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Main urinary biomarkers of golden berries (Physalis peruviana) following acute and short-term nutritional intervention in healthy human volunteers

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ABSTRACT

The metabolites entering the bloodstream and being excreted in urine as a result of consuming golden berries are currently unidentified. However, these metabolites potentially underlie the health benefits observed in various in vitro, animal, and human models. A nutritional intervention with 18 healthy human volunteers was performed, and urine was collected at baseline and after acute and short-term fruit consumption for 19 days. After UPLC-ESI/QToF-MS analysis, untargeted metabolomics was performed on the urine samples, and from the 50 most discriminant ions (VIP > 2) generated by a validated PLS-DA model (CV-ANOVA = 3.7e-35; R^2Y = 0.86, Q^2Y = 0.62 and no overfitting), 22 compounds were identified with relatively high confidence. The most discriminant metabolites confirmed by DHS/GC-MS^2 analysis of volatiles in urine were sesquiterpenes ($C_{15}H_{22}$): 3 stereoisomers, β-vatirenene, β-vetivenene, and β-vetispirene, and 2 isomers, eremophila-1(10),8,11-triene and α-curcumene. Another major urinary biomarker was 4β-hydroxywithanolide E and its phase II derivatives, which were observed in urine for all individual up to 24 h after the fruit was consumed; thus, the bioavailability of this biomarker in humans was demonstrated for the first time. Additionally, the excretion of certain acylcarnitines and hypoxanthine in urine increased after golden berry consumption, which may be associated with a detoxifying effect and may occur because fats were utilized rather than carbohydrates to meet the body's energy needs. The main biomarkers of golden berry consumption are specific to this fruit, confirming its potential for the functional food market.

1. Introduction

Golden berries are consumed worldwide, although commercial production is concentrated in only a few countries. Colombia is currently the world's leading producer and exporter of the fruit (Gomez et al., 2022). Formerly, golden berries were used almost exclusively in importing countries as decoration for dishes and desserts; however, the fruit is increasingly being consumed for its taste and nutritional and health benefits, which are becoming known to the general public. In fact, the golden berry has recently been labeled a "superfruit", a fruit with potential health-enhancing effects (Kupska et al., 2016), and there is increasing interest in its various unique nutritional constituents. Numerous reviews on the chemical composition and potential functional properties of golden berries have been conducted within the last decade (Puente et al., 2011; Olivares-Tenorio et al., 2016; Ramadan, 2011; Kasali et al., 2021, 2021; Puente et al., 2019; Ramadan, 2019). In summary, the fruit stands out for its content of withanolides, sucrose esters and terpenoids, three emblematic compounds of the Physalis species. The fruit is also an important source of other more common compounds, such as β-carotene (Etzbach et al., 2018); flavonols, mainly rutin and myricetin (Licodiedoff et al., 2013); and phenolic acids. However, withanolides, sucrose esters and terpenoids are most often

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cited as the primary compounds potentially responsible for the functional health qualities attributed to golden berries.

Withanolides belong to a group of compounds characterized by a 28-carbon (C28) steroidal backbone based on an ergostane structure bound to a lactone. There is a large variety of withanaloid compounds, but mainly derivatives of withanolide E and withanolide D were reported in the fruits. Physalolactone, which is a glycosylated withanaloid, and physagulin, another kind of withanaloid, have also been reported in fruits (Navarro-Hoyos et al., 2022). However, to date, it has remained unproven whether withanolide compounds are bioavailable. The other specific constituents of Physalis fruits are sucrose esters, a group of sugars esterified with saturated fatty acids. The main sucrose esters reported in Physalis are O-isobutanoylsucrose, di- and o-tri-o-isobutanoylsucrose and their derivatives. Finally, terpenoids are volatile and often determine the flavor and taste of fruits. In golden berries, all families of terpenes with 10 and 15 carbons are present Therefore, it is crucial to determine the bioavailability of these compounds and to identify related biomarkers in urine that are indicative of their intake. This information would establish their potential for bioactivity within the body.

Biomarkers of dietary intake have emerged as a significant and contemporary subject within the realm of food science. This advancement has been facilitated through nutrimetabolomic research, which involves the combination of metabolomic tools with dietary interventions conducted directly on human volunteers. However, despite a limited number of published studies on this subject, they remain insufficient in light of the extensive diversity of food products. Specific biomarkers from golden berry remain largely unexplored, even though their understanding is crucial for comprehending the potential health-functional attributes.

Untargeted metabolomics using high-throughput high-resolution mass detectors can highlight differences in metabolite composition between different biofluid states. Various biofluids can be used for this purpose, primarily plasma, serum, and urine. Urine has the advantage of being an easily accessible biological fluid that contains a considerable range of excreted metabolic by-products, including dietary compounds. It also has the enormous advantage of concentrating these exogenous metabolites and facilitating their continuous recovery over a long period, whereas blood sampling can only offer a snapshot at a given point in time.

A better understanding of the biomarkers of golden berry intake would significantly increase in the value of this fruit and open new markets, such as that of functional foods.. The objective of this study focuses on discovering these main urinary biomarkers of golden berry consumption through a nutritional intervention study with healthy human volunteers.

2. Methods

2.1. Materials

The plant material utilized in this experiment was exclusively sourced from the Dorada variety, registered by the Colombian research institute AGROSAVIA. The plants originated from a plantation situated in San Vicente, Antioquia (Colombia), at an elevation of 2161 m above sea level. Typically designated for export, these fruits were cultivated and managed in adherence to global standards. They were provided by the company Caribbean Exotics (Rionegro, Antioquia, Colombia), and subsequently packaged in perforated polypropylene containers of 250 g and 150 g, which were distributed to the volunteers.

2.2. Clinical trial

2.2.1. Volunteers

A group of 18 volunteers participated in this single-blind nutritional intervention, which has been described in detail elsewhere (Vaillant et al., 2021; Sierra et al., 2022). This nutritional intervention was previously approved by the ethical committee of the Institute of Health Sciences of CES University (Medellin, Colombia; ethical ID: 707, 20 September 2017) and registered with the International Clinical Platform (https://rpcec.sld.cu/trials/RPCEC00000268-En; accessed on 17 April 2018). Participants were assured of anonymity and confidentiality. All participants provided written informed consent. Briefly, the study involved the following periods: (1) washing (one week without golden berry consumption); (2) 12 h fasting baseline, in which placebo (250 ml of water sweetened with 25 g of sugar) was ingested at time 0 (Day 0) and urine was collected from 0 to 6 h, 6 h to 24 h, and at 24 h; (3) 12 h fasting before acute consumption of 250 g of golden berries at time 0 (Day 1) and collection of urine from 0 to 6 h, 6 h to 12 h, and 12 h to 24 h; (4) short-term intervention, free living with daily consumption at any time of the day of 150 g of golden berries for 18 days; and (5) on Day 19 at time 0, after 12 h fasting, the same placebo from Day 0 was ingested and followed by collection of urine from 0 to 6 h, 6 h to 12 h, and 12 h to 24 h. The quantities of fruit to be consumed were established in collaboration with the volunteers as part of a preliminary study. During this process, a maximum acceptable quantity of 250 g for acute consumption and 150 g for daily consumption over several days were deemed appropriate. On the days of sampling (Days 0, 1 and 19), the same standardized meals were provided to all the participants (breakfast, lunch, and dinner). The participants, employed by Nutresa company (Medellin, Colombia) were selected based on their commitment to exclusively utilizing the company's 24/7 catering services for their meals during the sampling phase. They were also given the option to bring home meals from the same restaurant. This method guaranteed a dependable provision of standardized meals throughout the sampling phase. Urine samples were collected in polypropylene bottles (1000 ml), which were immediately stored at 4 °C in a cool box during the collection phase. All urine was collected and pooled between consecutive time points 0 and 6 h, 6 h and 12 h, and 12 h and 24 h. Aliquots of pooled urine at each time point were centrifuged and then stored at −80 °C until analysis.

2.3. Metabolomic analysis

2.3.1. Sample treatment

Published guidelines for nutrimetabolomic studies (Ulaszewska et al., 2019) were strictly followed. Briefly, the thawed urine was centrifuged at 5000 g for 15 min, and a milliliter of supernatant was collected, to which 1 ml of Milli-Q water was added. Subsequently, the samples were filtered under pressure with microporous regenerated cellulose filters with a size of 0.22 μm (Minisart-R 25). A pool of all collected urine samples was prepared for use as quality control (QC) and drift correction. The samples and pools were injected into the HPLC-MS with injection of 2 quality controls (pools) for every 10 sample injections.

2.4. UPLC-ESI/QToF-MS analysis

Ultra-performance liquid chromatography coupled to an electrospray ionization source and a quadrupole time-of-flight mass spectrometry detector (UPLC/ESI-QToF-MS) was used. Chromatographic separation was performed using a 1.7 μm, 2.1 * 100 mm CSH 18 column (Acquity®) coupled to a 1.7 μm Vanguard CSH 18 precolumn in a UPLC (Waters) equipped with an automatic sampler and a quadrupole time-of-flight detector (ESI/QToF-MS). An elution gradient was employed using water (solvent A) and acetonitrile (solvent B), and both were adjusted with 0.1% (w/v) formic acid. The elution started at 0% B and continued as follows (time in min, concentration of B in % (v/v)): (initial, 100; (7, 10); (22, 95); (22,1; 0) and ended with a rebalancing stage (26. 0). The flow rate was adjusted to 0.45 ml/min with a total running time of 26 min. The temperature of the column was maintained at 30 °C, and the temperature in the autosampler chamber was controlled at 10 °C. The

volume of samples injected was 2 μL.

Positive and negative ESI were selected for full-scan (*m/z* 100–1500) data acquisition in continuous mode. The operating conditions of the ESI-QToF-MS were capillary voltage = 0.4 kV and sampling cone voltage = 40 V. Nitrogen was used as the cone gas and desolvation gas with flows of 0 L/h and 1000 L/h, respectively. Additionally, mass spectrometry analyses were performed with a constant collision energy of 6 eV. The scan time was adjusted to 0.5 s. The equipment was calibrated with leucine enkephalin (556.2771 Da) with an infusion period of 10 s and a flow of 12 μL/min. Some samples were reinjected to obtain the MS2 fragmentation pattern of selected ions. The MS2 fragmentation spectra were obtained through a complete scan from 50 to 1000 Da at the following collision energies: 10 eV, 20 eV, 30 eV, 40 eV and 50 eV in the case of ions with molecular weights>500 *m/z*. They were compared to the ESI-MS2 of authentic standards published in the literature or to those deposited in databases, such as Massbank of North America (MoNA, https://mona.fiehnlab.ucdavis.edu/) and HMDB (The Metabolomics innovation Center, University of Alberta, Canada).

2.5. DHS/GC–MS/MS analysis

Analysis by dynamic headspace solid-phase microextraction coupled to gas chromatography and mass spectrometry at different fragmentation energies (DHS/GC-MS2 analysis) was performed on urine spots. Five milliliters of urine was placed in a 10 ml glass vial. The sample was equilibrated to 30 °C, and then the headspace was swept with a nitrogen flow at 1 ml/min and stirred at 500 rpm. The volatile compounds were trapped on a Tenax TA using a Gerstel autosampler (Gerstel, Mülheim a der Ruhr, Germany) and dried with an additional purge flow at 100 ml/min at 50 °C for 2.5 min to remove residual water. The collected volatile compounds were then desorbed using an automatic thermal desorption unit (TDU) maintained at 30 °C (0.4 min) and then increased to 300 °C at 120 °C/min. The desorbed compounds were transferred to a CIS4 cooled injection system, in which compounds were cryofocused. The CIS4 temperature was raised from −10 °C to 300 °C at 12 °C/s and held for 5 min. Desorption and analysis were carried out on a GC–MS using a tandem gas chromatograph 7890B/MSD 5977 system (Agilent Technologies, Santa Clara, USA). Volatiles were analyzed on a DB-Wax column (60 m × 250 μm × 0.25 μm). Mass spectra were recorded in EI + mode at 70 eV within a range of 40 to 350 Da with a solvent delay time of 2 min and a scan speed of 4.52/s. The analyzer and source temperatures were 150 °C and 250 °C, respectively. Mass spectrometry data were analyzed using MassHunter software version B.08.00. Volatile compounds were identified by comparing their GC–MS/MS mass spectra to the NIST 08 library (Wiley, New Jersey, USA) using a match factor to assess similarity (calculated by MassHunter software). A match factor > 700 was used to decide whether a given peak was identified with relatively high confidence. A reverse match (RMatch) factor was also calculated, and it was required to be >800. RMatch is calculated by ignoring the peaks in the unknown's spectrum that are not in the library's known reference spectrum. Linear retention indices were calculated on capillary columns coated with polar stationary phase (DB-Wax) and compared with those of authentic reference standards reported in databases and literature, run on polar stationary phase under similar conditions.

2.6. Data processing

To center the chromatographic peaks, the measurements were taken in continuous mode; therefore, this information was retrospectively based on the *m/z* of the external reference mass. This was done with the MassLynx V3.1 SCN 639 program (Waters Inc., Milford, Massachusetts, USA). To continue with the analysis, the program Progenesis QI (Nonlinear Dynamics, Newcastle, UK) was used. In this stage, mass detection, deconvolution and alignment among samples were performed. Once the list of ions was obtained, the UPLC-ESI/QToF-MS equipment drift was corrected using LOESS-fit signal correction (Thonusin et al., 2017) by adjusting the intensity of the samples, accounting for the drift in the variable from quality control (pools). Then, only ions with a coefficient of variation lower than 30% in the quality control samples were selected. Additionally, only ions that were found in at least 80% of individuals from either group were examined.

2.7. Biological association networks

Datasets containing metabolite identifiers and their corresponding values (fold change) from the metabolomics experiment were uploaded into Ingenuity Pathway Analysis software (Ingenuity® Systems, https://www.ingenuity.com) to identify potential interactions with other biological molecules, as described previously (Vaillant et al., 2021). The p values were calculated using Fisher's exact test to determine the probability that the association between the metabolites in the dataset and the top pathway was due to chance alone. Data sources from Ingenuity expert findings were used with the "Core Analysis" function to interpret the data from the perspective of biological processes, pathways, and networks. For canonical pathway analysis, disease, and function, a − log (p value) > 2 was taken as the threshold, a Z score > 2 was defined as the threshold of significant activation, and a Z score < −2 was defined as the threshold of significant inhibition. Differentially expressed metabolite identifiers were defined as value parameters for analysis, and the relationship between metabolite alterations and related changes in biofunctions in the subcategories of molecular and cellular functions, physiological system development and function, and disease and disorders was identified. Differentially expressed metabolites with $p < 0.05$ were overlaid onto global molecular networks developed from information contained in the IPA knowledge base. Networks were then algorithmically generated based on their connectivity.

2.8. Statistical analysis

The ion data base obtained by UPLC-ESI/QToF-MS analysis was used to construct an experimental space that was explored by principal component analysis (PCA) using the SIMCA 15.0.2 program (Sartorius Stedim Data Analytics AB, Sweden). The space was then subdivided into groups of samples corresponding to baseline, acute consumption and short-term consumption of golden berries. These groups were subsequently subjected to a discriminant analysis of partial least squares (PLS-DA) after orthogonal signal correction (OSC). The acceptability of the model was validated by means of different proprietary parameters and ad hoc tests such as CV-ANOVA (Eriksson et al., 2008) and the test of permutations. This last test compares the goodness of fit (R^2Y and Q^2Y) of the original model with other models constructed by random permutations (n = 100) of the Y observations, and it is represented by a permutation plot. Variable importance of the projection (VIP) was calculated for each variable (ion) by summing the squares of the PLS loading weights, weighted by the amount of the sum of squares explained by each model component (Farrés et al., 2015). Variables (ions) were ordered according to the highest VIP among variables between baseline and acute and between baseline and short term with a p value <0.05 and with a p value inferior to their Benjamini-Hochberg critical p value (Glueck et al., 2008) calculated with a false discovery rate of 5%. For biological association networks, canonical pathway (CP) analysis was used to identify function-specific metabolites significantly present within the networks.

3. Results and discussion

All urine datasets obtained by UPLC-ESI/QToF-MS were analyzed using orthogonal signal correction-partial least square-discriminant analysis (OSC-PLS-DA). The model generated by comparing 3 groups, baseline (Day 0) after the washout period, acute consumption on Day 1,

and Day 19 after 18 days of constant consumption of golden berries, was deemed highly reliable by the usual tests (CV-ANOVA = 3.7e-35; R^2Y = 0.86, Q^2Y = 0.62, permutation test plot (n = 100) does not show overfitting (Figure S1)). Then, the OSC-PLS-DA model allowed us to establish a list of the top 50 discriminant ions among the three groups, which are presented in Table 1 and ordered by VIP scores. P values comparing baseline (B) with acute consumption (A) and baseline (B) with short-term consumption (S) are also presented as well as the average intensity in each group.

As shown in Table 1, the main discriminant ion in the PLS model after Physalis peruviana intake is 203.18 [M + H], which appears in Table 1 at 8 different retention times (rank 1, 3, 5, 6, 19, 23, 35, and 42). The assigned formula C15H22 corresponds to sesquiterpenoid isomers with a double bond. To determine the identities among various possible C15H22 sesquiterpene isomers with a higher level of confidence, a DHS/GC–MS2 analysis of a urine sample collected 6 h after Physalis peruviana ingestion was performed. This analysis confirmed the presence of eight C15H22 isomers, and among them, five were identified with high confidence (match value > 700) by comparison of their GC-MS2 spectra with authentic standards reported in the NIST database (similarity factor represented by polar linear retention indices (Table 2)): α-curcumene; the 3 stereoisomers β-vatirenene, β-vetivenene, and β-vetispirene; and eremophila-1(10),8,11-triene (Fig. 1).

All of these isomers, except *m/z* 203.1807 at RT 10.53, exhibit the same LC-MS2 fragmentation pattern (Table 1), corresponding to the bicyclic sesquiterpenoid structure with an eremophilane backbone (Fig. 1). Alpha-curcumene, detected in urine by DHS/CG-MS, was tentatively attributed to ion *m/z* 203.1807 at RT 10.53 because of the absence of the *m/z* 175.15 and 161.13 fragments typical of the bicyclic structure of the eremophilane backbone (Fig. 2).

All C15H22 sesquiterpenoids present a very similar pattern of excretion (Fig. 3), and they appeared in all individuals in urine collected between 0 and 12 h after fruit intake. This means that this compound is readily bioavailable with probable absorption in the upper GI tract, and it is cleared quite quickly almost 12 h after ingestion, even after almost 3 weeks of daily consumption.

Other ions among the top discriminant ions correspond to phase I and II reactions that occur in vivo to increase the polarity and the excretion of C15H22 sesquiterpenes. This includes ion *m/z* 413.21 [M + H] (rank 4) that shows a main fragment at *m/z* 219.17 consistent with glucuronide loss (-194.04 amu) and corresponds to the formula C15H22O, which can be attributed to a hydroxy or epoxy form of C15H22 sesquiterpenoids after hydroxylation or epoxidation of the alkene by human cytochrome P450. Experimental MS2 spectra with main fragments at *m/z* 201.16 and 135.11 confirm this assignment when compared to MS2 spectra generated in silico (Wang et al., 2021; Figure S2). Consequently, *m/z* 413.21 [M + H] can be tentatively attributed to the glucuronide form of the hydroxy or epoxy-β-vatirenene stereoisomers. Another ion corresponding to two phase I reactions is *m/z* 237.18 [M + H] (rank 9; C15H24O2), which has an MS2 spectrum similar to those of the preceding compound and shows a fragment at *m/z* 219.17 following the loss of a water molecule. This ion can be attributed to dihydroxy or hydroxy-epoxy-sesquiterpene.

Therefore, C15H22 sesquiterpenoid isomers and derivatives are among the most important biomarkers of golden berry intake, and their concentration peaks in urine soon after fruit ingestion. Sesquiterpenes are secondary metabolites of plants that, in addition to giving the fruit its specific aroma and slightly bitter taste, also have interesting functions for health, including anti-inflammatory, cardiovascular, gastroprotective and anticancer properties (Durán et al., 2021). Among the C15H22 sesquiterpenes, curcumene has been the most studied compound for its health effect, and it was found that curcumene has the potential ability to modulate cancer, neurodegeneration, diabetes, and inflammation (Obrenovich et al., 2010). Some authors have shown that curcumene (a derivative of curcumin) inhibits the activation of NF-kB and downstream signaling, reducing inflammatory stress and concomitant cytokine expression from adipose tissue, such as visceral fat (Gonzales and Orlando, 2008). Additionally, some data suggest that curcumene may improve cardiovascular function and insulin sensitivity (Obrenovich et al., 2010), which could also provide some clues to confirm the impact of golden berry consumption on insulin-associated signaling pathways observed previously in this same study (Vaillant et al., 2021).

The next discriminating ion is *m/z* 260.18 [M + H] (rank 7), which was assigned to L-hexanoyl carnitine with the formula C13H25NO4 according to the fragmentation pattern, which is in agreement with MS2 spectra published for the authentic compound by Mass bank (MoNA). L-hexanoyl-carnitine belongs to the acylcarnitine group, with the prominent fragment ion at *m/z* 85.03 reported in common for all acylcarnitines (Giesbertz et al., 2015). In fact, other acylcarnitines, such as undecanoyl-carnitine (*m/z* 330.26 [M + H], rank 14) and 3-hydroxynonanoyl carnitine (*m/z* 318.22 [M + H], rank 47), are among the top 50 most discriminant ions, and they were tentatively identified against the MS2 spectra of authentic compounds published by the Mass Bank (MoNA; Table 1). For all these acylcarnitines, urinary excretion appeared to be impaired immediately after fruit consumption, with an increase of>10-fold, in the case of L-hexanoyl-carnitine, in urine fractions collected up to 12 h. After 12 and 24 h, the excretion of acylcarnitines decreased and returned to normal in the urine collected 24 h after short-term consumption of fruit (Fig. 4).

Notably, healthy volunteers who participated in the study still consumed their normal diet with an estimated daily intake above 100 g of animal protein. The study was supervised by a nutritionist who assessed this quantity by reviewing food records and administering a questionnaire to volunteers. The concomitant consumption of golden berries appeared to increase the excretion of acylcarnitines, which is generally associated with the use of fat over carbohydrates to produce body energy (Stephens and Galloway, 2014). Increased urinary excretion of acylcarnitines is related to the enhancement of fatty acid oxidation and CoA recycling by removing short-chain acyl groups that accumulate in mitochondria. This increases free CoA levels, which are then available to continue lipid β-oxidation and the Krebs cycle, thereby releasing more ATP (Tonazzi et al., 2021). The esterification of L-carnitine to form acylcarnitine derivatives is part of the metabolism of fatty acids and the synthesis of urea under negative energy balance. Interestingly, the increased urinary excretion of acylcarnitine metabolites suggests a possible detoxifying effect in the elimination of ammonia, hypoxanthine and uric acid; therefore, it plays an important role in the catabolism of purines, avoiding the toxic effect of nitrogenous products (Tonazzi et al., 2021). The presence of purine bases, such as hypoxanthine, among the top 50 biomarkers after golden berry intake tends to confirm this statement. Hypoxanthine sulfate (C5H4N4O + SO3) was identified in negative mode (ion *m/z* 214.99 [M−H + SO3] (rank 38)) by comparison of experimental MS2 spectra with MS2 spectra of the authentic compound (MoNA). When biomarkers of golden berry intake and their fold change were uploaded to IPA software for bioinformatic computation analysis, a significant connection ($p < 0.05$) and effects on the life cycle related to canonical pathways, such as the mitochondrial L-carnitine pathway (Fig. S3A), L-carnitine biosynthesis adenine and adenosine salvage III, and adenosine nucleotide degradation II (Fig. S3B), were predicted.

The next most discriminant ion corresponded to 606.27 *m/z* [M + H] (rank 10) at RT 11.4 min, but it also had another discriminant isomer at RT 12.08 min (rank 17). Additionally, two isomers of ion *m/z* 624.28 [M + H + H2O] (rank 16 and 18) were observed. The MS2 fragmentation patterns of all these ions were similar, and they exhibited a main fragment at *m/z* 169.08 [M +] corresponding to γ-lactone ring loss followed by fragments typical of the fragmentation patterns of withanolides (Musharraf et al., 2011). These fragments were *m/z* 449.24 [M + H-3H2O] and 431.23 [M + H-4H2O] due to loss of water molecules, *m/z* 299.16 [M + H-Lact] due to the loss of lactone moieties and *m/z* 281.15 [M + H-Lac-H2O] and *m/z* 263.15 [M + H-Lac-2H2O] due to the loss of

Table 1
List of the most discriminant Ion (VIP > 2) in human urine considering a OSC-PLS model considering base-line, acute and short term consumption of golden berries.

Rank	Obs m/z[1] [M + H]/ [M−H]	RT (min)	Formula	VIP score	Tentative identification	M	Error (ppm)	Main MSMS fragments (% mas peak)	P-Value		Average relative intensity		
									B/A	B/C	B	A	S
1	203,1807	11,54	[C15H22 + H]	4,00	Sesquiterpene isomer	202.1730	4.5	203.18 (20); 161.13(30) 147.11(100); 119.10(60); 105.07(60);133.107(40);91.05 (20)	2,4E-07	4,0E-04	<50	12.490	490
2	552,3488	13,42		3,90	**Unknown**			130.05(100); 495.27(60);439.28 (60);375.17(60);331.18(90)	1,6E-09	5,0E-05	<50	535	165
3	203,1807	10,53	[C15H22 + H]	3,80	α-curcumene	202.1730	4.5	146.11(100);119.10(60);105.07 (60);133.107(40);91.05(20)	1,2E-07	1,6E-02	<50	327	<50
4	413,2168	11,88	[Glu-C15H22 + H + H2O]	3,80	hydroxy or epoxy-sesquiterpene glucuronide	412.2096	1.4	219.17(20);201.16(20);135.11 (100);107.08(90);93.07(90)	2,9E-10	3,2E-03	<50	5 257	1 665
5	203,1807	9,62	[C15H22 + H]	3,80	Sesquiterpene isomer	221.19	4	203.01808(30);147.11(100); 119.10 (90);105.07(80);133.107(50);91.05(20)	1,2E-06	4,9E-03	<50	488	<50
6	203,1807	11.12	[C15H22 + H]	3,70	Sesquiterpene isomer	202.1730	4,5	147.11(100);119.10(90);105.07 (80);133.107(50);91.05(20)	2,5E-06	8,0E-05	<50	417	<50
7	260,1872	7,51	[C13H26NO4 + H]	3,70	L-hexanoyl carnitine	259.1784	6	85.03(100);201.11 (20);144.10(10)	1,1E-10	1,8E-03	9 979	74 579	28 925
8	552,3459	12,8		3,60	**Unknown**			130.05(100); 495.27(60);439.28 (60);375.17(60);331.18(90)	3,9E-06	3,1E-01	<50	168	<50
9	237,1848	13,02	[C15H2402 + H]	3,40	Dihydroxysesquiterpne or hydroxy-epoxy-sesquiterpene	237.1849	0	219.1767 (30); 191.1150(30); 147.1158 (100);95.1027 (50)	5,1E-06	3,1E-01	207	825	259
10	606,2719	11,41	[C28H38O8-Cystein + H]	3,40	4β-hydroxywithanolide E- cystein	605.2657	1.3	30 eV:169.08(100);281.15(80); 299,16 (60); 449,23 (10); 123,08(50); 125,1 (30)	3,9E-06	5,5E-04	<50	502	65
11	276,0823	10,44		3,40	**Unknown**			201.0469 (100);183.0359(50)	4,9E-01	3,4E-05	885	1009	2220
12	778,4536	12,84		3,30	**Unknown**				5,0E-08	4,1E-02	<50	388	<50
13	163,0398	10,44	[C9H6O3 + H]	3,30	Hydroxycoumarine	162.0317	1.8	77.046 (100); 107.04 (40);91.05 (30);119.05(10)	>0.05	4,3E-06	<50	15	74
14	330,2635	10,2	[C18H35NO4 + H]	3,20	Undecanoyl carnitine	329,2566	−2,7	130.06(100);85.03(20)	>0.05	3,7E-04	374	550	1 157
15	568,3397	12.43		3,10	**Unknown**				4,9E-07	1,5E-02	<50	105	<50
16	624.2806	11,41	[C28H38O8 + Cystein + H + H20]	3,09	4β-dihydroxywithanolide E cystein	623.2762	5.4	606,27 (50); 169.08(100); 281.15(80); 299,16(50)	2,0E-04	3,7E-01	<50	1565	319
17	606,2732	12.09	[C28H38O8 + Cystein + H]	3,08	4β-hydroxywithanolide E cystein	605.2657	1.3	30 eV:169.08(100);281.15(80); 299,16 (60); 449,23 (10); 123,08(50); 125,1 (30)	5,8E-06	8,9E-02	<50	664	185
18	624.2806	11.64	[C28H38O8 + Cystein + H + H20]	3,07	4β-dihydroxywithanolide E cystein	623.2762	5.4	606,27 (50); 169.08(100); 281.15 (80);299,16(50)	8,1E-04	8,7E-03	<50	1 393	180
19	203.1809	13,3	[C15H22 + H]	3,07	Sesquiterpene isomer	202.1730	4.5	147.11(100); 105.07(60); 119.10(50); 133.107(40); 161.13 (30)	8,5E-07	1,9E-01	<50	723	<50
20	320.0198	3.85	[C10H9NO6 + SO3 + H]	3,06	**Unknown**			240.0507(30); 212.05(100); 194.04 (70); 176.03(50), 166.05(70)	6,5E-06	>0.05	<50	324	<50
21	486,3047	11,14		3,05	**Unknown**				2,4E-03	1,0E-02	<50	296	<50
22	519,1871	10,72		3,02	**Unknown**				1,3E-08	1,9E-02	348	2 133	542
23	543,257	12,82	[C28H38O8 + Na + H_2O]	3,01	4β-dihydroxywithanolide E	542,2491	0,2	503,26 (40); 449,23(20); 229,16(30); 281,15(20); 169,08(20)	2,4E-04	7,5E-04	<50	2321	1054
24	203,1809	12,76	[C15H22 + H]	3,00	Sesquiterpene isomer	202.1730	4.5	203.18 (40); 147.11(100); 119.10 (60);105.07(60);133.107(40); 161.13 (30), 186.05 (30)	1,1E-06	3,3E-02	<50	2 477	125

(*continued on next page*)

Table 1 (*continued*)

Rank	Obs m/z[1] [M + H]/ [M−H]	RT (min)	Formula	VIP score	Tentative identification	M	Error (ppm)	Main MSMS fragments (% mas peak)	P-Value B/A	P-Value B/C	Average relative intensity B	Average relative intensity A	Average relative intensity S
25	573,2831	13,42		3,00	**Unknown**				1,6E-05	1,0E-01	472	1069	608
26	325,1314	10,72		3,00	**Unknown**				8,0E-04	>0.05	3 094	5 780	3 411
27	**303.0120**	11.89	[C11H12O5 + SO3-H]	3,00	Sinapic acid sulphate	304,0252	17,7	223.05 (90); 208.03(100);164.03(50); 149.02(20; NOMA)	1,8E-07	4,1E-01	172 354	839 045	185 000
28	851,277	13,24		2,95	**Unknown**				3,9E-05	>0.05	<50	304	<50
29	566,1879	11,4		2,93	**Unknown**				8,9E-05	>0.05	597	3 156	655
30	525,2446	13,09	[C28H38O8 + Na]	2,93	4β-hydroxywithanolide E	524,2385	−3,2	503.2612(20); 299.16(50); 281.15 (20);169.08(100);125.09(10)	8,4E-04	2,5E-04	<50	14 304	4 003
31	559,2324	12,64	[C28H38O8 + K + H2O]	2,93	4β-dihydroxywithanolide E	558,223	2,8	299.16(50);281.15(20);169.08 (100);125.09(10)	3,7E-05	1,2E-05	<50	322	162
32	**219,1204**	7.20		2,93	**Unknown**				1,0E-12	2,4E-02	338	3467	608
33	**261.1594**	13.70		2,93	**Unknown**				9,0E-04	3,3E-07	<50	66	147
34	**411.1814**	12.17		2,88	**Unknown**				1,0E-13	3,5E-03	2533	8660	3402
35	**307.0023**	12.64		2,80	**Unknown**				9,6E-06	3,9E-07	64	370	745
36	203,1808	12,43	[C15H22 + H]	2,80	Sesquiterpene isomer	202.1730	0	147.11(100); 105.07(60); 119.10(50); 133.107(40); 161.13 (30)	1,9E-06	3,1E-01	<50	2091	176
37	**412.1709**	13.47		2,74	**Unknown**				6,81E-11	4,24E-01	6285	12,808	6360
38	**214.989**	12.26	[C5H4N4O + SO3-H]	2,63	Hypoxanthine sulphate	215.9953	7	92.01(100);65.02 (10)	3,01E-12	8,54E-03	1993	9602	2857
39	**412.1866**	11.95		2,63	**Unknown**				1,60E-09	3,03E-01	1388	3757	1282
40	**411.1793**	12.65		2,53	**Unknown**				5,00E-11	2,28E-01	13,178	32,195	14,579
41	**327.1010**	10.55		2,46	**Unknown**				2,87E-10	5,18E-05	2155	6525	4456
42	**535.1607**	10.44		2,44	**Unknown**				6,78E-11	6,06E-03	1668	9409	2619
43	203,1808	15,06	[C15H22 + H]	2,40	Sesquiterpene isomer	202.1730	0	147.11(100); 105.07(60); 119.10(50); 133.107(40); 161.13 (30)	2,00E-05	1,70E-04	<50	287	121
44	**519.1822**	13.02		2,35	**Unknown**				5,14E-07	8,39E-02	1196	4800	1367
45	**287.0889**	10.22		2,35	**Unknown**				7,18E-02	2,55E-04	6805	5389	14,655
46	**399.1340**	11.10		2,10	**Unknown**				5,62E-08	1,01E-02	3249	5679	3687
47	**135.0397**	12.26		2,10	**Unknown**				7,49E-10	5,50E-02	579	1365	733
48	318.2282	8.94	[C16H31NO5 + H]	2.04	3-hydroxynonanoyl carnitine	317,2202	0,6	176.07 (40); 130.0669(100); 85.02(60)	3,66E-04	8,83E-01	2 666	5 354	2 603
49	**461.2234**	14.63		2,03	**Unknown**				5,14E-02	6,04E-06	4207	3257	2170
50	**537.1756**	11.08		2,02	**Unknown**				1,48E-06	6,86E-02	1756	12,258	2160

Table 2
Sesquiterpenoids C15H22 identified by *DHS-SPME/GC-MS*[2] *analysis* in urine of volunteer 6 h after consumption of physalis peruviana.

#	Compound	RT	LRI [1]calc	LRI [2]teorico	Match	R-match	IUPAC name
1	Eremophila-1(10),8,11-triene	48,9	1833	1507	759	811	(1S,7S,8aR)-1,8a-Dimethyl-7-(prop-1-en-2-yl)-1,2,3,7,8,8a-hexahydronaphthalene
2	**Unknown**	49,2					
3	α-Curcumene	50,4	1862	1777	894	943	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-
4	β -Vetispirene	50,6	1866	1737	819	850	(5R,10R)-10-Methyl-6-methylene-2-(propan-2-ylidene)spiro[4.5]dec-7-ene
5	β-Vatirenene	53,7	1927	1852	711	814	8-Isopropyl-2,5-dimethyl-1,2,3,4-tetrahydronaphthalene
6	**Unknown**	56,1					
7	**Unknown**	56,8					
8	β-Vetivenene	59.5	2042	1868	819	850	(8R,8aS)-8,8a-Dimethyl-2-(propan-2-ylidene)-1,2,3,7,8,8a-hexahydronaphthalene

[1] Calculated for a DB-Wax column.
[2] Retention indices reported in literature for polar column.

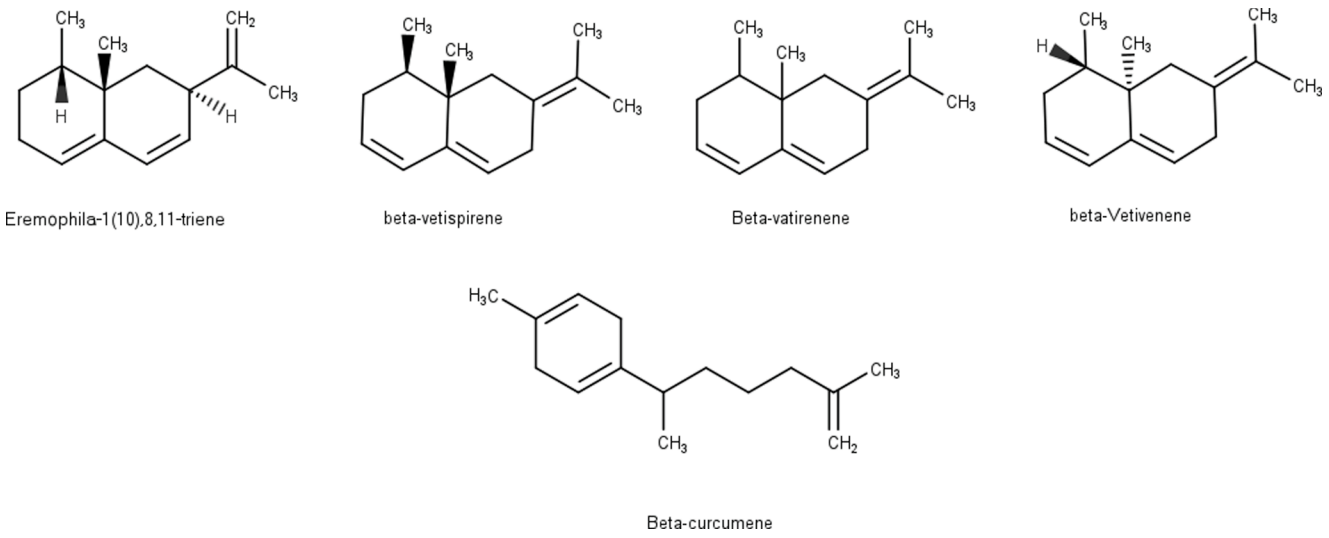

Fig. 1. $C_{15}H_{22}$ sesquiterpenoids isomers identified in urine spots collected 6 h after ingestion of goldenberries by DHS /CG-MS.

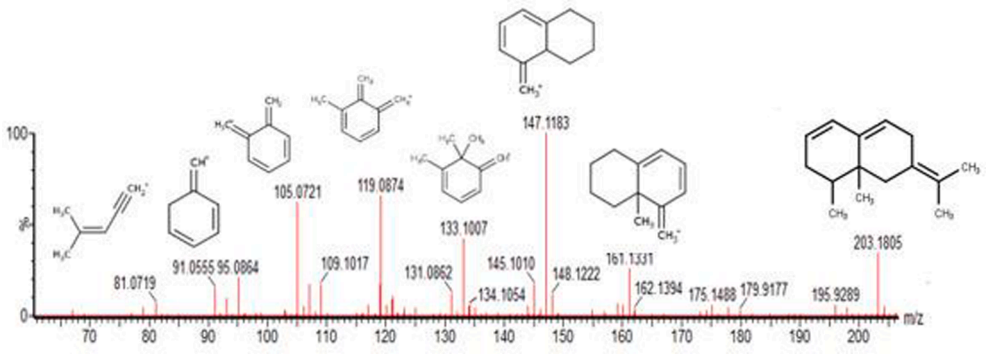

Fig. 2. MS^2 fragmentation pattern of bicyclic sesquiterpenoids (C15H22) based on eremophilane structure (CFM-ID) and possible structure of fragments.

both the lactone moiety and water. This fragmentation pattern was specific to 4β-hydroxywithanolide E (4βHWE; C28H38O8 *m/z* 503.26 [M + H]), as published previously (Ballesteros-Vivas et al., 2019; Musharraf et al., 2011).

Therefore, ions *m/z* 606.27 [M + H] and *m/z* 624.28 [M + H + H2O] were assigned with relatively high confidence to 4β-hydroxywithanolide E (4βHWE) and 4β-dihydroxywithanolide E conjugated to a cysteine residue (loss of 103.00 amu), as this conjugation probably occurred during the phase II reaction. This identity of 4βHWE was also confirmed by the presence of unconjugated ions among the top VIPs in urine with the same typical fragmentation pattern, such as ions 543.25 [M + Na + H2O] (rank 23), *m/z* 525.24 [M + Na] (rank 30), and *m/z* 559.24 [M + K + H2O] (rank 31). In the fruit extract, ions at *m/z* 503.26 [M + H] also appeared with a similar fragmentation pattern at a similar retention time of 12.7 min, confirming the presence of 4βHWE in the edible fruit (Figure S3). 4βHWE is reported as the main withanolide in the fruit of Physalis peruviana (Yang et al., 2020; Navarro-Hoyos et al., 2022). In addition, the ion *m/z* 606.27 [M + H] was also observed in the fruit at only one retention time (11.4 min), showing that cysteine conjugation may also occur in plants, as the amino acid composition of golden berry fruits is known for its high level of sulfur amino acids (cystine/methionine; Mokhtar et al., 2018). According to various authors (White et al.,

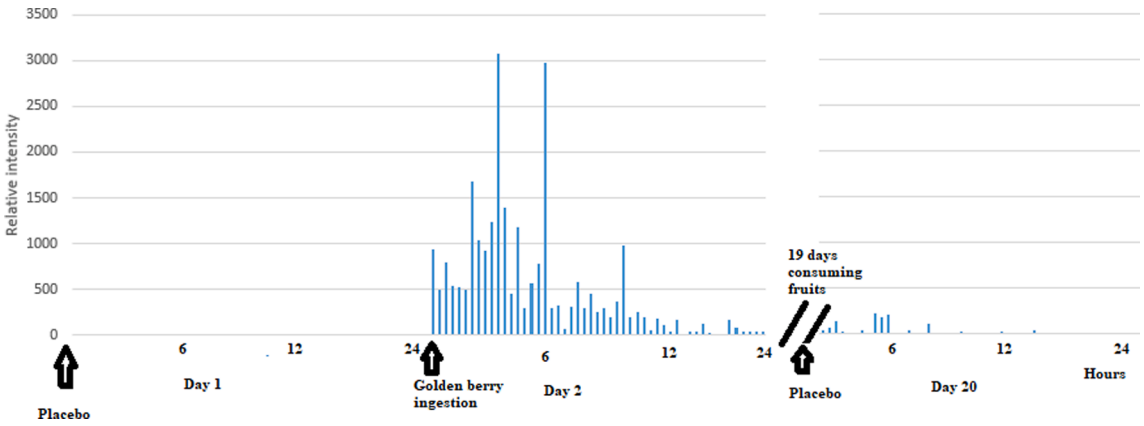

Fig. 3. Urinary excretion of the main isomer of sesquiterpenoid C15H22 (Tr = 11.54 min) by the 18 individuals after consuming golden berry fruit.

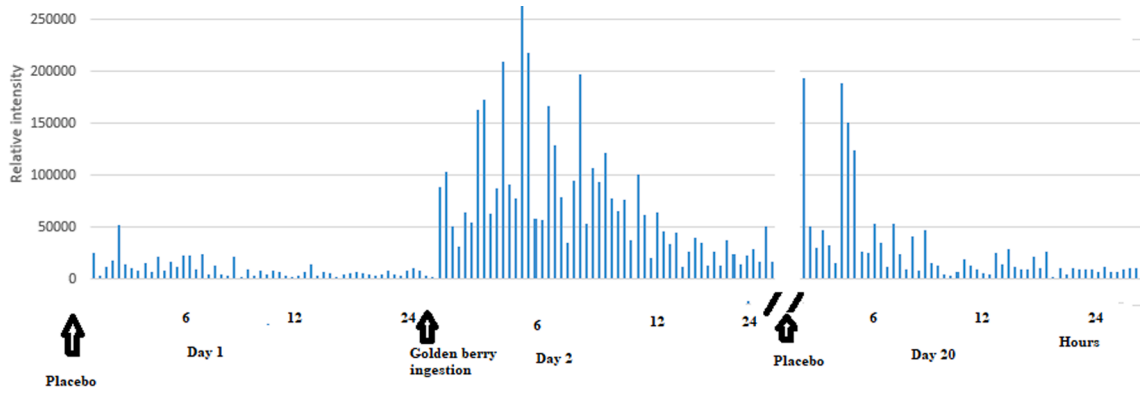

Fig. 4. Urinary excretion of L-hexanoyl carnitine by the 18 individuals after consuming golden berry fruit.

2016; Yang et al., 2020), cysteine residues are most often implicated to react with key electrophilic sites on the withanolide molecule during phase II reactions but also in the plant. The reaction of cysteine residue with 4βHWE could probably also explain why cysteine, L-cysteine, cystine and L-cystine were significantly impaired after acute consumption of golden berries with a significant reduction in plasma concentration as observed in this same clinical study (Vaillant et al., 2021).

As shown in Fig. 5, the excretion of 4βHWE derivatives was highest in urine collected between 0 and 6 h after ingestion, meaning that this withanolide is readily bioavailable almost immediately after ingestion. Similar to sesquiterpenoids, withanolide is quickly removed from the

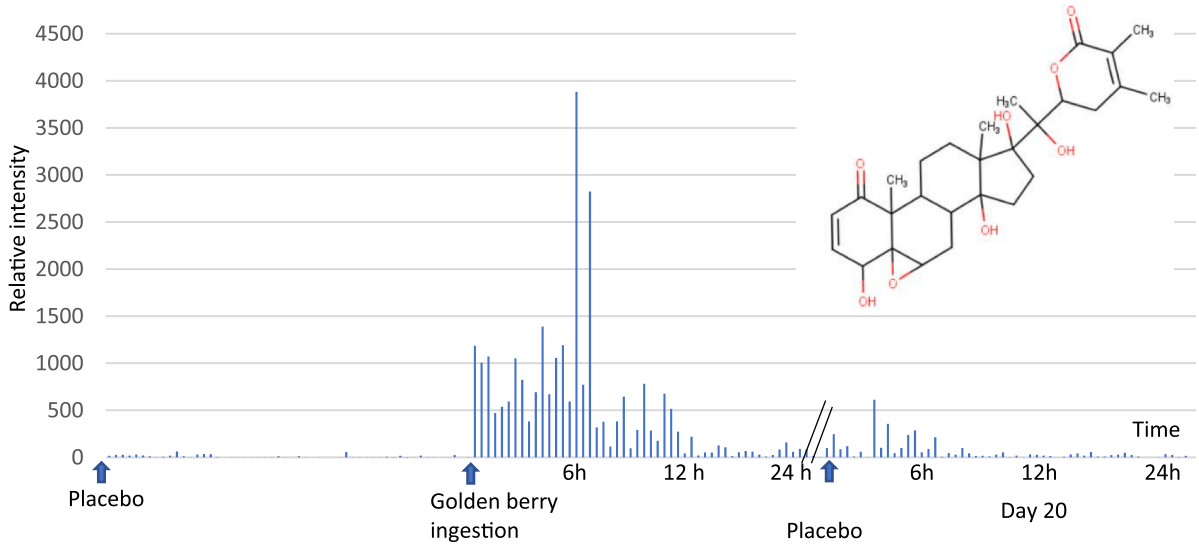

Fig. 5. Urinary excretion of 4β-hydroxywithanolide E (4βHWE; $C_{28}H_{38}O_8$) cysteine conjugated (Ion 606.27 *m/z* [M + H] at tr 11.4 min) in the 18 individuals after golden berry consumption.

body 12 h after acute consumption and 24 h after a period of constant consumption of the fruit for almost 3 weeks. Given its relatively high molecular weight, this compound is probably absorbed at the level of the upper GI tract.

This is the first time that the bioavailability of 4βHWE and its derivatives has been reported in humans, and this is a relevant result that could confirm the potential functional interest of golden berry fruit. The observation of urinary excretion of 4βHWE and its derivatives proves their systemic presence in the body with the capacity to potentially exert all the biological activity observed in vitro and in vivo. The withanolide 4βHWE has been linked repeatedly to a positive impact on chronic diseases, anti-inflammatory and anticancer activities in vitro and in cell and animal models (White et al., 2016; xxxx; Yen et al., 2010; Chiu et al., 2013; Wang et al., 2019; Park et al., 2016; Ye et al., 2019; Xu et al., 2021). This significant result could explain some of the results observed in different studies after golden berry consumption.

Next, tentatively identified ion *m/z* 163.039 [M + H] (rank 13) was discriminant only after short-term intervention. The exact mass and comparison of experimental MS2 published (Nguyen et al., 2020) was apparently consistent with assignation to hydroxycoumarin (C9H6O3), a phase I product of the metabolism of coumarin in the human body. Coumarin has recently been detected in considerable quantities in the calyxes of Physalis peruviana (Puente et al., 2019), but although some authors have repeatedly reported its presence in the fruits, it has not been possible to find an original article with the scientific basis for confirmation.

Finally, *m/z* 303.01 [M−H] observed in negative mode was tentatively assigned to sinapic acid sulfate [C11H12O + SO3] in accordance with MS2 spectra published by Mass Bank (NoMA). Sinapic acid is among the major biomarkers of hydroxycinnamate consumption. In golden berry fruits, p-coumaric acid and *trans*-ferulic acid have been detected (Vega-Gálvez et al., 2014), and both can eventually be transformed into sinapic acid after phase I hydroxylation.

Finally, it is worth discussing what was expected but was not observed in urine. Sucrose esters, which are known to be present in physalis fruits, did not generate any identifiable ions among all ions observed (significant and not significant in the PLS model). Sucrose esters are probably metabolized like sugars due to the nonspecific endogenous esterase present in the human body. These enzymes may cleave the link between short fatty acids and the sucrose moiety, liberating these compounds, which are subsequently metabolized. Therefore, the possible benefits of these compounds should be limited to the GI tract.

4. Conclusion

This research highlights the distinct nutritional value of golden berries, revealing that their consumption introduces uncommon compounds into the circulatory system. These compounds potentially account for the observed health benefits associated with their consumption. Importantly, the identification of key biomarkers for dietary intake opens a pathway to infer potential health impacts of a given food. This inference is supported by studies employing the same purified compounds in laboratory and animal settings, demonstrating discernible bioactivity. This analytical approach can be further strengthened by integrating endogenous biomarkers influenced by food consumption into established biological networks, elucidating plausible mechanisms of action. However, a primary limitation of this nutrimetabolomic methodology is the absence of established physiological concentrations for these biomarkers. Thus, subsequent investigations are essential to determine exposure doses for meaningful comparisons with studies involving pure compounds.

Finally, recognizing that biomarkers of dietary intake cannot be directly deduced from untargeted conventional food analyses is crucial. The case of golden berries exemplifies this: specific compounds, such as sesquiterpenes and withanolides, despite being present in low concentrations in the food matrix, emerge as the primary indicators of golden berry consumption. This underscores the critical need to enhance the recognition of biomarkers of dietary intake, enabling their incorporation in the assessment of food quality, impact of processing techniques, and even plant breeding considerations.

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CRediT authorship contribution statement

Fabrice Vaillant: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Visualization, Supervision, Project administration. **Sandra llano:** Investigation, Writing – review & editing. **Alberto Ángel Martín:** Formal analysis, Writing – review & editing. **Natalia Moreno-Castellanos:** Methodology, Validation, Formal analysis, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113443.

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