Synthesis of the Main Red Wine Anthocyanin Metabolite: Malvidin-3-*O*-β-Glucuronide

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The large diversity of flavonoid metabolites in vivo makes their proper detection and quantification difficult in the absence of pure standards. Indeed, flavonoids are extensively metabolized to different conjugated derivatives (methyl ethers, glucuronides, sulfates) that are likely to be the circulating forms able to reach their metabolic targets. To study the activity of these circulating metabolites, these compounds must be available as standards. However, most of these compounds are not commercially available and hence have to be prepared in the laboratory.

Amongst red-wine flavonoids, anthocyanins are the most difficult to track in vivo. The human metabolism of anthocyanins is poorly described in the literature, with scarce information on the biological effects of their metabolites. A new study led by Dr. Luis Cruz and Dr. Iva Fernandes at the University of Porto (Portugal) has recently investigated this issue.

"Anthocyanins are naturally occurring polyphenols belonging to the group of flavonoid compounds widely found in plants. These pigments are responsible for the color of many flowers, leaves, seeds, fruits and vegetables ranging from red to purple and blue," said Dr. Fernandes, who added: "In vitro and in vivo studies have demonstrated that anthocyanins may offer potentially beneficial effects to human health because of their biological properties such as anti-aging, anticancer, anti-inflammatory, anti-infection and anti-diabetes. Epidemiological evidence suggests that the ingestion of high proportions of anthocyanins in the diet may contribute to lowering the risk of cardiovascular events such as hypertension and stroke."

However, it is recognized that the in vivo anthocyanin bioactive forms do not always correspond to the native forms, but rather consist of conjugates or metabolites originating from the original anthocyanins after absorption.[1] "Anthocyanins are rapidly absorbed in the stomach and small intestine and then they appear in plasma and urine as intact, methylated, glucurono- and/or sulfoconjugated forms.[2,3] This enterohepatic recycling opens a new field of interest that remains a challenge: unravelling the biological properties of anthocyanin metabolites," explained Dr. Fernandes.

Dr. Fernandes said: "In 2009, an enzymatic approach to obtain anthocyanin metabolites seemed the best option for this type of flavonoid in order to overcome their low stability under the conditions used for their chemical synthesis (pH and temperature).[4] Some anthocyanin metabolites (methylated, glucuronides and glutathione adducts from cyanidin- and delphinidin-3-glucosides) were obtained through that enzymatic approach."[4]

Some of these standards – such as delphinidin-3-glucoside-, cyanidin-3-glucoside- and petunidin-3-glucoside-methylated metabolites – were then tested against several biological properties, antiradical and reducing properties and also their antiproliferative effects against different cancer cell lines (MKN-28, Caco-2 and MCF-7).[5] "The methylated metabolites were found to still retain significant radical scavenging activity and reducing activity, suggesting that they could act as potential antioxidants in vivo," said Dr. Fernandes, adding: "The conjugation with methyl groups decreased or did not alter the antiproliferative effect of the native anthocyanins. This report investigated the biological meaning of the new circulating molecules that are produced by the human body in response to the ingestion of phytochemicals."

Other important cyanidin-3-glucoside metabolites, namely methylated and glucuronylated in positions 4′ and 7, respectively, were chemically synthesized by the same research group, following the strategy of aldol condensation reaction between a salicylic aldehyde and an acetophenone derivative, which had been previously prepared.[6,7] This strategy allowed the research team to obtain useful amounts of these standards for rapid and correct identification in biological samples.

Dr. Fernandes explained: "Recently, in a crossover trial with healthy volunteers who ingested a blackberry puree, the purified standards allowed our group to identify the position of insertion of the glucuronyl group and methyl group in the metabolites detected in plasma and urine samples.[8] Indeed, cyanidin-7′-*O*-glucuronyl-3-glucoside was identified for the first time in human plasma and urine samples. Moreover, identification of the attached methyl group's position was anticipated from comparison with the natural standard peonidin-3-*O*-β-D-glucoside and the currently available standard cyanidin-4′-*O*-methyl-3-glucoside, previously obtained by hemi-synthesis."[4]

According to Dr. Fernandes, previous studies evaluating the bioavailability of red-wine anthocyanins had only looked for the main native anthocyanin (Mv3glc) in plasma and urine, underestimating the total anthocyanin content.[9,10] "In

the work of Garcia-Alonso et al., where volunteers consumed 180 mg of red wine anthocyanin extract in a sugar-sweetened yogurt," she said, "the authors were already able to identify some anthocyanin metabolites in plasma."[11]

"Identification of the metabolites originating in vivo is often compromised, which restricts the comprehension of the metabolic pathways followed by anthocyanins after absorption," said Dr. Fernandes. She continued: "Usually, it is only possible to determine the type of conjugation that occurs after anthocyanin absorption, but it is not possible to ascertain the position where that insertion takes place. This is quite important since different molecules may have different biological activities."

In a work registered in clinicaltrials.gov under the code NCT02975856, a similar human trial was performed with the ingestion of red wine as source of anthocyanins, especially malvidin-3-*O*-β-glucoside (Mv3glc).[12] After analysis of urine and plasma samples, glucuronidation was clearly identified as the main metabolic pathway, although the detailed mechanism was still missing.

"Firstly, and inspired by the in vivo enzymatic assay, the enzymatic hemi-synthesis was performed with the co-addition of glucosidase and UGT protein source in the presence of the main red wine anthocyanin," explained Dr. Fernandes. This approach was not very successful, since after glucose removal the anthocyanins started to precipitate in the buffer system and degraded rapidly. "The other possibility was to collect the corresponding peak, purify it and perform NMR analysis to ascertain its structure; however, this would be almost impossible due to the low sample quantities and quite hard and time-consuming work," said Dr. Fernandes, who continued: "To overcome these limitations, synthesis of the most probable main conjugate to be detected after red wine ingestion (malvidin-3-*O*-β-glucuronide)[13] was successfully performed (Figure 1) following the chemical synthesis strategy previously developed by our research group."[6,7]

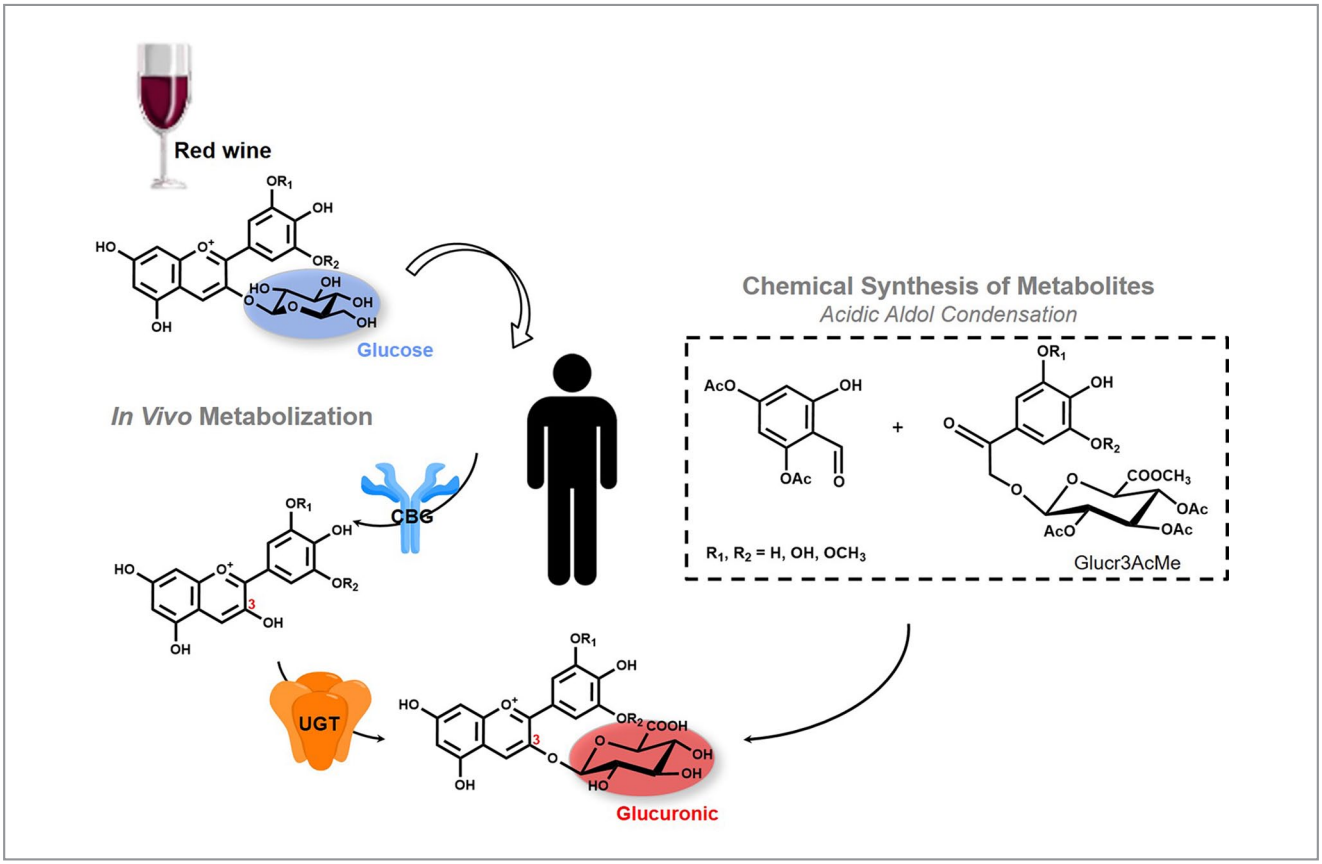

Figure 1 Schematic representation of the metabolic pathway of red wine anthocyanins during absorption and the chemical synthesis approach followed to obtain the main red wine pigments. CGB: Cytosolic-β-glucosidase; UGT: Uridine 5′-diphospho-glucuronosyltransferase; Glucr3AcMe: 2,3,4-tri-*O*-acetyl-α-D-glucopyranuronic acid methyl ester.

The authors concluded: "The combination of efforts between different areas of knowledge is remarkably fruitful. In this particular case involving our laboratory, knowledge of the chemical and biological behavior of polyphenols allowed us to overcome some of the major limitations associated with the identification of this class of flavonoids in human biological samples."

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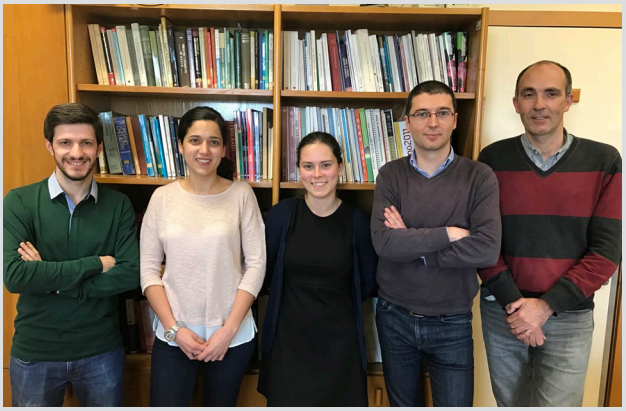
About the authors



From left: Dr. L. Cruz, Dr. I. Fernandes, A. Évora, Prof. N. Mateus, Prof. V. de Freitas

Luís Cruz graduated in chemistry (2004), and obtained his M.Sc. in chemistry (2006) and Ph.D. in chemistry (2010) at the Faculty of Sciences of the University of Porto (FCUP, Portugal) under the supervision of Professor Victor de Freitas. He was assistant professor of the organic chemistry laboratory of the 2nd year of chemistry graduation. He carried out postdoctoral research activities at the Food Chemistry group of REQUIMTE-LAQV Research Center (Portugal) during the period of 2010–2016. He is currently auxiliary investigator in the same group. He has been a team member in several projects funded by national institutions and has been supervising under- and post-graduate students (with M.Sc. and Ph.D. degrees). He is now principal investigator of the project entitled 'Development of novel anthocyanin-lipophilic bioactives for technological applications'. His research interests include: (a) chemical synthesis of anthocyanin metabolites for biological studies, (b) design of natural and bio-inspired flavyliuns as photosensitizers for energetic applications (e.g. dye-sensitized solar cells), (c) recycling and lipophilization of anthocyanins from agro-food wastes towards novel stable colorants for applications in the food, pharmaceutical and cosmetics industries, and (d) study of physical-chemical, antioxidant and biological features of novel anthocyanin-derived pigments.

Iva Fernandes graduated in biochemistry in 2006 and obtained her Ph.D. in chemistry in December 2012, both at the University of Porto (FCUP, Portugal). She is currently a postdoctoral research at the REQUIMTE/LAQV at the Faculty of Sciences of the University of Porto under the research line of recycling of polyphenols from industrial wastes and their putative applications as nutraceuticals or cosmetic ingredients. She has been involved in various research projects, funded essentially by the Portuguese government. Those projects involved different areas of research, such as the development, optimization and validation of methods for the analysis and characterization of polyphenols and metabolites in biological samples, synthesis of metabolically relevant phenolic metabolites and evaluation of polyphenol bioavailability, metabolism and biological activity, using both in vitro/ex vivo models and in vivo approaches, including human intervention trials. Recently, she became the leading researcher for the development of a new nanotechnology approach for targeted absorption of nutraceuticals towards healthy human nutrition. She is coauthor of 35 papers in peer-reviewed journals, including three chapters in international reference books and around 40 communications to congresses. She is also a guest editor for the International Journal of Molecular Sciences.

Ana Évora is an M.Sc. student in biochemistry at the Faculty of Sciences of the University of Porto (FCUP, Portugal), having obtained her B.Sc., also in biochemistry, at the same institution in 2015. Currently, she is working in the Department of Chemistry and Biochemistry (DBQ) of FCUP as a B.Sc. fellow, studying the absorption, stability and bioactivity of new anthocyanin derivatives towards their application in the cosmetics and food industries. More recently, she participated in two national congresses in the field of chemistry with a conference poster, and also in IJUP (the meeting of student researchers from the University of Porto) with an oral presentation. While at college, she concluded her B.Sc. degree in Hungary under the ERAMUS+ program, performing her curricular internship.

Nuno Mateus graduated in biochemistry in 1997 and obtained his Ph.D. in chemistry in 2002, both at the University of Porto (Portugal). He has been teaching (food chemistry and industrial biochemistry, among other courses) at the University of Porto since 2001 and is currently an associate professor at the Department of Chemistry and Biochemistry of the Faculty of Sciences at the University of Porto. Included in the section of organic chemistry of the department, his field of research concerns food chemistry and biochemistry, essentially food polyphenols and in particular red wine chemistry (grape and wine polyphenolic composition; chemical pathways occurring during wine ageing; detection, isolation and characterization of newly formed polyphenols as well as their putative industrial applications, etc.). He has been collaborating with local industrial companies (especially Port wine companies) and has been involved in several research projects funded essentially by the Portuguese government. One of his main areas of research deals with the synthesis and characterization of anthocyanin-derived pig-

ments detected in Port wines and their putative applications as colorants in the food industry. More recently, he has been coordinating projects dealing with biological properties of red wine polyphenols towards some cancers and age-related diseases. He is now also exploring a new research line focused on the application of industrial wastes of the food industry into novel applications in the cosmetics industry.

Victor Freitas graduated in chemistry from the Faculty of Sciences of the University of Porto (FCUP, Portugal) in 1989. In 1995, he obtained his Ph.D. in biological and medical sciences from the University of Bordeaux II (France), specializing in oenology. After his Ph.D., he returned to the Department of Chemistry and Biochemistry (DQB) of FCUP where he has been developing his teaching and research activities. He is currently full professor at the University of Porto and member of the REQUIMTE-LAQV Research Centre where he has been developing an independent area of research involving polyphenol compounds. He is president of the 'Groupe Polyphénols' society since July 2016 (www.groupepolyphenols.com).

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