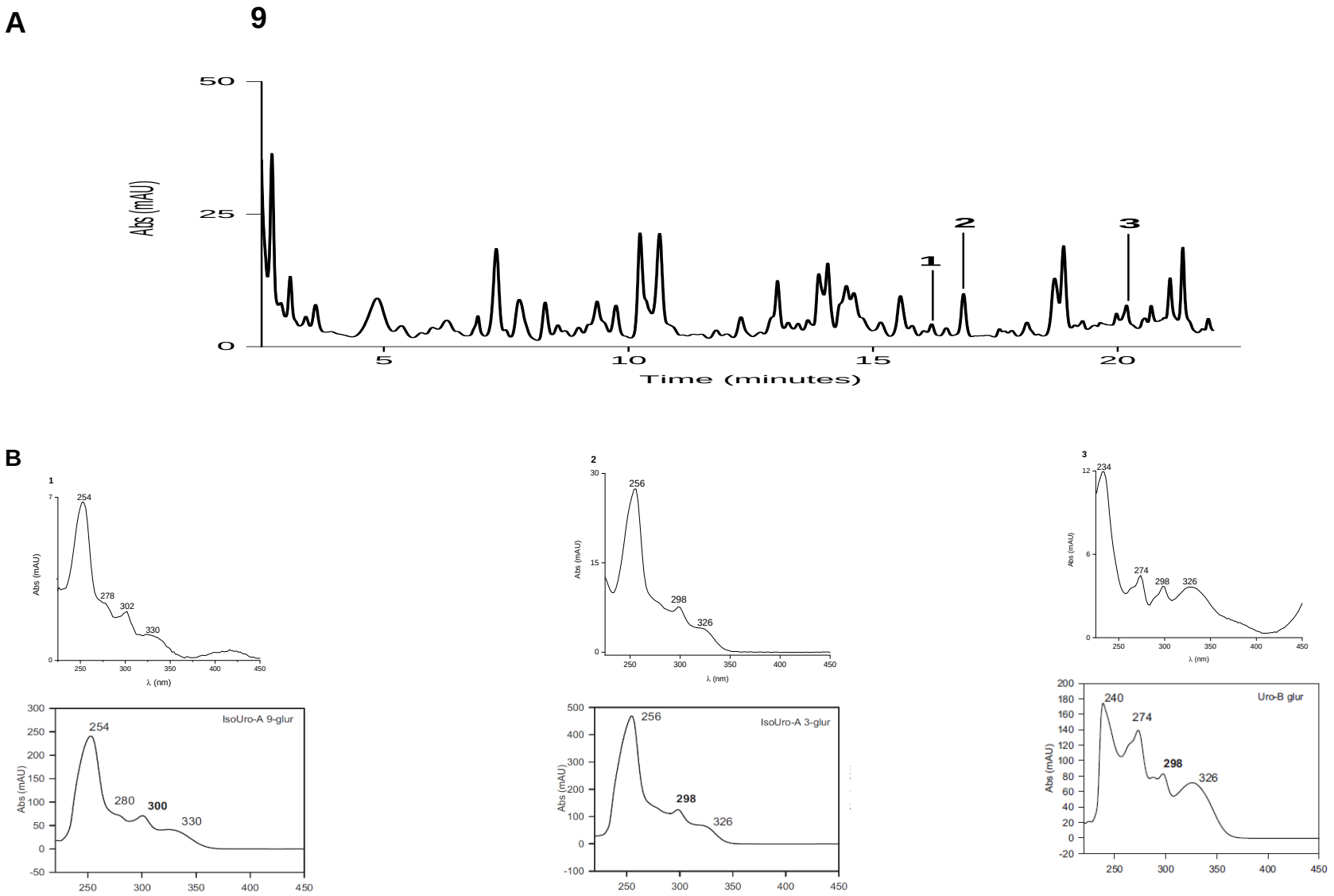
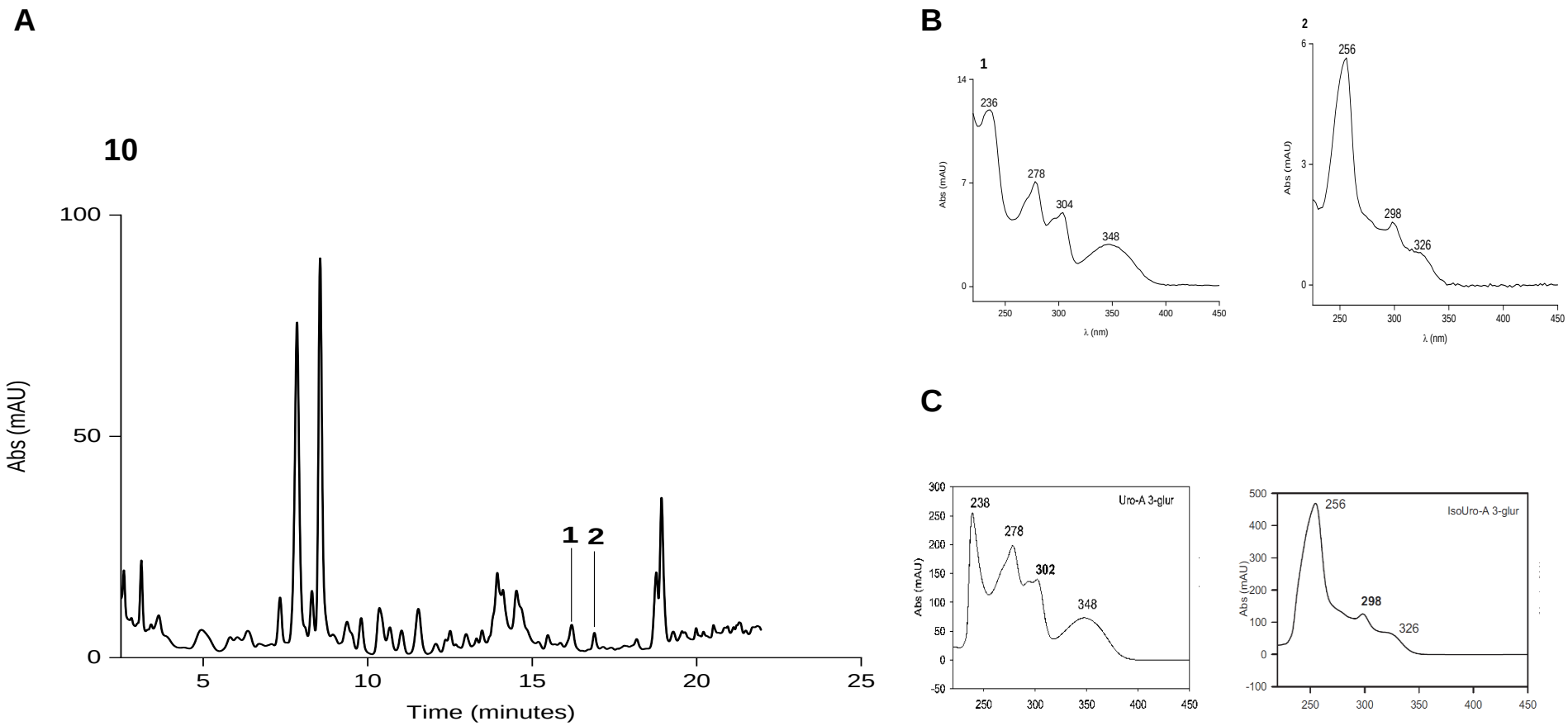


Chromatographic profile at 305 nm (A); UV spectra of urolithin metabolite in urine (B); Typical UV spectra of the urolithin-A 3 glucuronide (C).



Chromatographic profile at 305 nm (A); UV spectras of urolithins metabolites in urine (B); Typical UV spectra of the isourolithin A 9-glucuronide, isourolithin A 3-glucuronide and urolithin B-glucuronide (C)



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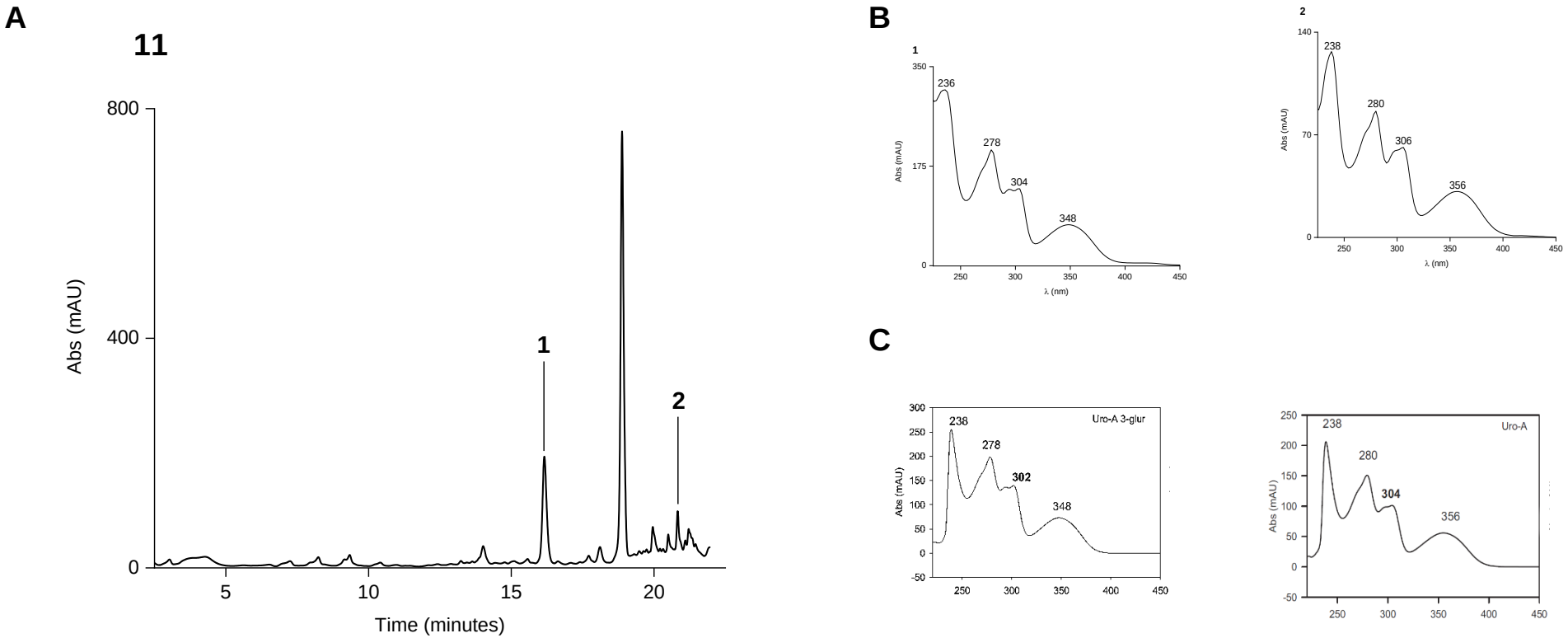
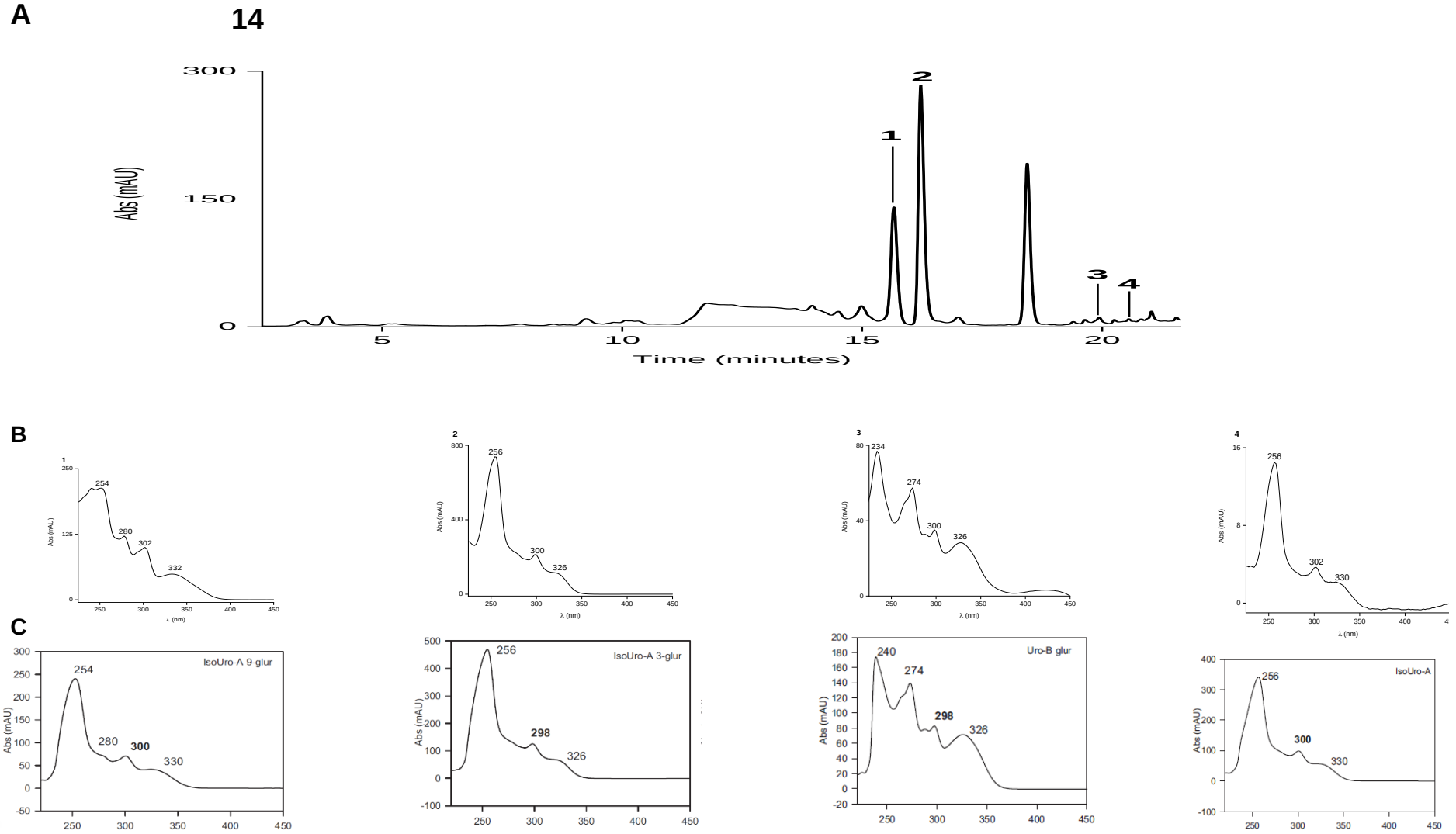


Chromatographic profile at 305 nm (A); UV spectras of urolithins metabolites in urine (B); Typical UV spectra of the urolithin-A 3 glucuronide and urolithin-A(C)



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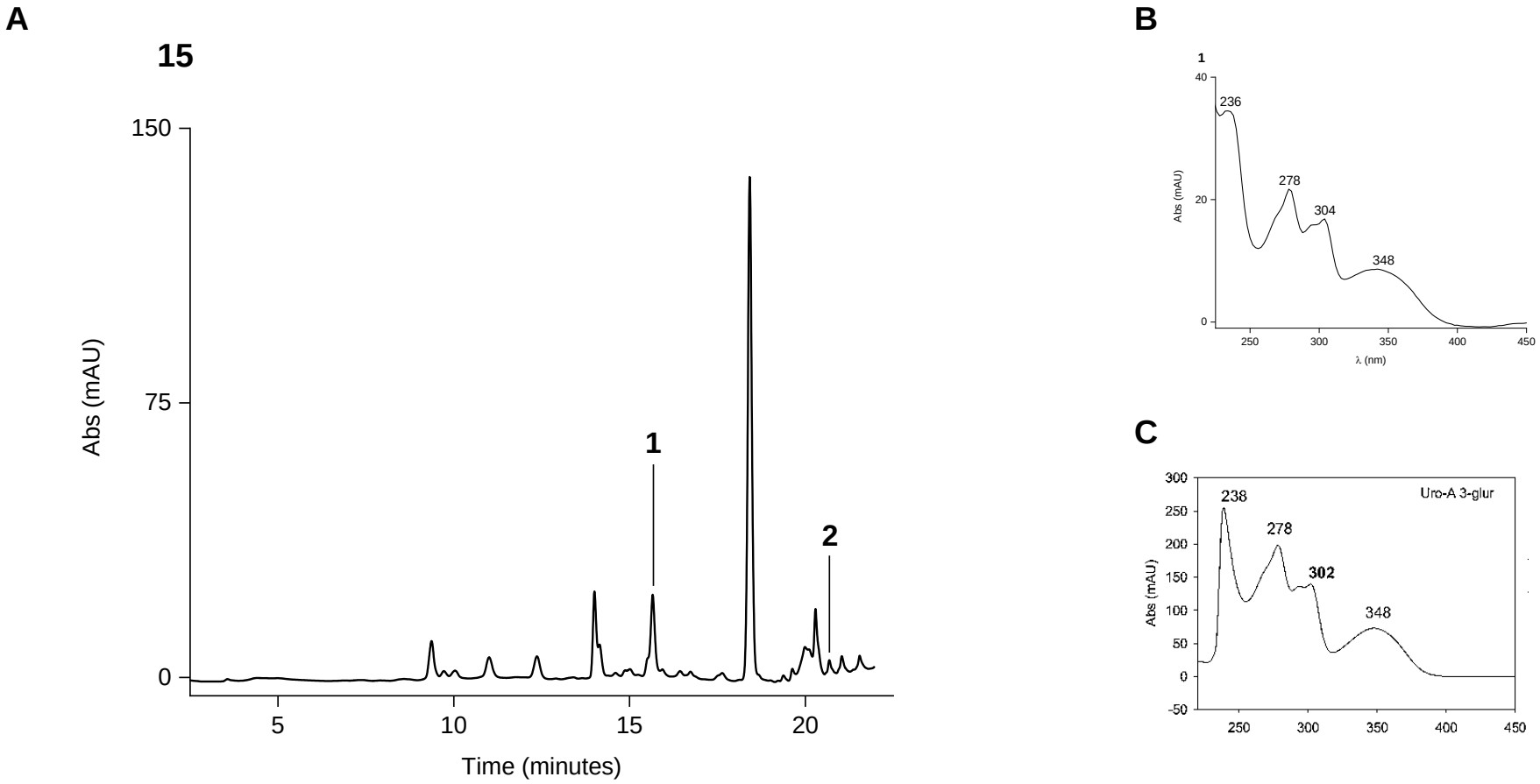

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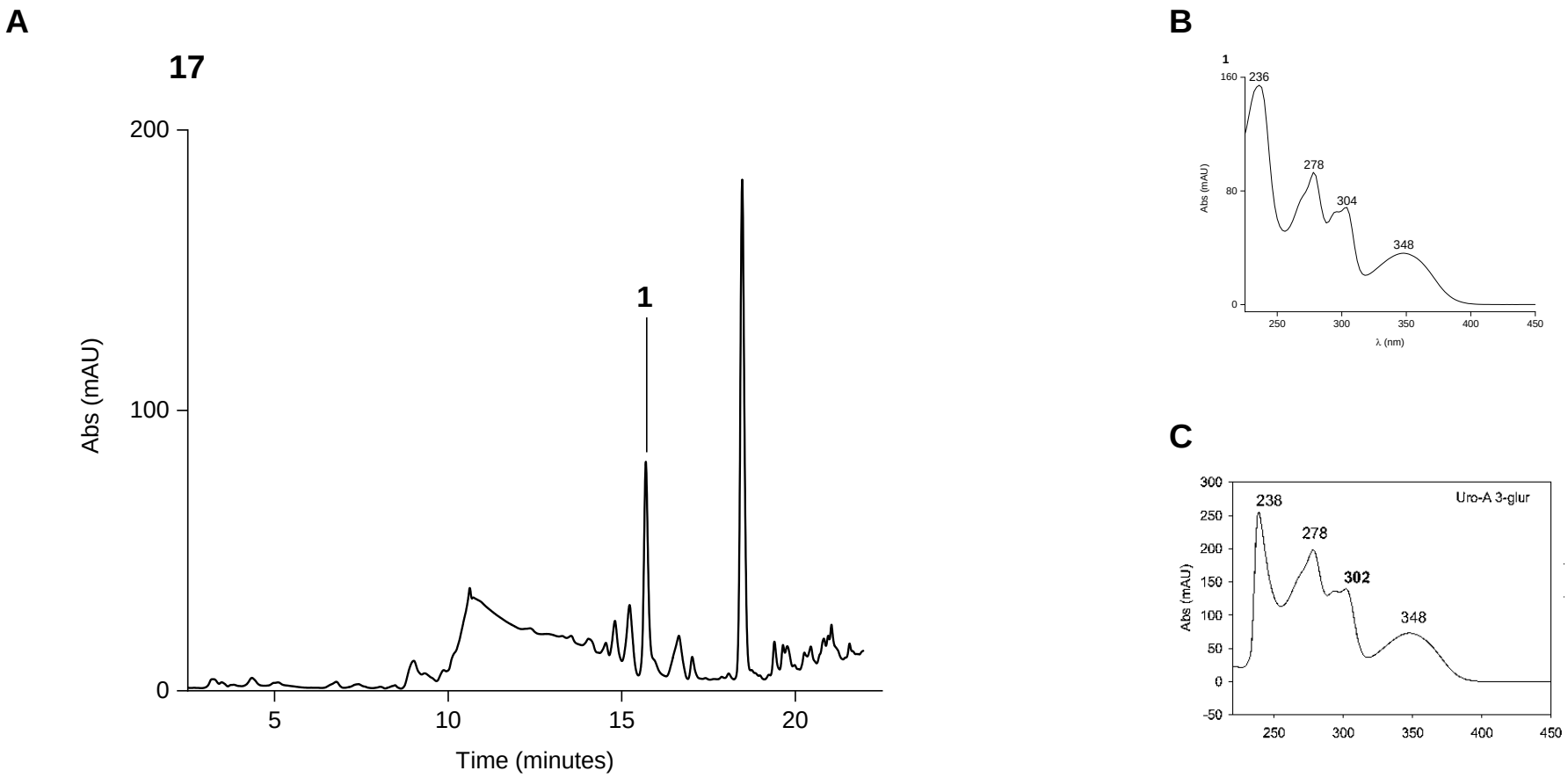

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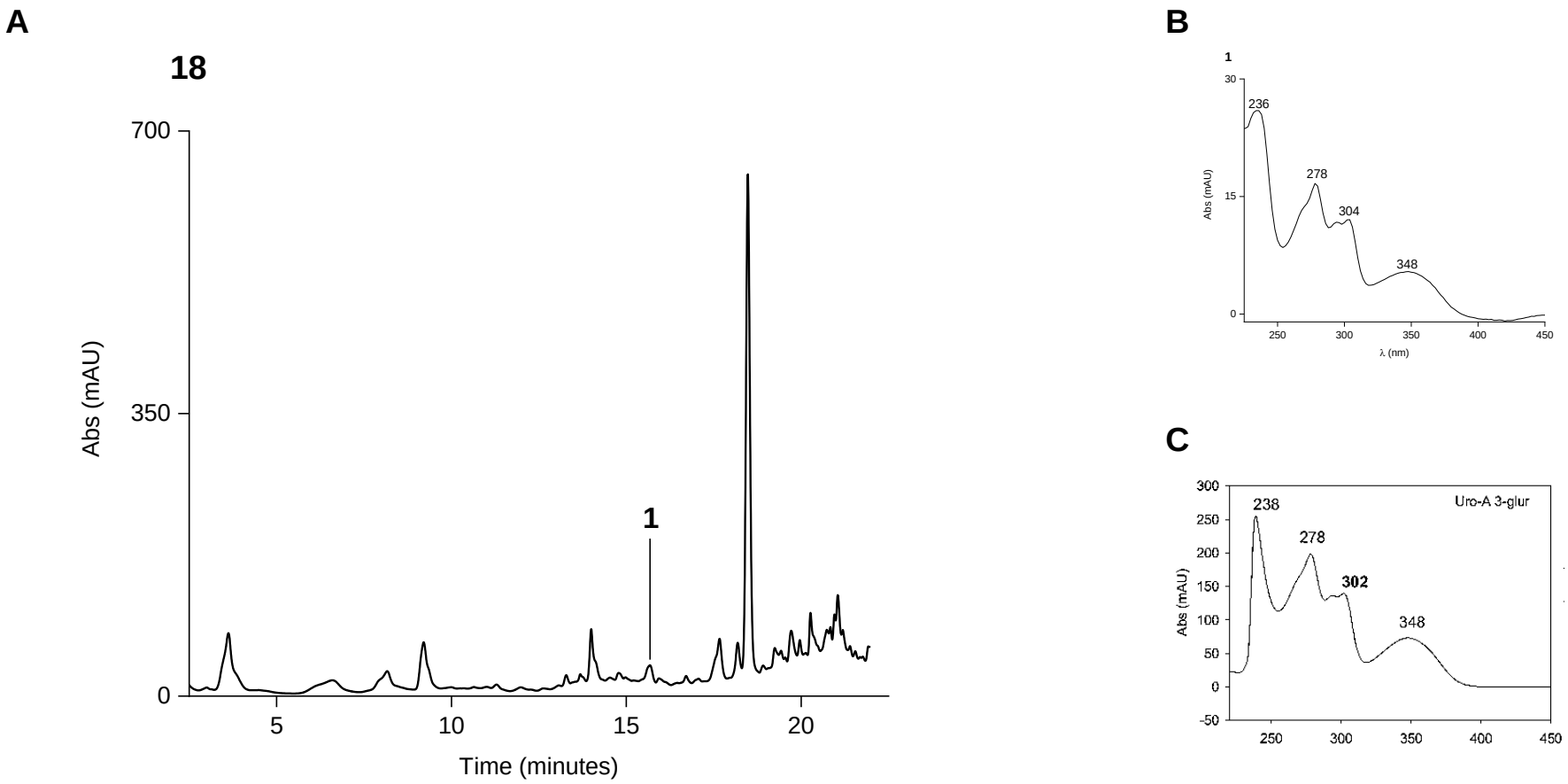

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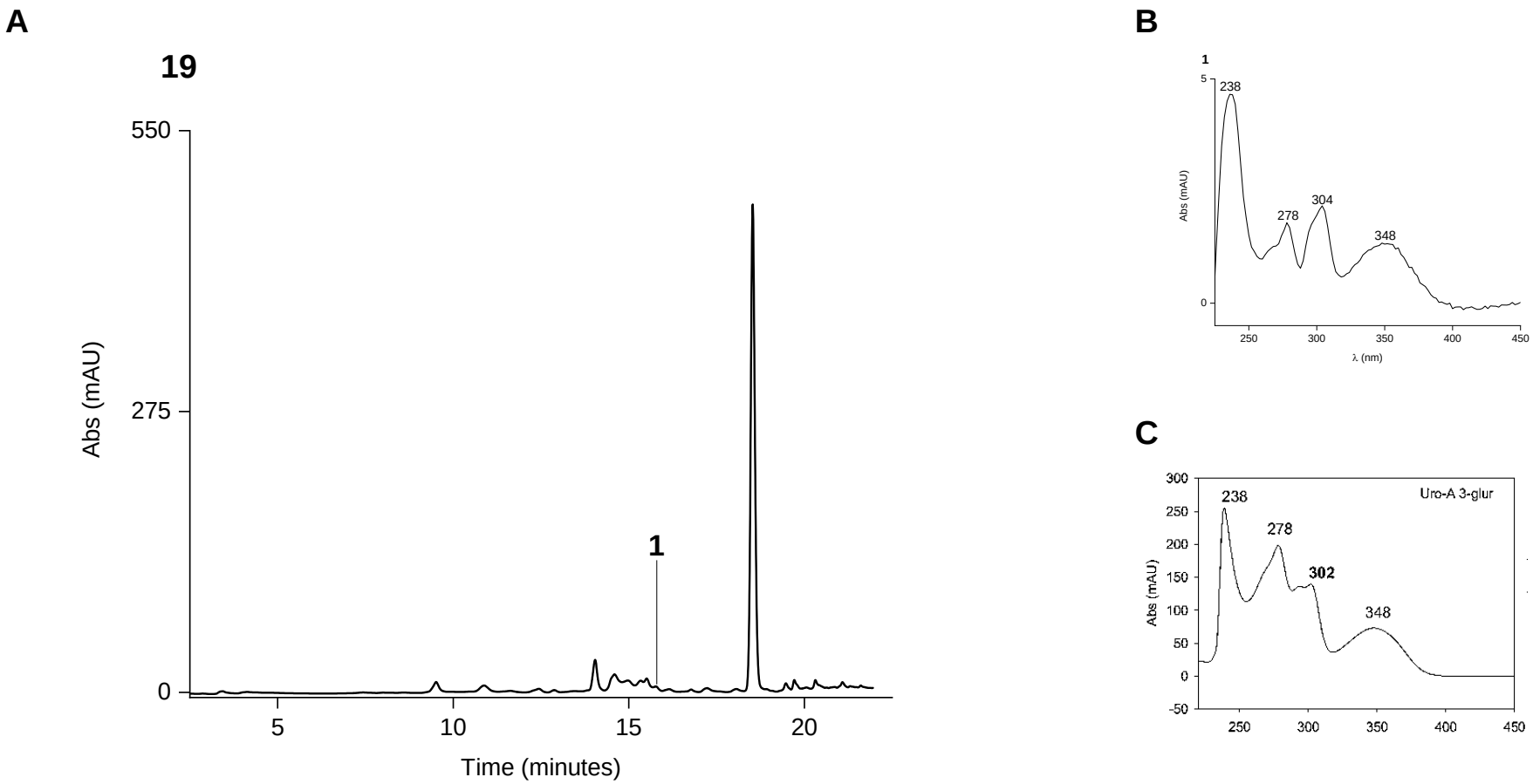

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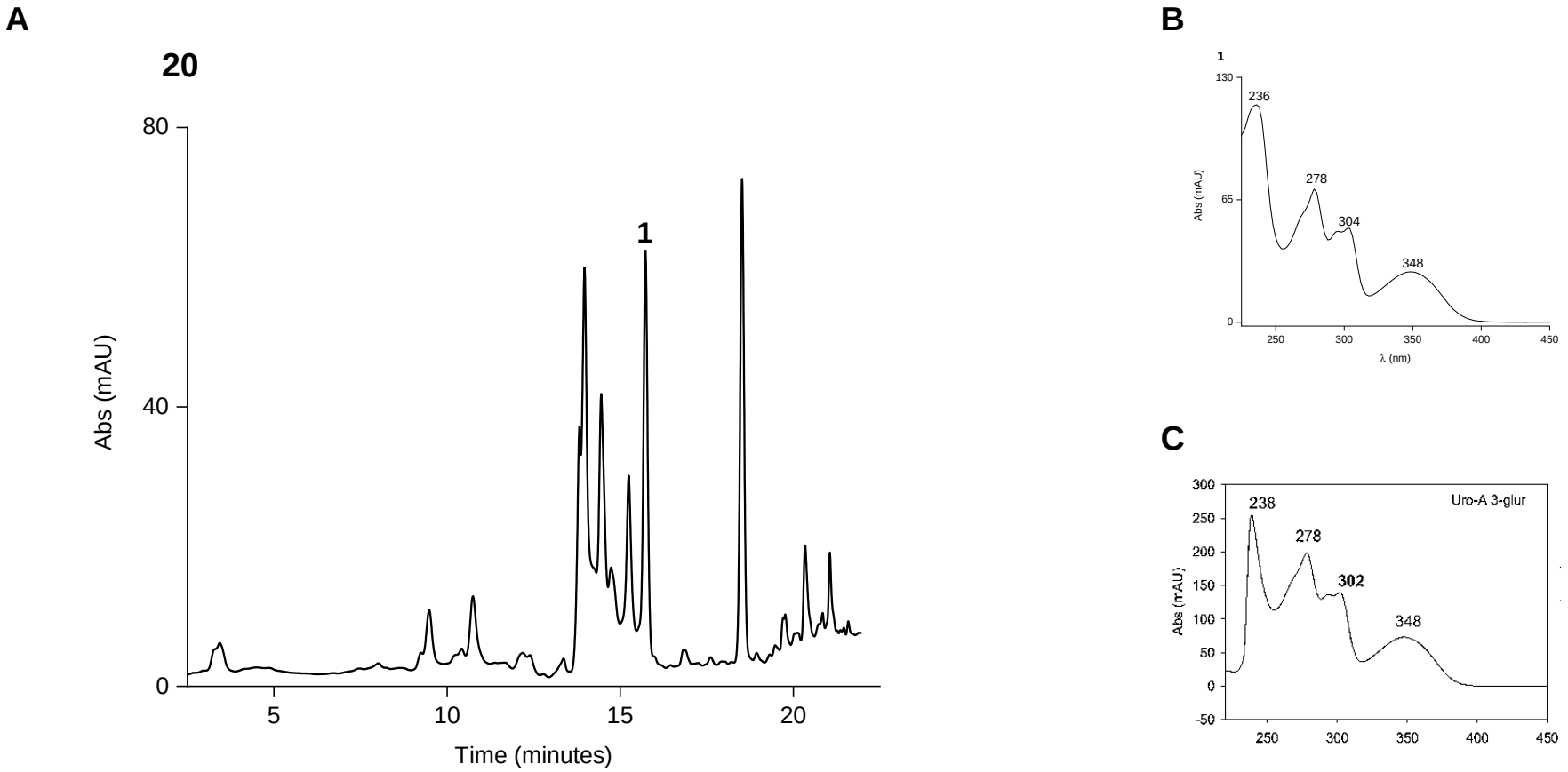

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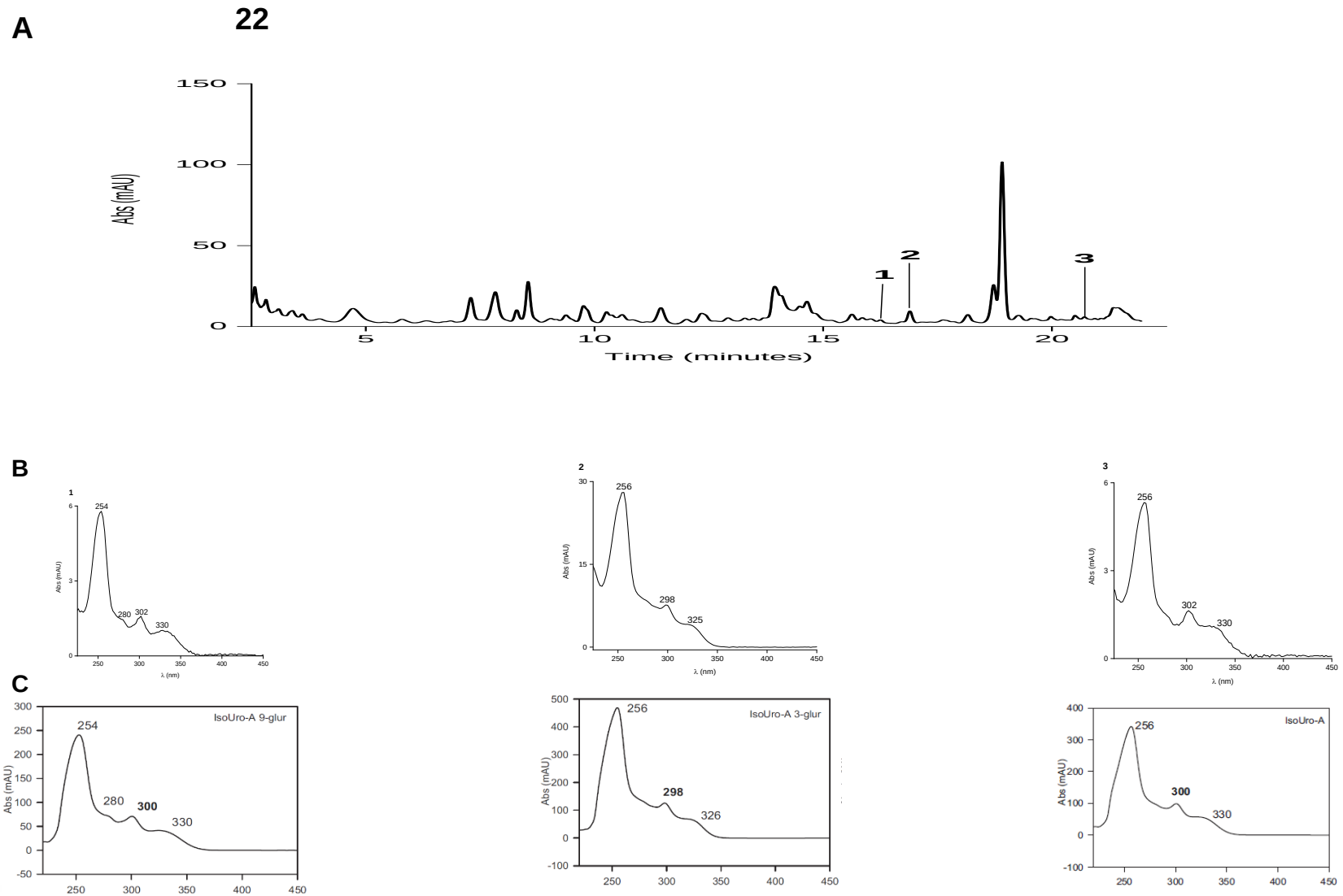

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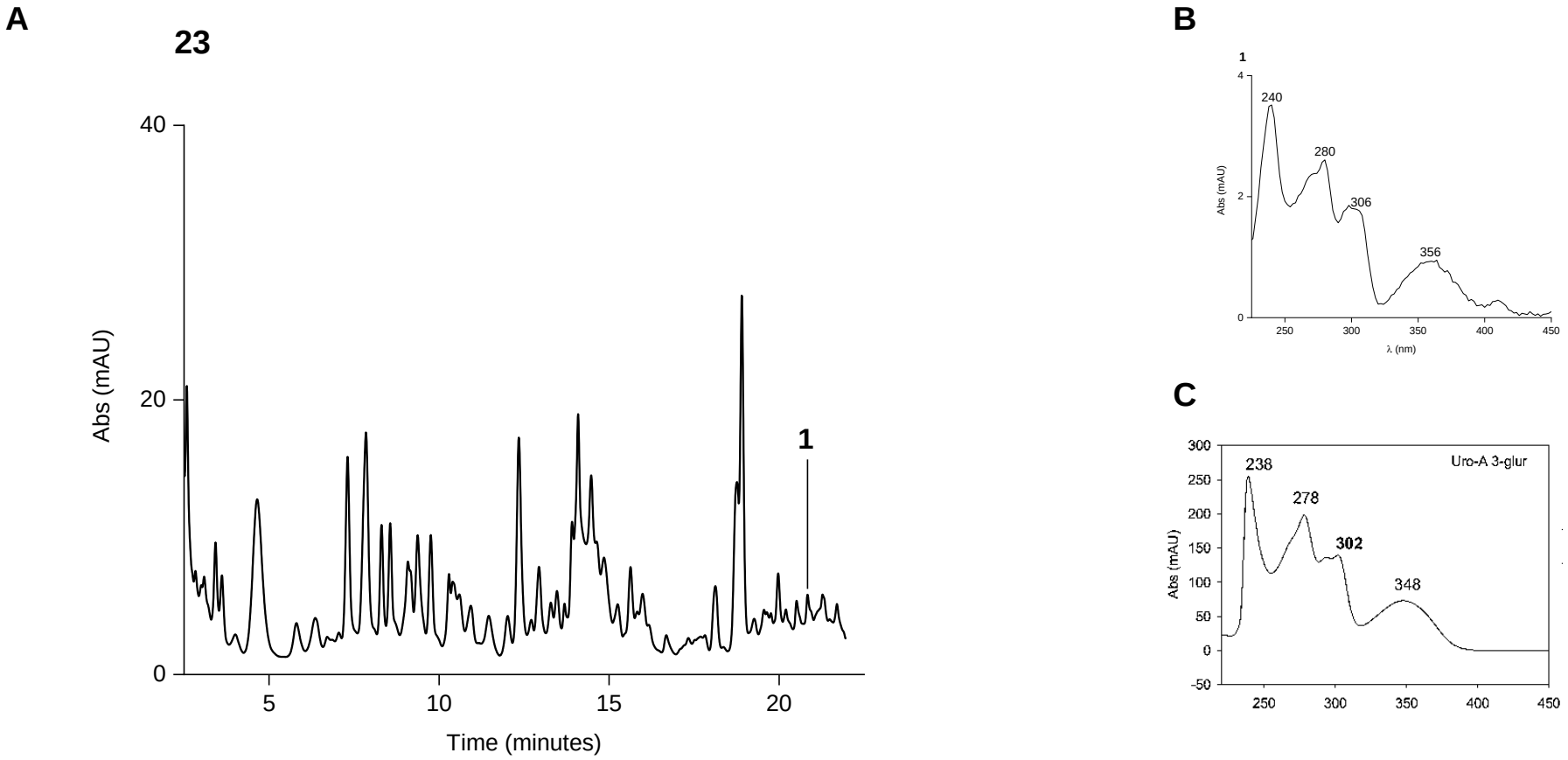

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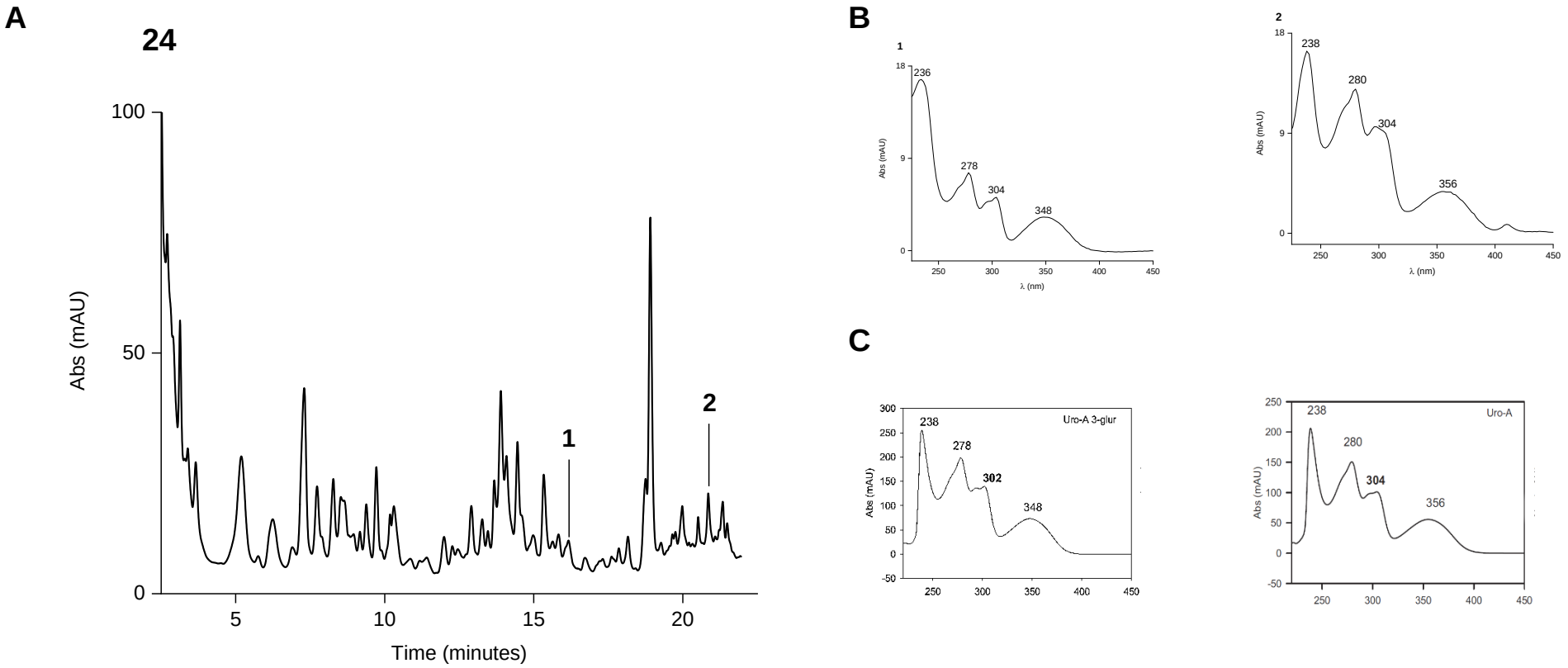

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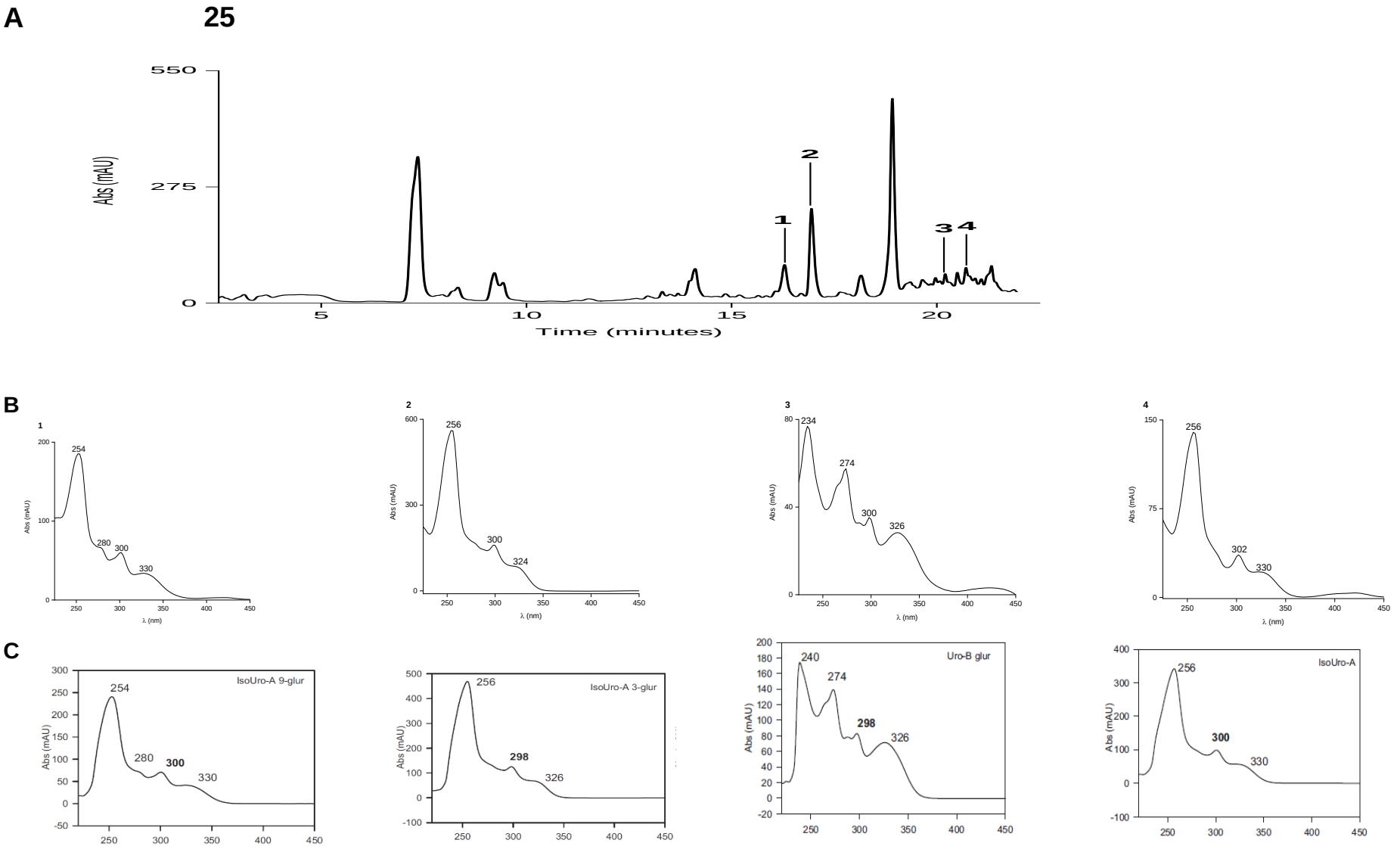

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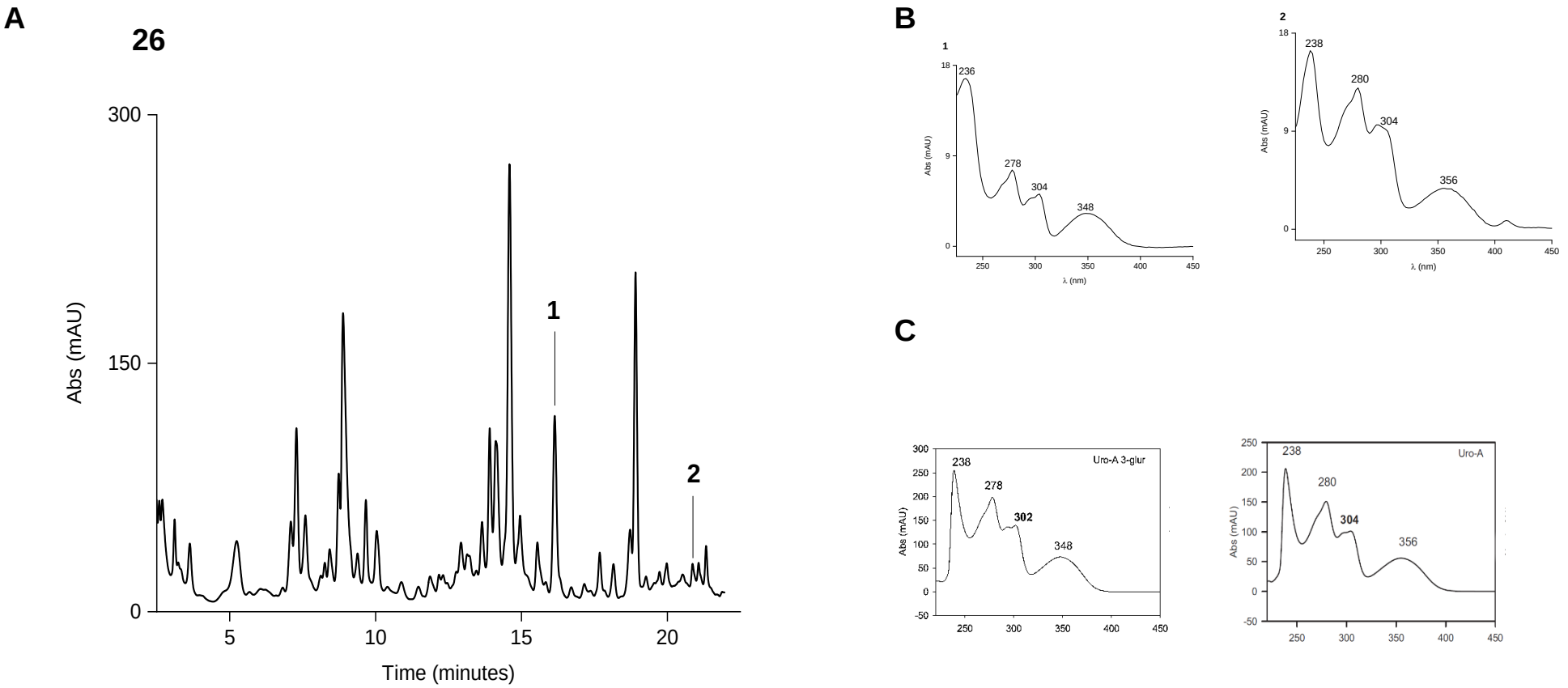

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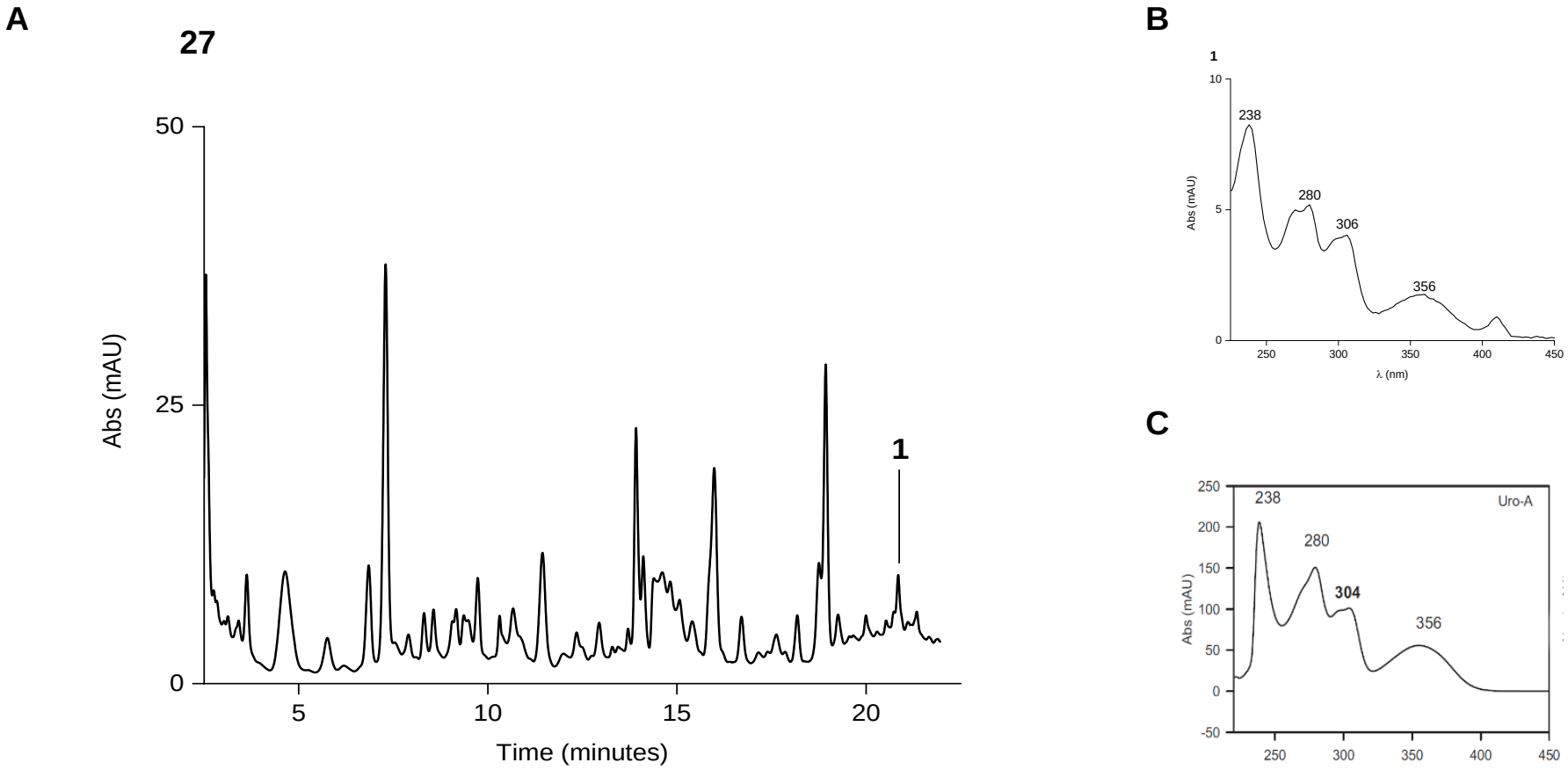

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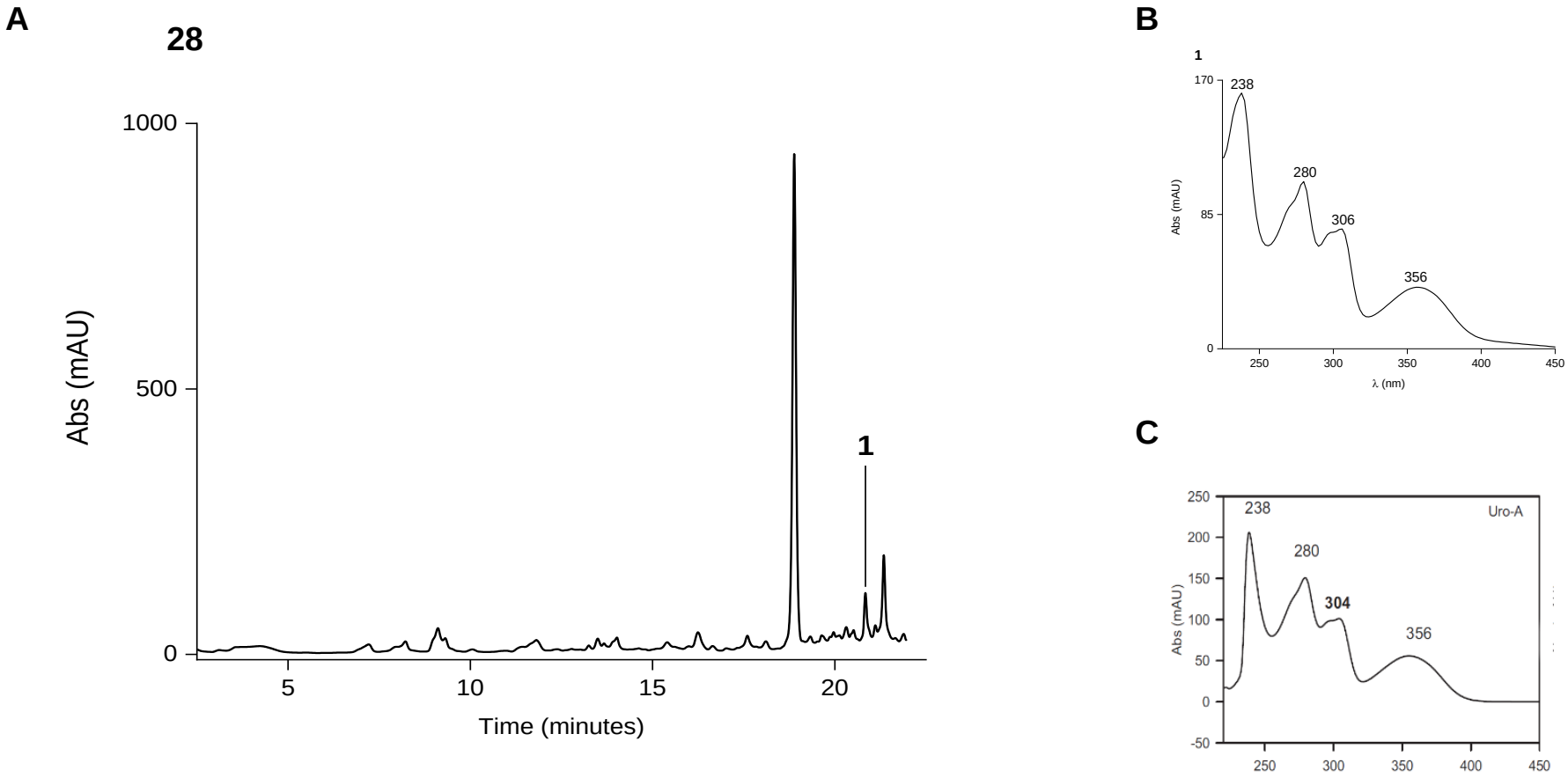

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