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# Metabolites and tissue distribution of resveratrol in the pig

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**List of Abbreviations:** **A**, Aorta tissue; **B**, Bile; **Br**, Brain; **CeC** and **CeT**, Cecum content and tissue, respectively; **Cr**, Cerebellum; **CSF**, Cerebrospinal fluid; **DC** and **DT**, Duodenum content and tissue; **DCC** and **DCT**, Distal colon content and tissue; **DH-RES**,  $\alpha,\beta$ -dihydroresveratrol (dihydroresveratrol); **H**, Heart; **IC** and **IT**, Ileum content and tissue; **J1C** and **J1T**, Jejunum-1 content and tissue; **J2C** and **J2T**, Jejunum-2 content and tissue; **J3C** and **J3T**, Jejunum-3 content and tissue; **JP**, Jugular vein plasma; **K**, Kidneys; **LgD**, *Longissimus dorsi* muscle; **Ln**, Lungs; **Lv**, Liver; **L**, Lymph; **LDL**, Low density lipoprotein; **LYN**, Superficial inguinal lymphatic node; **MF**, Mesenteric fat; **Ov**, Ovaries; **PCC** and **PCT**, Proximal colon content and tissue; **PF**, Perirenal fat; **Pn**, Pancreas; **PP**, Portal vein plasma; **QqQ**, UHPLC-triple quadrupole; **RBC**, Red blood cells; **RC** and **RT**, Rectum content and tissue; **RES**, *trans*-resveratrol (3,5,4'-trihydroxy-*trans*-stilbene; resveratrol); **SF**, Subcutaneous fat; **SM**, *Semimembranosus* muscle; **Sp**, Spleen; **StC** and **StT**, Stomach content and tissue; **U**, Urine; **UBL**, Urinary bladder tissue; **Ut**, Uterus; **WBC**, White Blood Cells.

**Keywords:** Bioavailability / disposition / metabolites / polyphenol / dihydroresveratrol

## **Abstract**

**Scope:** *trans*-Resveratrol (RES) and(or) its metabolites exert many *in vivo* effects. Our aim was to study the metabolism and tissue distribution of RES using the pig, a mammal physiologically close to humans.

**Methods and results:** Forty-seven tissues, organs and fluids were analyzed at 6 h after intragastric RES administration (5.9 mg/kg body weight) using HPLC-MS/MS. Twelve RES and seven dihydroresveratrol (DH-RES) metabolites were detected. DH-RES was the main metabolite in cecum, colon and rectum whereas RES-3-*O*-glucuronide was the most abundant one in fluids and organs. Approximately 74.5% of the total RES administered was recovered in the form of RES, DH-RES and derived metabolites (65.1% along the gastrointestinal tract, 7.7% in urine, 1.2% in bile and 0.5% in organs). We report here, for the first time, the occurrence of RES ribosyl-sulfate derivative, DH-RES diglucuronide, DH-RES sulfoglucuronide and DH-RES disulfate as well as the metabolic profile of RES and DH-RES in the aorta, lymph, lymph node, ovaries, uterus, cerebellum, pancreas, urinary bladder tissue, fat and muscle.

**Conclusion:** This study contributes to clarify the metabolism and tissue distribution of RES and could help to further understand the mechanisms underlying its effects.

## 1 Introduction

The natural occurring polyphenol resveratrol (3,5,4'-trihydroxy-*trans*-stilbene; RES) has been reported to exert many different health-promoting effects including antioxidant, anti-inflammatory, antitumor, anti-platelet aggregation, cardioprotective and aging-delay effects [1]. However, despite the reported beneficial effects, RES is poorly bioavailable as it is rapidly absorbed, metabolized and excreted in humans and different animal models [2-7]. Therefore, this apparent paradox (high activity but low bioavailability) involves that the real bioactive RES metabolites as well as the mechanisms underlying RES properties are not yet fully understood [1, 8].

Most studies regarding RES metabolism have focused on the analysis of RES and derived metabolites in plasma, urine or feces. However, there is a general lack of studies looking at the distribution of RES in organs, fluids or tissues upon oral or intravenous administration [9, 10]. RES tissue distribution has only been reported so far in rodents [11-16, amongst others] and one study in rabbits [17].

It is known that the pig gastrointestinal system and the associated gut microbiota are closer to the human digestive system than that of rodents [18, 19]. Despite research on pigs require large and rather expensive facilities, its use has been reported to be a valuable tool when investigating the metabolism of nutrients as well as phenolic compounds such as phenolic acids [20], flavan-3-ols [21], anthocyanins [22, 23], ellagitannins [24] and quercetin [25, 26]. Recently, we have reported the pharmacokinetics of RES in pigs [7]. In the pig, the metabolic profile of RES in plasma revealed the presence of RES diglucuronide, two isomers of RES sulfoglucuronide, two isomers of RES glucuronide, RES sulfate, and RES, being RES-3-*O*-glucuronide the most abundant plasma metabolite.

Taking into account the lack of studies regarding the tissue distribution of RES in big mammals, physiologically closer to humans than rodents, the aim of this study was to explore extensively the metabolism and tissue distribution of RES in the pig.

The results presented here could shed some light on the apparent 'RES paradox' (high activity but low bioavailability) since we show that RES and its derived metabolites are widely distributed in different pig tissues and biological fluids, many of them, not previously reported. In addition, the metabolic profile of RES in different organs, tissues and fluids, including some new RES and DH-RES metabolites, are reported here for the first time.

## 2 Materials and Methods

### 2.1 Chemicals

*trans*-Resveratrol (resveratrol, RES, 3,5,4'-trihydroxy-*trans*-stilbene, >99% purity) and the internal standard quercetin (>98%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Organic solvents such as methanol (MeOH), acetone, acetonitrile, etc. were obtained from Merck (Darmstadt, Germany). Milli-Q system (Millipore Corp., USA) ultra pure water was used throughout this experiment. Tiletamine-zolazepam (Zoletil 50) was purchased from Virbac España S.A. (Esplugues de Llobregat, Spain) and sodium pentobarbital (Dolethal) from Vétoquinol (Alcobendas, Madrid, Spain).

### 2.2 Synthesis of resveratrol metabolites

*trans*-Resveratrol-3-*O*- $\beta$ -D-glucuronide (RES glucuronide, >95% purity) was prepared from RES following a reported synthesis [27].  $\alpha,\beta$ -Dihydroresveratrol (DH-RES, >95% purity) was synthesized as described earlier by catalytic hydrogenation of RES [28]). Finally, the metabolite  $\alpha,\beta$ -dihydroresveratrol-3-*O*- $\beta$ -D-glucuronide (DH-RES glucuronide) was prepared by hydrogenation of the same protected resveratrol 3-*O*- $\beta$ -D-glucuronopyranosyl intermediate used in the preparation of resveratrol-3-*O*- $\beta$ -D-glucuronide (3, 4'-*O*-*tert*-butyl-5-*O*-(2,3,4-tri-*O*-acetyl- $\beta$ -D-glucuronopyranosyl) resveratrol). Final deprotection to obtain DH-RES glucuronide was carried out in two steps, silyl deprotection with tetrabutylammonium fluoride in tetrahydrofuran and acetyl cleavage with sodium carbonate in a methanol-water mixture. Purity was >95%.  $d_H$  (300 MHz, D<sub>2</sub>O) 6.96 (d, J = 8.0Hz, 2 H, Harom), 6.68 (d, J = 8.1 Hz, 2 H, Harom), 6.44, 6.40, 6.31 (3s, 3 H, Harom), 4.80 (d, J = 7.0 Hz, 1 H, H-1), 3.74 (m, 1 H, H-5), 3.52 (m, 3 H, H-3, H-4, H-2), 2.75 (s, 4 H, 2x CH<sub>2</sub>Ar);  $d_{13C}$  (75 MHz, D<sub>2</sub>O) 176.6 (C=O), 160.2, 159.2, 156.4, 145.5, 134.0, 130.5, 116.1, 110.9, 109.5, 102.9, 102.5 (C-1), 77.7, 76.5, 74.6, 73.5 (C-2, C-3, C-4, C-5), 39.5, 37.9 (CH<sub>2</sub>Ar). ESI-MS (ES<sup>-</sup>): Calcd. for C<sub>20</sub>H<sub>22</sub>O<sub>9</sub> (M-H) 405.1, found: 405.0.

All reactions were monitored by TLC on precoated Silica-Gel 60 plates F254 (Merck), and detected by heating with Mostain (500 mL of 10% H<sub>2</sub>SO<sub>4</sub>, 25g of (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>•4H<sub>2</sub>O, 1g Ce(SO<sub>4</sub>)<sub>2</sub>•4H<sub>2</sub>O). Products were purified by flash chromatography with Merck Silica gel 60 (200-400 mesh). High resolution FAB (+) mass spectral analyses was obtained on a Micromass AutoSpec-Q spectrometer

(Micromass, Manchester, UK). NMR spectra were recorded on either a Bruker Avance 300 or ARX 400 or Bruker Avance DRX 500 MHz [300 or 400 MHz ( $^1\text{H}$ ), 75 or 100 ( $^{13}\text{C}$ ) (Bruker Biospin GmbH, Rheinstetten, Germany), at room temperature for solutions in  $\text{CDCl}_3$ ,  $\text{D}_2\text{O}$  or  $\text{CD}_3\text{OD}$ . Chemical shifts are referred to the solvent signal. Further NMR experiments (COSY, TOCSY, ROESY, and HMQC) were done when necessary to assign the compound. Data were processed using manufacturer software, raw data were multiplied by shifted exponential window function prior to Fourier transform, and the baseline was corrected using polynomial fitting.

### **2.3 Animals and study design**

All experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 86/609/EC). Experimental design, included in the Spanish National Research Project BFU2007-60576, was approved by the Ethics Committee of the University of Murcia (Murcia, Spain) and by the Bioethics Committee-CSIC (Madrid, Spain). Five female pigs (cross-breed 25% Landrace x 25% Large White x 50% Duroc) were provided by the Veterinary Teaching Farm of the University of Murcia (sanitary registry number 302315). Housing and animal interventions were carried out in the same Teaching Farm. The pigs ( $80 \pm 8$  kg and 7 months old) were fasted overnight with free access to water. Animals were tranquilized with tiletamine-zolazepam (3.125 mg/kg body weight) to allow animal handling during intragastric RES administration. The dose of RES was prepared as previously described and adjusted to 6.25 mg/kg body weight (500 mg for an 80 kg animal). To determine the actual final dose, the amount of RES remaining in the probe after intragastric administration was evaluated [7]. One animal was used as control and received only water. All animal interventions were carried out at the same hour in the morning.

Six hours after RES administration, the pigs were sacrificed using an overdose of sodium pentobarbital and complete necropsies were carried out. All organs, the content of hollow organs as well as different body fluids were weighted and measured. The following samples were obtained: tissue and content from stomach, duodenum, jejunum (3 segments), ileum, cecum, proximal colon, distal colon and rectum, plasma from jugular and portal veins, urine, lymph, bile, cerebrospinal fluid, superficial inguinal lymphatic node, brain, cerebellum, heart, liver, kidney, lung, spleen, pancreas, uterus, urinary bladder,

ovary, three adipose (perirenal, mesenteric and subcutaneous) and two skeletal muscle tissue types (*Longissimus dorsi* and *Semimembranosus*), erythrocytes, white blood cells and serum LDL particles. The organs were thoroughly rinsed with PBS to avoid external blood contamination.

Blood samples were collected just before euthanasia from the jugular vein and immediately after the sacrifice from the portal vein in BD Vacutainer lithium heparin tubes (BD, Franklin Lakes, NJ). To separate the plasma, the collected blood samples were immediately centrifuged at 3000g for 10 min at 4 °C in a Sigma 1-13 microcentrifuge (Braun Biotech. Int., Melsungen, Germany). The plasma samples were immediately frozen at -80 °C for further analyses.

Erythrocytes were isolated from pig blood (4 mL) according to Nakamura et al. [29]. White blood cells (WBC) were isolated from pig heparinized blood (3 mL). Blood was diluted 1:1 with PBS and processed by density gradient centrifugation with Histopaque-1077 and Histopaque-1119 (Sigma-Aldrich, Madrid, Spain) according to the manufacturer instructions.

Urine, bile, lymph and cerebrospinal fluid were collected by puncture, with sterile syringes and needles, from the urinary bladder, gallbladder, the cisterna chyli and the subarachnoid space through the atlantooccipital articulation, respectively. After collection, all fluid samples were placed into sterile containers and frozen at -80 °C for further analyses.

LDL isolation from 4 mL of pig serum was carried out according to the protocol of Vieira et al. [30] but using 68,000 rpm for 90 min at 4 °C in a Beckman 70.1 Ti rotor.

## **2.4 Sample preparation**

Plasma (300 µL), lymph (150 µL), cerebrospinal fluid (200 µL) and bile (100 µL) samples were extracted using acetonitrile and processed according to Azorín-Ortuño et al. [7]. The supernatant was evaporated under vacuum in a SpeedVac Concentrator Savant SPD121P (Thermo Scientific, Alcobendas, Spain). The pellet was re-dissolved in 150 µL methanol. Methanolic samples were diluted 1:1 (v:v) with acidified (0.2% formic acid) ultrapure Milli-Q water (Millipore Corp., Bedford, MA), filtered through a 0.45 µm membrane filter Millex-HV13 (Millipore Corp.) and an aliquot was analyzed by HPLC.

Whole blood (300 µL) was processed according to Biasutto et al. [31].

Urine samples were diluted 5-fold with ultrapure water, filtered through the Millex filter and injected into the HPLC equipments.

The content (1 g) of the stomach, duodenum, jejunum (3 segments), ileum, cecum, proximal colon, distal colon and rectum were processed according to Espín et al. [24].

The skeletal muscle tissues (*Longissimus dorsi* and *Semimembranosus*), erythrocytes, WBC as well as the organs (tissues from the above parts of the gastrointestinal tract, superficial inguinal lymphatic node, brain, cerebellum, heart, liver, kidneys, lungs, spleen, pancreas, uterus, urinary bladder, aorta and ovaries) were processed (0.5 g) according to Azorín-Ortuño et al. [32].

Adipose (perirenal, mesenteric and subcutaneous) tissues (1 g) were processed following the protocol of González et al. [33].

LDL particles (700  $\mu$ L) with a mean protein content of 0.9 mg/mL protein were extracted according to de la Torre-Carbot et al. [34].

In all the cases, sample manipulation was performed avoiding the direct light exposure to prevent the possible photochemical isomerization of *trans*-resveratrol to the *cis*- form. The internal standard quercetin (20  $\mu$ M) was routinely used in all the samples.

## 2.5 LC-MS/MS analyses

The identification of RES and derived metabolites was achieved by HPLC-DAD-ESI-MS/MS, UHPLC-triple quadrupole (QqQ) MS detection and HPLC-Q-TOF.

The LC-DAD-ESI ion trap system was a 1100 series HPLC-DAD device (Agilent Technologies, Waldbronn, Germany) equipped with an ion-trap mass spectrometer (Agilent). The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. Mass scan (MS) and MS/MS daughter spectra were measured from  $m/z$  100 up to 800  $m/z$ . Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, and the collision energy was set at 50%. Mass spectrometry data were acquired in the negative ionization mode. Chromatographic separations were achieved on a 250 x 4 mm i.d., 5  $\mu$ m, C<sub>18</sub> Mediterranea Sea column (Teknokroma, Barcelona, Spain) using water:formic acid (99:1, v:v) (A) and acetonitrile (B) as the mobile phases at a flow rate of 1 mL/min. The gradient started with 5% B in A to reach 55% B at 30 min, 90% B at 31 min for 5 min and



returning to the initial conditions (5% B). For quantifying DH-RES and derived metabolites in UV, the conditions were the same but using a 250 x 4.6 mm i.d., 3  $\mu$ m, C<sub>18</sub> 100 Å Phenomenex column (Phenomenex<sup>®</sup>, Torrance, CA, USA). The samples were diluted in water (1:1) and different sample volumes (from 50 to 80  $\mu$ L, depending on the sample) were injected.

The UHPLC-MS triple quadrupole system (QqQ) consisted of a 1290 Infinity HPLC series (Agilent) equipped with a 6460 series triple quadrupole mass spectrometer (Agilent). Chromatographic separations were carried out at room temperature on a 100 x 3 mm i.d., 2.7  $\mu$ m, C<sub>18</sub> Poroshell 120 column (Agilent) using water:formic acid (99.9:0.1, v:v) (A) and acetonitrile:formic acid (99.9:0.1, v:v) (B) as the mobile phases at a flow rate of 0.6 mL/min. The gradient started with 15% B in A to reach 50% at 15 min, 90% at 18 min for 5 min and returning to the initial conditions (15% B). The injected sample volume was 10  $\mu$ L. The optimum mass spectrometer parameters for detection of the available metabolites (DH-RES, RES and their corresponding glucuronides) were optimized connecting directly the column inlet to the Jet Stream source. The source parameters were the following: capillary voltage -3500 V, charging potential -500 V, nebulizer pressure 40 (psi), auxiliary gas heated to 275 °C at a flow rate of 9000 cm<sup>3</sup>/min. MS data were collected in multiple reaction monitoring (MRM) mode by monitoring specific transitions of parent and product ions for each metabolite: RES triglucuronide 755/227, RES diglucuronide 579/227, RES glucuronide 403/227, RES sulfoglucuronide 483/227, RES trisulfate 467/227, RES disulfate 387/227, RES sulfate 307/227, RES 227/185, DH-RES triglucuronide 757/229, DH-RES diglucuronide 581/229, DH-RES glucuronide 405/229, DH-RES sulfoglucuronide 485/229, DH-RES trisulfate 469/229, DH-RES disulfate 389/229, DH-RES sulfate 309/229 and DH-RES 229/187.

The Q-TOF equipment consisted of a HPLC-DAD-MS system (1200 series, Agilent) equipped with a UHD Accurate-Mass Q-TOF (6540A series, Agilent). The column, flow rate, injected volume and gradient conditions were the same as those described above for the UHPLC-QqQ equipment. The optimum mass spectrometer parameters for detection of both RES and DHR-RES and their corresponding 3-*O*-glucuronides (at 80  $\mu$ M) were optimized, connecting directly the column inlet to the Jet Stream source. The source parameters were the following: capillary voltage -3500 V, charging potential -500 V, nebulizer pressure 40 (psi), auxiliary gas heated to 275 °C and a flow rate of 9000 cm<sup>3</sup>/min. The parameters of the mass spectrometer were similar to those optimized in the UHPLC-QqQ

equipment. The acquiring spectra were in the range from 100 to 800  $m/z$  in negative mode and spectra time in MS mode 0.5 s. The acquisition mode was in auto MS<sup>2</sup>, the MS<sup>1</sup> ranged from 100 to 900 Da and the MS<sup>2</sup> ranged from 70 to 700 Da while the scan rate was set at 0.3 s. Finally, the collision energy slope was 7V and the collision energy offset was 5V. The Mass Hunter tool (Agilent) was used for the calculation of elemental composition of compounds. This tool lists and rates possible molecular formulas consistent with the accurate mass measurement and the true isotopic pattern (TIP).

The different RES and DH-RES metabolites were identified by their UV spectra, molecular mass, daughter ions, fragmentation pattern and specific transitions in the HPLC-DAD-ESI-MS/MS, UHPLC-MS-QqQ and HPLC-Q-TOF equipments. The different organs and fluids from the control pig were spiked with the four available standards (RES, RES 3-*O*-glucuronide, DH-RES and DH-RES 3-*O*-glucuronide) at six different concentrations (from 0.05 to 10  $\mu\text{M}$ ). The curves were characterized by regression coefficients of  $R^2 = 0.999$  or above. The compounds RES and RES 3-*O*-glucuronide as well as DH-RES and DH-RES 3-*O*-glucuronide were quantified in the 1100 series equipment using UV detection at 320 and 276 nm, respectively, and using their corresponding available standards. The rest of RES-derived and DH-RES-derived metabolites were quantified using UV detection and the commercial RES and DH-RES as external standards, respectively. The mean recovery efficiency of the standards from the different samples was  $85 \pm 4\%$ , ranging from  $80 \pm 5\%$  (in the case of RES 3-*O*-glucuronide in colon content) to  $99 \pm 3\%$  (in the case of RES 3-*O*-glucuronide in plasma). The intra-day and inter-day precision (coefficient of variation; CV) was calculated by triplicate for each available standard and concentration assayed (1, 5 and 10  $\mu\text{M}$ ) in three representative samples (colon content, liver and plasma). The intra-day precision ranged from 0.35% (10  $\mu\text{M}$  RES 3-*O*-glucuronide) to 7.03% (10  $\mu\text{M}$  DH-RES 3-*O*-glucuronide) in colon content samples; from 0.51% (10  $\mu\text{M}$  RES) to 4.44% (10  $\mu\text{M}$  DH-RES 3-*O*-glucuronide) in plasma and finally, from 0.53% (10  $\mu\text{M}$  RES) to 4.39% (10  $\mu\text{M}$  RES 3-*O*-glucuronide) in liver samples. The inter-day (assessed in three different days) ranged from 0.14% (10  $\mu\text{M}$  RES) to 6.18% (10  $\mu\text{M}$  DH-RES 3-*O*-glucuronide) in colon content samples; from 0.92% (10  $\mu\text{M}$  RES) to 4.07% (10  $\mu\text{M}$  DH-RES) in plasma samples and finally, from 0.42% (10  $\mu\text{M}$  DH-RES) to 6.65% (10  $\mu\text{M}$  RES 3-*O*-glucuronide) in liver samples. The accuracy was measured as the mean percentage of error of the measured concentration to the theoretical concentration of each standard. The best accuracy was obtained

with the plasma extraction protocol (% bias) that ranged from -2.26% (10  $\mu$ M RES 3-*O*-glucuronide) to 2.35% (10  $\mu$ M DH-RES 3-*O*-glucuronide). In the case of both colon content and liver samples the accuracy was lower than in plasma, ranging from 18.77% (10  $\mu$ M RES 3-*O*-glucuronide) to 21.41% (10  $\mu$ M RES) in colon content samples and finally, from 12.87% (10  $\mu$ M DH-RES 3-*O*-glucuronide) to 14.97% (10  $\mu$ M RES 3-*O*-glucuronide) in liver samples. The limits of detection in UV ranged from 0.025  $\mu$ M (RES) to 1.5  $\mu$ M (DH-RES 3-*O*-glucuronide). The limits of quantification in UV ranged from 0.1  $\mu$ M (RES) to 3  $\mu$ M (DH-RES 3-*O*-glucuronide). The CV was always lower than 10%.

When the low amount of metabolites prevented UV quantification, the compounds RES, RES-3-*O*-glucuronide, DH-RES and DH-RES-3-*O*-glucuronide were quantified in the QqQ equipment using the corresponding available standards. The limits of detection in QqQ ( $\mu$ M) ranged from 0.025 (RES) to 0.05 (DH-RES). The limits of quantification in QqQ ( $\mu$ M) ranged from 0.05 (RES) to 0.25 (DH-RES). The coefficient of variation was always lower than 10%. In the case of other non-available standards, the relative abundance for these metabolites were scored in the QqQ system as high (+++); medium (++) and low (+). ‘Low values’ were those that permitted proper compound identification according to both ion fragmentation and transitions; ‘medium’ and ‘high values’ exceeded the ion intensity of ‘low values’ by around 10-fold and 100-fold (or higher), respectively. These values were used to assess the relative abundance of the same metabolite in different organs and fluids but not to compare abundance among different metabolites. For ion intensity comparison, the ionization of the mass spectrometers was daily checked using RES, DH-RES, RES-3-*O*-glucuronide and DH-RES-3-*O*-glucuronide standards.

### **3 Results**

#### **3.1 RES and DH-RES metabolites identification**

The metabolites were characterized by their UV spectra and MS analyses using ion trap, UHPLC-triple quadrupole (QqQ) and HPLC-Q-TOF. Nineteen metabolites were detected in different pig organs, tissues or fluids after intragastric RES administration (Table 1). The combination of different analytical approaches was important to support the identification of metabolites. For example, sulfoglucuronide and diglucuronide isomers that coeluted using the QqQ equipment (Table 1) were separated and

characterized using the 1100 Agilent ion-trap equipment (results not shown). In addition, the use of control samples (organs and fluids) from a pig without RES administration was crucial to discard confounding ions and specific transitions not related to RES-derived metabolites that were found in different samples analyzed from the control pig (results not shown).

The metabolites detected in this study were RES, *cis*-RES, nine RES-conjugates (glucuronide, sulfate and sulfoglucuronide derivatives), the microbiota-derived metabolite DH-RES and six DH-RES conjugates (also glucuronide, sulfate and sulfoglucuronide derivatives) (Table 1). One additional metabolite with RES UV-like spectrum was also detected. This metabolite, compound **9**, was tentatively identified as a RES ribosyl-sulfate derivative according to its ion mass (439  $m/z$ ) and daughter ions (359, 307, 227) (Table 1).

The compounds **10** (RES 3-*O*-glucuronide), **12** (DH-RES 3-*O*-glucuronide), **17** (RES) and **18** (DH-RES) were fully identified by direct comparison with the available standards. The rest of metabolites were tentatively identified according to their UV spectra, ion mass and daughter ions. The UV spectra was very useful to distinguish between RES-like metabolites (maximum at 305 and 320 nm) and DH-RES-like metabolites (bell-shaped spectrum with maximum at 276 nm). The compound **11**, another glucuronide conjugate, was indirectly identified as the other possible more common glucuronide isomer, RES 4'-*O*-glucuronide. In the case of the rest of metabolites, the tentative identification was assessed by a combination of analyses using ion trap and specific transitions in the QqQ. The lack of available standards for many metabolites as well as their small amount prevented their isolation and full characterization by other spectroscopic means. To the best of our knowledge, metabolites **3** (DH-RES diglucuronide), **4** (DH-RES sulfoglucuronide), **9** (RES ribosyl-sulfate) and **16** (DH-RES disulfate) are reported here for the first time (Table 1).

Table 1 is also useful to observe the relative distribution of each metabolite in all the pig organs, tissues and fluids analyzed. Some metabolites such as compounds **10** (RES 3-*O*-glucuronide), **13** (RES sulfate), **14** (DH-RES sulfate) and **17** (RES) were widely distributed in many different tissues and fluids. On the contrary, other metabolites such as compounds **15** (RES disulfate) and **16** (DH-RES disulfate) were detected only in urine (U) and bile (B) and metabolite **3** (DH-RES diglucuronide) was only detected in urine using QqQ (Table 1).

### 3.2 Distribution of RES-derived metabolites in the pig gastrointestinal tract

RES was intragastrically administered followed by various washes of the probe. This was important to ensure a maximum delivery of RES into the stomach due to its low solubility in water. Otherwise, upon oral administration, even if using hydroalcoholic or cyclodextrins solutions, a significant part of RES would remain in the esophagus (results not shown) and this would prevent correct quantification of the mass balance and calculation of RES recovery. The actual RES dose administered in the present study was calculated by subtracting the RES quantity that remained in the probe after intragastric administration from the initial amount prepared and delivered (500 mg for a 80 kg animal). The final total intragastric RES quantity administered was 472 mg RES (5.9 mg/kg body weight; equivalent to a similar human dose) [35].

Table 2 shows the different RES-derived metabolites detected in both the lumen and the gastrointestinal tissues of the pig gastrointestinal tract (GIT) from stomach to ileum, 6 h after of intragastric RES administration. An increasing amount of RES conjugates was found from stomach to ileum, with maximum levels of compounds detected in the ileum. The most abundant metabolite was the RES sulfate derivative (**13**), although other RES conjugates such as **1** (diglucuronide), **5** and **6** (sulfoglucuronides), and **10** and **11** (glucuronides) were also very abundant. Other minor RES conjugates such as the metabolites **7** and **9** were also detected using QqQ. It should be noted that approximately 42 mg RES (**17**) (8.9% of the initial RES amount administered) was still present in the pig GIT after 6 h of its intragastric administration (Table 2). RES was also found in the stomach (14.2 mg; 3% of the initial RES amount given) and decreased along the GIT until the ileum, where the amount rose again to 21 mg (4.25% of the initial RES amount given) (Table 2). A small amount of *cis*-RES (**19**) was also detected along the GIT. Although exposure to direct light was carefully avoided during the sample processing, a small isomerization from the *trans*- to the *cis*- form cannot be discarded. The microbiota-derived metabolite DH-RES and some DH-RES conjugates, compounds **4** (DH-RES sulfoglucuronide), **8** (DH-RES trisulfate), **12** (DH-RES 3-*O*-glucuronide), **14** (DH-RES sulfate) and **18** (DH-RES) were also detected at trace levels in the GIT from stomach to ileum (Table 2).

Overall, it is noticeable that a total amount of  $228.40 \pm 119.82$  mg (lumen content) and  $8.2 \pm 4.1$  mg (tissues) RES-derived metabolites (including DH-RES metabolites) were recovered from stomach to ileum. This meant that approximately 50% the initial RES amount administered was still present in the GIT, mainly in the ileum (33.7%), in the form of RES and derived metabolites (Tables 2 and Figure 1).

At this time point, 6 h after RES administration, the cecum (Ce) was also an important reservoir for RES and its metabolites ( $\sim 42$  mg) (Table 3). However, in this case, the most abundant compounds were the microbiota derived metabolite DH-RES (**18**) ( $27.04 \pm 11.87$  mg) and RES (**17**) ( $14.74 \pm 5.06$  mg) (Table 3). Other minor RES and DH-RES conjugates (**12**, **13** and **14**) as well as *cis*-RES (**19**) were also detected (Table 3). The amount of metabolites decreased in the proximal colon (PC) to  $\sim 22$  mg and was substantially lower in the distal colon (DC) and rectum (R) (Table 3). Therefore, approximately 15% of the initial RES administered was recovered in the pig large intestine, mostly in the cecum (Table 3, Figure 1). The transit time from stomach to ileum was quite similar in all the animals studied (results not shown), however, the transit time from cecum to rectum was very different for each pig, yielding a variable distribution of RES and DH-RES along the large intestine of the animals. This variability is illustrated in Figure 2. For example, in pig number 1 (P1 in Figure 2), RES and DH-RES were detected only in the cecum, whereas in pig number four (P4 in Figure 2) these metabolites were mainly detected in the distal colon (DC) and rectum (R) (Figure 2).

### 3.3 Distribution of RES-derived metabolites in pig fluids, LDL and blood cells

Urine (U) and bile (B) were the reservoirs in which the highest diversity of metabolites was found, with eighteen and fifteen different metabolites detected, respectively (Table 4). The volume of both urine and bile was determined and the total amount of metabolites was quantified, reaching a total amount of 36.3 mg in urine (7.7% of the initial RES amount administered) and 5.6 mg in bile (1.2% recovery) (Figure 1). The most abundant metabolite found in urine was compound **10** (RES 3-*O*-glucuronide) whereas, in bile, the most abundant one was compound **6** (RES sulfoglucuronide isomer-2) followed by compounds **10** and **13** (Table 4).

Regarding the presence of metabolites in plasma, the concentration was higher in the portal vein plasma (PP;  $3.8 \pm 0.92$   $\mu$ M total metabolites) than in jugular vein plasma (JP;  $1.19 \pm 0.36$   $\mu$ M). The most

abundant metabolites were **10**, **12** and **13** in both plasma from PP and JP. The compound **17** (RES) was also quantified in the case of PP (Table 4).

Regarding the rest of the analyzed samples, lymph (L;  $1.1 \pm 0.12$   $\mu$ M total metabolites) and cerebrospinal fluid (CSF) as well as LDL, red blood cells (RBC) and white blood cells (WBC), the concentration of metabolites detected at this time point (6 h) was low (Table 4). To the best of our knowledge, this is the first report on the metabolic profile of RES and its metabolites in lymph, CSF and PP.

### **3.4 Distribution of RES-derived metabolites in systemic pig organs and tissues**

Ten different RES-derived metabolites (including those derived from DH-RES) were detected in the eighteen organs and tissues analyzed (Table 5). Six hours after intragastric administration of RES, approximately 0.5% of the initial quantity was recovered in these reservoirs in the form of metabolites (Figure 1). For those organs and tissues in which we were able to quantify the total amount of the metabolites, the highest values were found in the kidneys and liver followed by the rest of organs and tissues in which much lower quantities were detected. Regarding those reservoirs with unknown total weight, such as lymph node (LYN), muscle and fat, the amount of metabolites detected was very low and expressed as  $\mu$ g/g (Table 5).

Cerebellum (Cr), liver (Lv), ovaries (Ov) and pancreas (Pn) were the organs in which a wider variety of metabolites were detected. It is also noteworthy the detection of six different metabolites in both perirenal fat (PF) and *Longissimus dorsi* muscle (LgD) (Table 5).

The most abundant metabolites in systemic pig organs and tissues were the compounds **10** (RES 3-*O*-glucuronide), **13** (RES sulfate) and **17** (RES). RES (**17**) and the compound **10** were the most abundant metabolites detected in pig brain and cerebellum, respectively (Table 5).

## **4 Discussion**

A number of studies dealing with the bioavailability and metabolism of the molecule *trans*-resveratrol (17, RES) in humans and in rodents suggest that RES has a low bioavailability as it is rapidly metabolized and excreted, mainly via urine [5, 9, 10, 36]. These studies report both inter- and intra-species differences together with a high variability in the metabolic profiles which may be caused by different variables such as gender, age, polymorphisms (phase I and II enzymes, transporters, etc.), different administration procedures, sample processing, analytical protocols, type of drugs and protocols used in anesthesia, etc as recently discussed [7]. The main RES-derived metabolites identified so far in humans and rodents are the sulfates, disulfates, trisulfates, glucuronides, diglucuronides and sulfoglucuronides derivatives as well as the microbiota-derived DH-RES and its corresponding sulfate and glucuronide conjugates [2, 6, 9, 10, 14, 16, 36-38]. The recovery of RES metabolites in previous studies, after oral RES administration, was very variable [36]. This depended on the dose and route of administration, the analytical approach, the time of analysis post-administration as well as the organs and fluids finally analyzed. The highest recoveries reported (higher than 95%) were attained by measuring total radioactivity in both urine and feces [10, 36].

Besides this amount of information, there is a lack of studies looking at RES and RES metabolites presence and specific distribution in most organs and tissues and thus the aim of the present study was to explore more in depth the metabolism of intragastrically administered RES in the pig. A complete study regarding the tissue distribution of RES metabolites, at different time-points, after RES administration is not common although achievable in rodents. Recently, a detailed study on the gastrointestinal or transit kinetics of RES in the GIT at different time-points after RES administration, has been reported in mice [39]. However, given the size and characteristics of the pig as an animal model and the high-cost facilities required for the experimentation with this animal, it is reasonable to choose only one post-ingestion time. Our approach included a systematic analysis at a single time-point post-ingestion using different equipments. We finally chose 6 h as post-ingestion time analysis to increase the chances of finding microbiota-derived metabolites (DH-RES), based on previous studies carried out in the pig with other polyphenols [20] as well as on the data obtained for RES in our previous mice study [39]. Under this approach, we focused on the identification of possible new *in vivo* reservoirs for RES or derived metabolites. This could cover, at least partially, the gap regarding the lack of studies of metabolism and



tissue distribution of RES in animals (such as the pig) physiologically much closer to humans than rodents. Bearing in mind all the above, we admit that this is a specific picture of RES and derived metabolites distribution in the pig 6 h after a single intragastric RES administration and therefore, the distribution as well as the type and amount of metabolites could differ at other time-points or upon chronic consumption.

Most of the metabolites identified in the present study are in agreement with those previously published in the literature. However, we have identified a number of new RES and DH-RES-derived metabolites, i.e. the metabolites **3** (DH-RES diglucuronide), **4** (DH-RES sulfoglucuronide), **9** (RES ribosyl-sulfate) and **16** (DH-RES disulfate) (Table 1). Regarding the compound **9**, the occurrence of ribosyl conjugates, as another mechanism of xenobiotic metabolism in mammals, has been reported in several occasions on N-containing xenobiotics, and the N-ribosyl derivatives were tentatively described. In some cases, combination of this ribosyl substitution with the addition of hydroxyl-sulphate has also been reported [40]. Our data suggest that this minor metabolite is a ribosyl derivative of RES by conjugation in one of the phenolic hydroxyls, and in addition, there is a sulphate conjugation on another phenolic hydroxyl. Therefore, this is the first time that pentosyl (most probably ribosyl) conjugates of phenolics, RES in this case, are reported in nature.

The RES-microbiota derived metabolite dihydroresveratrol (**18**, DH-RES) and its conjugates have been largely underestimated. In 2004, Walle et al. [4] already highlighted the possible importance of these microbial compounds in the RES metabolism. This underestimation has been due to the use of UV detection at 305 nm or 320 nm (maxima of absorbance for RES spectrum) whereas the maximum UV for DH-RES is 276 nm, a bell-shaped spectrum with very low absorption at either 305 or 320 nm [10, 41]. In addition, the molar absorptivity of DH-RES-like metabolites is significantly lower than that of RES-like metabolites which hampers their detection.

In our study, the levels of DH-RES and its conjugates in the pig GIT from stomach to ileum were very low although a number of DH-RES metabolites (**8**, **12**, **14**, **18**) were detected (Table 2). In contrast, RES metabolites were very abundant, specially in the ileum (Table 2). It is also noticeable the presence of a relatively high concentration of RES (**17**) in the stomach content 6 h after RES administration as well as the occurrence of *cis*-RES (**19**) and conjugates from both RES (**5**, **7**, **13**) and, to a lesser extent, from

DH-RES (**12**, **14**). The presence of RES conjugates in the stomach could be related to the previously reported ability of gastric cells to metabolize phenolics, including RES [42]. However, the presence of a minor amount of microbial DH-RES metabolites in the stomach content cannot be easily explained. A tentative explanation could be the presence of a small amount of microbial groups from stomach to ileum involved in the formation of DH-RES from RES. The abundance of RES conjugates in the pig ileum could be due to the biliar secretion of the enterohepatic circulation together with an active efflux of conjugates to the lumen content (Table 2) which has been reported in previous studies [4, 43]. However, the RES profile changed from the ileum (with abundant conjugates such as sulfoglucuronides, glucuronides and sulfates) to cecum (mainly aglycones and higher amount of DH-RES than RES) (Table 3, Figure 2). Although the distribution of the microbiota along the GIT involved in the conversion from RES (**17**) to DH-RES (**18**) is not known yet, the above changes could be explained by the significant increase in the cecum of the microbial groups involved in the production of DH-RES. Moreover, the lower presence of conjugates in the cecum and colon could be due to a decrease in the efflux of metabolites to the lumen caused by a lower amount of the RES transporters BCRP and MRP2, as previously reported in the rat [44]. The expression of these transporters has been reported to be maximal in the ileum [45]. Finally, another reason to explain the change in the metabolic profile from ileum to cecum could be the substantial increase in the glucuronidase and sulfatase activities associated to the large intestine microbiota [45]. The predominant presence of DH-RES in the pig large intestine is in agreement with Alfaras et al. [46] who identified DH-RES as the most abundant metabolite in the colon rat upon oral RES administration. This is specially relevant considering the colon cancer chemopreventive activities attributed to RES. Recently, the distribution of RES and some of its conjugates has been reported in colorectal tissue of patients that consumed eight daily doses of resveratrol [47]. Interestingly, RES and some conjugates (glucuronides, sulfates and sulfoglucuronide) were detected in that study. However, the possible occurrence of DH-RES and derived conjugates was not explored. Therefore, the presence of DH-RES and derived metabolites cannot be discarded in the colorectal tissue of the patients enrolled in the above study, which could add to the controverted issue of the identification of the actual molecules responsible for the biological activity attributed to RES.

In the present pig study, the occurrence of metabolites in the bloodstream was low at this post-ingestion time (Table 4), in accordance with previous pharmacokinetic studies in both humans [6] and pigs [7]. The portal vein mainly drains blood from the GIT to capillary beds in the liver. Accordingly, the amount of RES metabolites in plasma from the portal vein (PP) approximately doubled the amount of metabolites present in jugular vein plasma (JP). In addition, the relative contribution of RES (**17**) to the metabolic profile was also more relevant in the case of PP than in JP (Table 4).

A rich metabolic profile with high concentration of RES metabolites was found in the pig bile (B) which reinforced the active enterohepatic circulation of RES (Table 4). In contrast, the presence of DH-RES metabolites in bile was much lower (Table 4) which could be due either to a low enterohepatic circulation or to the post-ingestion time analysis (6 h), probably not enough for producing DH-RES metabolites. Both possibilities could explain the low amount of DH-RES metabolites detected in the content of the GIT from stomach to ileum after 6 h of intragastric RES administration (Table 2).

Interestingly, we also report here for the first time the metabolic profile of RES in the lymph which approximately matched that of plasma (Table 4). Although the lymphatic system plays an important role in the absorption of dietary lipophilic compounds, it is not usually investigated in metabolic studies with polyphenols, which many of them are amphiphilic molecules. Our results are in agreement with those reported by Terao and Murota [48] that detected quercetin metabolites mostly in the rat lymph after intragastric administration of quercetin. The authors also supported their results by the ability of the stomach to metabolize quercetin [42, 48], which was in accordance with our study with the presence of RES metabolites in the stomach. On the contrary, Chen et al. [49] mainly detected unmetabolized quercetin and rutin in the lymph upon intraduodenally administration of these flavonoids. The conclusions drawn by these authors have been previously questioned due to the methodological approach followed in that study [50].

As expected, the presence of RES and DH-RES metabolites in systemic organs and tissues was very small and amounted up to 0.5% of the initial dose of RES administered (Table 5; Figure 1). RES and derived metabolites were already extensively distributed in the pig body 6 h after RES administration with the highest amounts detected in the kidneys (K) and the liver (Lv). The main compound detected in pig brain was RES (**17**) in accordance to previous studies on rats [15]. This is of interest in relation to the

modulation of several blood flow variables in humans 45 min after RES (500 mg) intake [51] and indicates that RES itself could be the bioactive molecule in brain. Although the RES amount found in the pig brain was very low (0.18  $\mu\text{g/g}$ ; 22  $\mu\text{g}$  in the entire brain), the detection of higher amounts cannot be discarded at shorter post-ingestion times.

The occurrence of RES and derived metabolites (including DH-RES and its conjugates) in perirenal, mesenteric and subcutaneous fats (PF, MF and SF, respectively) as well as in skeletal muscle such as *Longissimus dorsi* (LgD) and *Semimembranosus* (SM) muscles (Table 5) is important due to the increasing evidence of the link between RES intake and fat mobilization [52] as well as the attenuation of sarcopenia [53].

The most abundant circulating RES metabolites have been reported to be RES 3-*O*-sulfate in humans [6] and RES 3-*O*-glucuronide in pigs [7]. This is noticeable taking into account the physiological similarities between both species. In the present study, RES 3-*O*-glucuronide (compound **10**), was the most abundant metabolite in plasma from jugular and portal veins (Table 4) which confirmed the previous report [7]. In addition, compound **10** was also the main metabolite in systemic organs and urine (Tables 4 and 5). In contrast, RES sulfate (compound **13**) was the most abundant metabolite in the lumen of the pig GIT, especially in the ileum, followed by RES sulfoglucuronide isomer-2 (compound **6**). A possible explanation could lie in the pig ATP-binding cassette transporters, involved in the disposal of resveratrol [54], which could be especially efficient in the efflux of sulfate conjugates from the enterocyte to the GIT lumen. This would decrease the absorption and systemic distribution of RES sulfates as well as to increase their accumulation in the pig intestine, in agreement with the results found in our study.

In summary, we report here a comprehensive study regarding the metabolism and tissue distribution of RES in the pig, an animal physiologically close to humans. This is the first report regarding the occurrence of RES and DH-RES metabolites in aorta tissue (A), urinary bladder tissue (UBI), cerebellum (Cr), ovaries (Ov), uterus (Ut), pancreas (Pn), fat (PF, MF and SF) and muscle (LgD and SM). In addition, new RES and DH-RES metabolites are also reported here for the first time. It should be noted that the specific RES and DH-RES metabolites distribution described here could be different at other post-ingestion time-points. For example, shorter post-ingestion time analyses such as 1 h (approximately

coincident with plasma  $T_{\max}$ ) could yield lower DH-RES metabolites (if any), but possibly a higher RES metabolites detection in systemic organs. The research for reaching this challenging goal is warranted.

The information included in this study may be very useful to move forward in the knowledge about *trans*-resveratrol bioactivity and beneficial health effects as well as in the understanding of the mechanisms underlying its properties.

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## **Conflict of interest statement**

All authors declare no conflict of interests.

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## Figures legend.

**Fig. 1.** Recovery of RES-derived metabolites in pig organs, fluids and tissues 6 h after intragastric RES administration.

**Fig. 2.** *trans*-Resveratrol (RES) and dihydroresveratrol (DH-RES) distribution in the large intestine of pig from cecum to rectum. This Figure complements Table 2 and illustrates the different transit time of metabolites along the large intestine 6 h after intragastric RES administration. Open bars (RES); cross-hatched bars (DH-RES). P1 to P4 designates different pigs (n=4).

**Table 1.** RES and DH-RES metabolites detected in the pig after intragastric RES administration.

Metabolite	#	Rt (min)	MS (M-H)	MS-MS	Occurrence*
RES diglucuronide-1	1	1.4	579	403, 227	DC, J1C, J2C, J3C, IC, U, B
RES diglucuronide-2	2	1.4	579	403, 227	U, B, JP
DH-RES diglucuronide	3	1.4	581	405, 229	U
DH-RES sulfoglucuronide	4	1.8	485	405, 309, 229	J3C, Lv, Pn, U, B
RES sulfoglucuronide-1	5	1.9	483	403, 307, 227	StC, DC, J1C, J2C, J3C, IC, J1T, J2T, J3T, IT, Cr, K, Lv, Ov, Pn, U, B, JP
RES sulfoglucuronide-2	6	1.9	483	403, 307, 227	DC, J1C, J1T, J2C, J2T, J3C, IC, DT, J3T, IT. Cr, K, Lv, Ov, Pn. U, B
RES trisulfate	7	2.3	467	307, 227	StC, DC, J1C, J2C, J3C, IC, StT, DT, J1T, J2T, J3T, IT. A, Cr, H, Lv, Ln, Ov, U, B, PP, JP
DH-RES trisulfate	8	2.4	469	309, 229	J3C, IC, IT, U, B
RES ribosyl sulfate	9	3.3	439	359, 307, 227	IC, U, B
RES 3- <i>O</i> -glucuronide	10	4.4	403	227	StC, DC, J1C, J2C, J3C, IC, StT, DT, J1T, J2T, J3T, IT, CeT, PCT, DCT, A, UBl, Br, Cr, H, K, Lv, Ln, Ov, Pn, Sp, Ut, LYN, PF, MF, SF, LgD, U, B, PP, JP, L, LDL
RES 4'- <i>O</i> -glucuronide	11	4.5	403	227	DC, J1C, J2C, J3C, IC
DH-RES 3- <i>O</i> -glucuronide	12	5.1	405	229	StC, DC, J1C, J2C, J3C, IC, StT, DT, J1T, J2T, J3T, IT, CeT, PCT, DCT. A, Cr, H, K, Lv, Ln, Ov, Sp, U, B, PP, JP, L, LYN, PF, MF, SF, LgD
RES sulfate	13	5.5	307	227	StC, DC, J1C, J2C, J3C, IC, CeC, PCC, DCC, RC, StT, DT, J1T, J2T, J3T, IT, CeT, PCT, DCT, RT, A, UBl, Br, Cr, H, K, Lv, Ln, Ov, Pn, Sp, Ut, LYN, PF, MF, SF, LgD, SM. U, B, PP, JP, L, CSF, RBC, WBC
DH-RES sulfate	14	5.6	309	229	StC, DC, J1C, J2C, J3C, IC, CeC, PCC, DCC, RC. StT, DT, J1T, J2T, J3T, IT, CeT, PCT, DCT, RT, A, UBl, Cr, H, K, Lv, Ln, Ov, Sp, LYN, PF, MF, LgD, SM, U, B, PP, JP, L, RBC
RES disulfate	15	6.5	387	307, 227	U, B
DH-RES disulfate	16	6.5	389	309, 229	U, B
RES	17	7.1	227	185	StC, DC, J1C, J2C, J3C, IC, CeC, PCC, DCC, RC, StT, DT, J1T, J2T, J3T, IT, CeT, PCT, DCT, RT, A, UBl, Br, Cr, H, K, Lv, Ln, Ov, Pn, Sp, Ut, LYN, PF, MF, SF, LgD, U, B, PP, JP, L, CSF, LDL, RBC, WBC
DH-RES	18	7.5	229	187	IC, CeC, PCC, DCC, RC, IT, CeT, PCT, DCT, RT, U
<i>cis</i> -RES	19	8.9	227	185	StC, DC, J1C, J2C, J3C, IC, CeC, PCC, DCC, RC, StT, DT, J1T, J2T, J3T, IT, CeT, PCT, DCT, Cr, Lv, PF, MF, LgD, U, JP, L, CSF, LDL, RBC, WBC

\*A, Aorta tissue; B, Bile; Br, Brain; CeC and CeT, Cecum content and tissue, respectively; Cr, Cerebellum; CSF, Cerebrospinal fluid; DC and DT, Duodenum content and Tissue; DCC and DCT, Distal colon content and tissue; H, Heart; IC and IT, Ileum content and tissue; J1C and J1T, Jejunum-1 content and tissue; J2C and J2T, Jejunum-2 content and tissue; J3C and J3T, Jejunum-3 content and tissue; JP, Jugular vein plasma; K, Kidneys; LgD, *Longissimus dorsi* muscle; Ln, Lungs; Lv, Liver; L, Lymph; LDL, Low density lipoprotein; LYN, Superficial inguinal lymphatic node; MF, Mesenteric fat; Ov, Ovaries; PCC and PCT, Proximal colon content and tissue; PF, Perirenal fat; Pn, Pancreas; PP, Portal vein plasma; RBC, Red blood cells; RC and RT, Rectum content and tissue; SF, Subcutaneous fat; SM, *Semimembranosus* muscle; Sp, Spleen; StC and StT, Stomach content and tissue; U, Urine; UBl, Urinary bladder tissue; Ut, Uterus; WBC, White blood cells. Rt are referred to the QqQ assays.

**Table 2.** Total amount (mg) of RES and derived metabolites in the gastrointestinal tract (GIT) from stomach to ileum 6 h after intragastric RES administration.

GIT part (g)	Lumen Content								Tissue					
	1	5	6	10	11	13	17	QqQ	5	6	10	13	17	QqQ
<b>Stomach (St)</b> WC: 193±38 WT: 440±40	ND	1.34±0.21	ND	2.43±0.77	ND	1.06±0.41	13.40±2.06	7 (++) 12 (+++) 14 (+) 19 (+++)	ND	ND	0.11±0.06	0.11±0.06	0.79±0.50	7 (+) 12 (0.005) <sup>a</sup> 14 (+) 19 (+)
<b>Duodenum (D)</b> WC: 61±19 WT: 255±95	0.07 <sup>a</sup>	0.22±0.14 <sup>b</sup>	0.88±0.69 <sup>b</sup>	0.51±0.22	0.79±0.74 <sup>b</sup>	2.95±2.73	2.41±1.36	7 (++) 12 (+) 14 (++) 19 (++)	ND	0.06±0.01 <sup>b</sup>	0.09±0.22	0.08±0.03	0.10±0.05	7 (++) 12 (++) 14 (+) 19 (+)
<b>Jejunum-1 (J1)</b> WC: 165±96 WT: 270±15	0.31±0.15	1.27±0.41	0.40±0.17	2.40±0.69	0.30±0.15 <sup>b</sup>	1.60±0.52	0.53±0.16	7 (++) 12 (0.024) <sup>a</sup> 14 (++) 19 (+)	ND	ND	0.04±0.03	0.09 <sup>a</sup>	0.08±0.05	5+6 (++) 7 (++) 12 (+) 14 (++) 19 (+)
<b>Jejunum-2 (J2)</b> WC: 118±23 WT: 263±16	0.49±0.27 <sup>b</sup>	1.51±0.95	0.24±0.04	2.19±0.79	0.47±0.26 <sup>b</sup>	1.98±1.08	0.55±0.14	7 (++) 12 (+) 14 (++) 19 (+)	ND	ND	0.06±0.03	0.12 <sup>a</sup>	0.03 <sup>a</sup>	5+6 (++) 7 (++) 12 (++) 14 (++) 19 (+)
<b>Jejunum-3 (J3)</b> WC: 116±25 WT: 281±20	1.76±1.27	6.40±4.56	3.28±1.94	10.90±5.92	1.52±1.11 <sup>b</sup>	7.67 ± 5.18	2.87±1.00	4 (+) 7 (+++) 8 (++) 12 (0.043) <sup>a</sup> 14 (+++) 19 (++)	0.18 <sup>a</sup>	0.08±0.01 <sup>c</sup>	0.19±0.06	0.45±0.30	0.21±0.14	7 (++) 12 (++) 14 (++) 19 (+)
<b>Ileum (I)</b> WC: 177±41 WT: 525±125	6.28±2.96 <sup>c</sup>	23.93±13.38 <sup>c</sup>	16.88±8.99 <sup>c</sup>	19.28±12.72	11.93±4.21 <sup>b</sup>	55.31±30.47	19.99±10.79	7 (+++) 8 (++) 9 (+) 12 (+++) 14 (+++) 18 (++) 19 (++)	0.47±0.38 <sup>b</sup>	0.55±0.23 <sup>b</sup>	0.67±0.26	2.59±1.41	1.04±0.42	7 (+++) 8 (+) 12 (+++) 14 (+++) 18 (++) 19 (++)
<b>Total metabolites (GIT content) = 228.4 ± 119.82</b>								<b>Total metabolites (GIT tissues) = 8.19 ± 4.08</b>						

Values (quantified using UV detection) are expressed as total mg (mean±SE) taking into account the total weight of the lumen content and of tissue from the different GIT parts; \*WC, weight content; WT, weight tissue; In QqQ, when not quantified, the relative abundance of each metabolite was scored as high (+++); medium (++) and low (+) (these values compare the abundance for the same metabolite in the different GIT parts but not among different metabolites); ND, not detected in UV. **1** RES diglucuronide-1; **2** RES diglucuronide-2; **4** DH-RES sulfoglucuronide; **5** RES sulfoglucuronide-1; **6** RES sulfoglucuronide-2; **7** RES trisulfate; **8** DH-RES trisulfate; **9** RES ribosyl sulfate; **10** RES 3-*O*-glucuronide; **11** RES 4'-*O*-glucuronide; **12** DH-RES glucuronide; **13** RES sulfate; **14** DH-RES sulfate; **17** RES; **18** DH-RES; **19** *cis*-RES; n = 4 except in <sup>a</sup>n = 1, <sup>b</sup>n = 2 and <sup>c</sup>n = 3.

**Table 3.** Total amount (mg) of RES and derived metabolites in the gastrointestinal tract (GIT) from cecum to rectum 6 h after intragastric RES administration.

GIT part (continued) (g)	Lumen Content			Tissue				
	17	18	QqQ	10	13	17	18	QqQ
<b>Cecum (Ce)</b> WC: 342.5±102.8 WT: 175±25	14.06±4.75	26.40±11.81	<b>13 (+)</b> <b>14 (++)</b> <b>19 (+)</b>	0.02±0.01	0.05±0.02	0.68±0.31	0.64±0.06	<b>12 (0.007)<sup>a</sup></b> <b>14 (+)</b> <b>19 (+)</b>
<b>Proximal colon (PC)</b> WC: 445±105 WT: 330±10	6.42±5.96 <sup>c</sup>	13.55±8.87 <sup>c</sup>	<b>13 (++)</b> <b>14 (++)</b> <b>19 (++)</b>	ND	0.063±0.008	0.54±0.27	1.52±0.34	<b>10 (0.02)<sup>a</sup></b> <b>12 (0.008)<sup>a</sup></b> <b>14 (++)</b> <b>19 (+)</b>
<b>Distal colon (DC)</b> WC: 165±65 WT: 310±90	0.79±0.02 <sup>b</sup>	1.92±0.97 <sup>b</sup>	<b>13 (+)</b> <b>14 (+)</b> <b>19 (+)</b>	ND	ND	0.06±0.01	1.12 <sup>a</sup>	<b>10 (0.015)<sup>a</sup></b> <b>12 (++)</b> <b>13 (++)</b> <b>14 (+)</b> <b>19 (+)</b>
<b>Rectum (R)</b> WC: 40±20 WT: 85±15	0.26 <sup>a</sup>	2.67 <sup>a</sup>	<b>13 (+)</b> <b>14 (+)</b> <b>19 (+)</b>	ND	ND	0.01±0.004	0.52 <sup>a</sup>	<b>13 (+)</b> <b>14 (+)</b>
<b>Total metabolites (lumen): 66.07±32.4</b>			<b>Total metabolites (tissues): 5.29±1.05</b>					

Values (quantified using UV detection) are expressed as total mg (mean ± SE) considering the total weight (g) of each lumen content and tissue. In QqQ, when not quantified, the relative abundance of each metabolite was scored as high (+++); medium (++) and low (+) (these values compare the abundance for the same metabolite in the different tissues but not among different metabolites); ND, not detected in UV. **10** RES-3-*O*-glucuronide; **12** DH-RES glucuronide; **13** RES sulfate; **14** DH-RES sulfate; **17** RES; **18** DH-RES; **19** *cis*-RES; n = 4 except in <sup>a</sup>n = 1, <sup>b</sup>n = 2 and <sup>c</sup>n = 3.



**Table 4.** Concentration of RES and derived metabolites in different fluids, LDL and blood cells of pigs 6 h after intragastric RES administration.

Fluids	1	2	5	6	7	9	10	13	17	QqQ
Urine (U) (1172±262) mL	Det	2.36±0.12 (2.79±0.53)	0.41±0.02 (0.49±0.09)	3.06±0.53 (3.72±1.01)	Det	Det	21.38±3.62 (24.11±0.97)	2.99±1.25 (3.83±1.59)	Det	<b>3 (+); 4 (+); 8 (++);</b> <b>12 (0.85)<sup>a</sup>; 14 (+++);</b> <b>15 (++);</b> <b>16 (++)</b> <b>18 (0.52)<sup>a</sup>; 19 (++)</b>
Bile (B) (42.0±9.5) mL	Det	9.60±2.60 (0.40±0.11)	9.21±4.00 (0.39±0.17)	33.75±13.81 (1.42±0.58)	13.40±2.92 (0.56±0.12)	7.55±2.44 (0.32±0.10)	28.63±1.08 (1.20±0.05)	26.70±9.53 (1.12±0.40)	2.22±0.22 (0.10±0.01)	<b>4 (+); 8 (++)</b> <b>12 (0.10)<sup>a</sup>; 14 (+++);</b> <b>15 (++);</b> <b>16 (++)</b>
Portal vein plasma (PP)	ND	ND	ND	ND	ND	ND	0.67±0.27 1.67±0.67 µM	0.17±0.04 0.56±0.13 µM	0.11±0.03 0.47±0.12 µM	<b>7 (++);</b> <b>12 (0.44;</b> <b>1.1 µM)<sup>a</sup>; 14 (++)</b>
Jugular vein plasma (JP)	ND	Det <sup>b</sup>	Det <sup>b</sup>	ND	ND	ND	0.28±0.05 0.69±0.13 µM	0.08±0.02 0.24±0.05 µM	ND	<b>7 (+); 12 (0.09;</b> <b>0.22 µM)<sup>a</sup>; 14 (+)</b> <b>17 (0.012; 0.05 µM)</b> <b>19 (+)</b>
Lymph (L)	ND	ND	ND	ND	ND	ND	0.10±0.02 0.25±0.05 µM	0.05±0.002 <sup>b</sup> , Det <sup>a</sup> 0.17±0.01 µM <sup>b</sup>	0.024, Det <sup>a</sup> 0.1 µM	<b>12 (0.21)<sup>a</sup>; 14 (+)</b> <b>19 (+)</b>
Cerebrospinal fluid (CSF)	ND	ND	ND	ND	ND	ND	ND	ND	ND	<b>13 (+); 17 (+)</b> <b>19 (+)</b>
LDL (ng/mL)	ND	ND	ND	ND	ND	ND	Det	ND	Det	<b>10 (+)<sup>a</sup>; 12 (+)</b> <b>17 (++);</b> <b>19 (+)</b>
Red Blood Cells (RBC) (ng/mL)	ND	ND	ND	ND	ND	ND	ND	ND	ND	<b>13 (+); 14 (+)</b> <b>17 (+); 19 (+)</b>
White Blood Cells (WBC) (ng/mL)	ND	ND	ND	ND	ND	ND	ND	ND	Det	<b>13 (+); 17 (++)</b> <b>19 (+)</b>
<b>Total metabolites (mg): 36.33±5.94 (urine) and 5.60±1.54 (bile)</b>										

Values (quantified using UV detection) are expressed as the mean ± SE µg/mL. In PP, JP and L, the values are also expressed as µM. LDL, RBC and WBC values are expressed as ng/mL. In the case of urine and bile, the total volume of the fluid was known and the values expressed between parenthesis are total mg (mean ± SE). In QqQ, when not quantified, the relative abundance of each metabolite was scored as high (+++); medium (++) and low (+) (these values compare the abundance for the same metabolite in the different fluids but not among different metabolites); Det, detected but not quantified; ND, not detected in UV. **1** RES diglucuronide-1; **2** RES diglucuronide-2; **5** RES sulfoglucuronide-1; **6** RES sulfoglucuronide-2; **7** RES trisulfate; **8** DH-RES trisulfate; **9** RES ribosyl sulfate; **10** RES-3-*O*-glucuronide; **12** DH-RES glucuronide; **13** RES sulfate; **14** DH-RES sulfate; **15** RES disulfate; **16** DH-RES disulfate; **17** RES; **18** DH-RES; **19** *cis*-RES; n = 4 except in <sup>a</sup>n = 1, <sup>b</sup>n = 2 and <sup>c</sup>n = 3.

**Table 5.** RES and derived metabolites distribution in the different pig organs and tissues examined 6 h after intragastric RES administration.

Organ and tissue	Weight (g)	10	13	17	QqQ
Kidneys (K)	293±26	0.610±0.207	0.167±0.095	0.020±0.014 <sup>c</sup>	<b>5+6 (+); 12 (0.014)<sup>a</sup>; 14 (+)</b>
Liver (Lv)	1603±26	0.516±0.215	0.365±0.145 <sup>c</sup>	0.185±0.057 <sup>c</sup>	<b>4 (+); 5+6 (++)</b> ; <b>7 (++)</b> <b>12 (0.020)<sup>a</sup>; 14 (++)</b> , <b>19 (+)</b>
Lungs (Ln)	483±17	0.038 ± 0.006	0.026±0.004 <sup>b</sup> , Det <sup>a</sup>	0.042±0.014 <sup>b</sup> , Det <sup>a</sup>	<b>7 (+); 12 (0.008)<sup>a</sup>; 14 (+)</b> <b>19 (+)</b>
Cerebellum (Cr)	12±1	0.053	ND	0.001	<b>5+6 (+); 7 (+); 12 (0.006)<sup>a</sup></b> <b>13 (++)</b> ; <b>14 (+++)</b> ; <b>19 (+)</b>
Heart (H)	420±37	0.020±0.004 <sup>b</sup> , Det <sup>b</sup>	0.011	Det <sup>b</sup>	<b>7 (+); 12 (0.006)<sup>a</sup>; 14 (+)</b>
Brain (Br)	118±5	ND	ND	0.022, Det <sup>a</sup>	<b>10 (+); 13 (+)</b>
Aorta Tissue (A)	38±4	0.019±0.003	0.006, Det <sup>b</sup>	0.002, Det <sup>b</sup>	<b>7 (+); 12 (0.005)<sup>a</sup>; 14 (+)</b>
Ovaries (Ov)	40±5	0.008 ± 0.001	0.004±0.0003	0.001±0.0002 <sup>c</sup> , Det <sup>a</sup>	<b>5+6 (+); 7 (+); 12 (0.002)<sup>a</sup></b> <b>14 (+)</b>
Uterus (Ut)	88±4	0.009±0.001 <sup>c</sup>	0.005, Det <sup>a</sup>	0.001, Det <sup>a</sup>	–
Urinary Bladder tissue (UBl)	60±10	0.012	Det <sup>a</sup>	Det <sup>a</sup>	<b>14 (++)</b>
Pancreas (Pn)	103±8	Det <sup>b</sup>	Det <sup>a</sup>	0.005	<b>4 (+); 5+6 (+); 10 (0.001)<sup>a</sup></b>
Spleen (Sp)	122±14	Det <sup>c</sup>	Det <sup>b</sup>	Det <sup>a</sup>	<b>10 (0.005)<sup>a</sup>; 12 (0.001)<sup>a</sup></b> <b>14 (+)</b>
<b>Total metabolites (mg) = 2.21 ± 0.77</b>					
Lymph Node (LYN)*	–	0.057, Det <sup>a</sup>	Det <sup>b</sup>	Det <sup>b</sup>	<b>12 (0.07)<sup>a</sup>; 14 (++)</b>
Perirenal Fat (PF)*	–	Det <sup>a</sup>	ND	0.089±0.022	<b>10 (0.017)<sup>a</sup>; 12 (0.023)<sup>a</sup></b> <b>13 (++)</b> ; <b>14 (++)</b> ; <b>19 (+)</b>
Mesenteric Fat (MF)*	–	0.025, Det <sup>a</sup>	ND	0.015, Det <sup>a</sup>	<b>12 (0.120)<sup>a</sup>; 13 (+)</b> ; <b>14 (++)</b> <b>19 (+)</b>
Subcutaneous Fat (SF)*	–	Det <sup>b</sup>	ND	0.019, Det <sup>a</sup>	<b>10 (0.020)<sup>a</sup>; 12 (0.030)<sup>a</sup></b> <b>13 (+)</b>
<i>Longissimus dorsi</i> (LgD)*	–	ND	ND	0.02±0.003 <sup>b</sup>	<b>10 (0.01)<sup>a</sup>; 12 (+)</b> <b>13 (++)</b> ; <b>14 (++)</b> ; <b>19 (++)</b>
<i>Semimembranosus</i> Muscle (SM)*	–	ND	ND	ND	<b>13 (+); 14 (+)</b>

Results (quantified using UV detection) are expressed as total mg taking into account the weight of the organ or tissue (mean±SE). \*Results are expressed as µg/g (the total weight was not determined). Det., detected but not quantified. ND, not detected in UV. In QqQ, when not quantified, the relative abundance of each metabolite was scored as high (+++); medium (++) and low (+) (these values only compare the abundance for the same metabolite in the different organs or tissues but not among different metabolites). **4** DH-RES sulfoglucuronide; **5** RES sulfoglucuronide-1; **6** RES sulfoglucuronide-2; **7** RES trisulfate; **10** RES-3-*O*-glucuronide; **12** DH-RES glucuronide; **13** RES sulfate; **14** DH-RES sulfate; **17** RES; **19** *cis*-RES; n=4 except in <sup>a</sup>n=1, <sup>b</sup>n=2, <sup>c</sup>n=3.