The potential of resveratrol to act as caloric restriction mimetic appears to be limited – insights from studies in mice

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Is resveratrol a caloric restriction mimetic?

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Abstract

Caloric restriction (CR) has been shown repeatedly to prolong lifespan in laboratory animals with its benefits dependent on molecular targets forming part of the nutrient signaling network including the NAD-dependent deacetylase SIRT1. It has been hypothesized that the stilbene resveratrol (RSV) may counteract age- and obesity-related diseases similarly to CR. In yeast and worms, RSV-promoted longevity also depended on SIRT1. While it remains unclear whether RSV can prolong lifespan in mammals, some studies in rodents supplemented with RSV have reported lowered body weight (BW) and fat mass, improved insulin sensitivity, lowered cholesterol levels, increased fitness and mitochondrial biogenesis. Molecular mechanisms possibly leading to such changes include altered gene transcription as well as SIRT1, AMP-activated kinase (AMPK) and PPAR gamma coactivator 1-alpha (PPARGC1A) activation. However, some mouse models did not benefit from RSV treatment to the same extent as others. We conducted a literature search on PUBMED (April 15th, 2020) for trials directly comparing RSV application to CR-feeding in mice. In most studies retrieved by this systematic PUBMED search, mice supplemented with RSV did not show significant reduction of BW, glucose or insulin. Moreover, in some of these studies, RSV- and CR-treatment affected molecular targets differently and/ or findings on RSV and CR impact varied between trials. We discuss RSV-induced changes in gene transcription hypothesized to partly counteract age-related alterations. Although there may possibly be a moderate effect of RSV supplementation on parameters such as insulin sensitivity towards a more CR-like profile in mice, data are inconsistent. Likewise, RSV supplementation trials in humans report controversial findings. While we consider that RSV may, under certain circumstances, moderately mimic some aspects of CR, current evidence does not fully support its use to prevent or treat age- or obesity-related diseases.

Keywords:

Sirtuin, polyphenol, healthspan, diabetes mellitus type II, lunularin, dihydroresveratrol

Teaser Text

Impact of caloric restriction on hormonal, transcriptional and other molecular changes in mice is compared to resveratrol application. Findings are related to data from human supplementation trials.

Introduction

Resveratrol sources and structure

Resveratrol (RSV[1]) is a secondary plant metabolite found in dietary sources such as berries and other fruits including peanuts and cocoa (1-4) with considerable amounts in grapes, and consequently in wine (5). The stilbene RSV forms *cis* and *trans* derivatives that are mostly found as glucosides in plants (5). While wine drinkers can consume more than 1 mg/d RSV (6), in the US population, average RSV uptake is estimated to be around 80 µg/d (7). Supplementation of synthetic RSV up to 150 mg/d has been regarded as safe by the European Food Safety Authority (8). However, at doses of 2.5 g or higher, RSV supplementation may cause mild to moderate gastrointestinal symptoms (9). RSV is metabolized by gut microbiota (to dihydroresveratrol and possibly other metabolites such as lunularin) and is quickly sulfonated or glucuronidated after resorption from the intestines, leading to very low levels of non-conjugated RSV in mice and humans (10, 11).

Caloric restriction as putatively lifespan-extending intervention

Studies employing model organisms ranging from yeast to mice found that RSV may possibly prolong lifespan (reviewed in (12)). The only non-genetic intervention that has repeatedly promoted longevity is a reduction of feed intake (caloric restriction (CR)). Compared to *ad-libitum* feeding, CR prolonged lifespan in model organisms including yeast, mice and rhesus monkeys (13-15). In mammals, CR was shown to reduce adipose tissue mass and body weight (BW) and decrease leptin, insulin, insulin-like growth factor-1 (IGF1) and cholesterol plasma levels. Furthermore, CR lowered markers of inflammation such as TNF, which is expressed under the control of NFKB1 (15, 16) and may even favor healthy aging in humans (17). The

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AGE - advanced glycation end products; AMPK - AMP-activated kinase; BW - body weight; CHD - coronary heart disease; CR - caloric restriction; CRM – caloric restriction mimetic; CRP – C reactive protein; EOD - every other day feeding regimen; GH - growth hormone; HbA1c - glycated hemoglobin; HFD - high fat diet; IGF1 - insulin-like growth factor-1; IGF1R - IGF1 receptor; MTOR - mechanistic target of rapamycin; NAFLD – non-alcoholic fatty liver disease; PPARGC1A - PPAR gamma coactivator 1-alpha; ROS - reactive oxygen species; RSV – resveratrol; SIRT1 - silent mating type information regulation 2 homologue 1; T2DM - type II diabetes mellitus; WAT - white adipose tissue; WC- waist circumference[1]

WHO defines healthy aging as “the process of developing and maintaining the functional ability that enables wellbeing in older age” (18). Aging, however, increases susceptibility towards many common diseases (19, 20) such as coronary heart disease (CHD), type II diabetes mellitus (T2DM), cancers, dementia and painful conditions (21). Chronic inflammation may further contribute to the pathogenesis of age-related diseases (22) and late in life, increased frailty is observed (23).

Pathways implicated in aging

Nutrients activate insulin and IGF-1 receptors (IGF1R) as well as downstream signaling pathways. Interestingly, heterogenetic knockout of *Igfr1* increased lifespan in mice (24). Upon inhibition of IGFR1 downstream mechanistic target of rapamycin (MTOR), the lysosomal degradation pathway autophagy is induced (25). Of interest, genetically inducing autophagy made mice longer-lived as well as more insulin-sensitive and tolerant towards oxidative damage (26). Another molecular nutrient-sensing enzyme is NAD-dependent deacetylase silent mating type information regulation 2 homologue 1 (SIRT1) (27). Its over-expression in yeast, worms and mice increased lifespan (28-32). Moreover, SIRT1 can activate PPAR gamma coactivator 1-alpha (PPARGC1A), a major regulator for mitochondrial biogenesis (27).

Consistent with CR counteracting age-related changes in cellular signaling, it improved insulin sensitivity and autophagy in aged organisms (33). Remarkably, the phenotypes of CR and transgenic SIRT1 mice resemble each other (28), and SIRT1 was necessary for CR-induced lifespan extension (32, 34, 35). In long-lived mice with genetically distorted signaling of growth hormone (GH1), which controls IGF1 production, CR could not further promote lifespan extension (36). Thus, CR appears to activate SIRT1 and inhibit IGF1, thereby affecting their downstream signaling (37).

Since the number of overweight humans worldwide and, consequently, premature onset of age-related diseases are increasing (38), molecules possibly mimicking CR (CRMs) without having to decrease nutrient intake may seem like a hopeful measure to prolong human lifespan. According to National Institute on Aging (NIA) based researchers, a CRM candidate

substance should not “significantly reduce long-term food intake”, mimic “metabolic, hormonal, and physiological effects of CR”, activate stress response pathways observed in CR and promote CR-like effects on life- and healthspan (39). In **Figure 1**, such effects and further desired properties (i.e. safety / lack of side effects) of ideal CRM candidates are depicted.

Resveratrol and putative lifespan extension

Of interest, RSV-induced lifespan increase in yeast, worms and flies depended on their SIRT1 homologue (30, 32). Because RSV supplementation resembled CR-induced lifespan extension in these settings, it has been hypothesized that RSV may mimic CR (32). However, it remains unclear whether RSV supplementation can increase lifespan in mice (40-45). Therefore, we have reviewed the data from studies directly comparing CR with RSV that analyze aging- and obesity-related parameters. Since the number of studies in mammalian non-mouse models was too small to compare trials reasonably, we have limited our systematic search to CR-RSV trials in mice. Prior to reviewing this data, we give an overview of studies that have supplemented RSV in mice without including a CR control. At the end of this narrative review with systematic component, we relate (inconsistent) findings from studies in rodents to data from human trials.

Molecular targets of resveratrol in rodents

In an early study on lifespan and RSV in male C57BL/6 mice, the stilbene prolonged lifespan when supplemented at 0.4 g/kg to a high fat diet (HFD) with 60% energy from fat. Moreover, stilbene supplementation lowered fed and fasted insulin as well as fasted glucose levels while increasing the number of mitochondria. In line with this finding, after six months of supplementation, hepatic PPARGC1A had a lower acetylation status in RSV- than in non-supplemented mice. Yet, feed consumption and body temperature were unchanged. (43). Moreover, RSV attenuated obesity-induced damage to heart and liver in the mice on a HFD (60% calories from fat (43)). Lagouge et al. (46) also found an increase in muscular mitochondria and brown adipose tissue (BAT) as well as improved insulin sensitivity in RSV-supplemented C57BL/6 on a HFD with 40% energy from fat. In contrast to Baur et al.(43),

Lagouge and colleagues (46) used a ten times higher dose of RSV (0.4 g/kg and 4 g/kg diet, respectively) and found lower BWs in RSV-treated mice compared to the controls since the stilbene-supplemented mice gained weight more slowly than the control mice. The RSV-fed Lagouge male C57Bl/6J mice (46) also had more active muscular PPARGC1A which was upregulated on mRNA, protein and SIRT-mediated post-transcriptional level via de-acetylation. While RSV supplementation of mice on a standard diet (in contrast to mice on a 60% energy from fat HFD) did not increase lifespan, it induced transcriptional changes similar to the pattern detected in animals following an every other day feeding regimen (EOD) (47).

Using *Ampk* knock out mice, Um et al. (48) showed that RSV-induced benefits relied on this kinase responding to lowered nutrient supply and forming part of the SIRT1 regulatory network (49). In contrast to *Ampk* knock out mice, RSV-supplemented wild-type mice showed increased metabolic rate, lowered BW as well as improved insulin sensitivity and glucose tolerance. In skeletal muscle, mitochondrial biogenesis and *Ppargca1* mRNA levels were increased in RSV- as compared to non-supplemented animals (48). However, phosphorylation of AMPK as measure of its activation shows high inter-individual differences within experimental groups when analyzed *in-vivo* and was not significantly altered in liver, muscle or white adipose tissue (WAT) of mice receiving RSV at 4 g/kg diet for 4 or 13 weeks (50). *In-vitro* studies observed AMPK phosphorylation when RSV was administered at 25µM to Chinese hamster ovarian cells (43). This concentration is not reached *in-vivo* since RSV fed at 4 g/kg diet led to concentrations below 2µM (41, 46).

In order to study whether RSV may slow down aging, the stilbene was applied to a mouse model for the premature aging Werner syndrome. A mutation of the gene *Wrn* in a C57BL/6 background led to increased BW, visceral fat, liver steatosis, triglyceride and glucose levels as well as insulin resistance when compared to wild-type mice (51). In the Werner mice, applying RSV at 0.4 g/kg diet from weaning to 5 or 9 months of age attenuated liver steatosis, hyperglycemia and insulin resistance without lowering TG levels, visceral fat mass or BW compared to non-supplemented mutant mice. Moreover, RSV supplementation did not attenuate inflammation in the prematurely aging mice and did not expand their lifespan (51).

In a study with organ-specific *Ppargca1* knockout mice, RSV-supplemented (4 g/kg diet) animals depended on muscular PPARGCA1 for increased mitochondrial biogenesis. In wild-type mice, RSV showed a rather moderate influence on insulin and glucose levels, decreased total cholesterol plasma levels and possibly hepatic TGs. Dietary application of RSV (4 g/kg diet) was further compared to feeding a chemical SIRT1-inducer. Interestingly, improved glucose homeostasis after supplementing the SIRT1 inducer did not depend on PPARGCA1. This indicates that RSV may not be a sole SIRT1 inducer. The effect of RSV in wild-type mice was highly tissue dependent and *Ppargca1* mRNA levels in muscle did not seem to be affected significantly. While *Ppargca1* transcription was decreased in liver, it was increased in WAT (50).

Conversely, in another study in mice on a HFD, feeding RSV at 4 g/kg diet did not increase mitochondria number or affect PPARGCA1 protein levels in the muscle. When the same authors used rats as a model, they similarly observed no changes in mitochondria number or PPARGCA1 (41).

In a Wistar-based steatosis rat model, RSV at 0.2 g/kg BW for 18 weeks attenuated the HFD-induced rise in total cholesterol, TGs and BW. Additionally, down-regulation of autophagy-related *Map1lc3b* mRNA levels in HFD- as compared to standard-diet-fed animals was counteracted and hepatic *Sirt1* mRNA levels were elevated by RSV feeding (52). An overview of molecular targets putatively affected by RSV, its microbial products and host-modified products is depicted in **Figure 2**.

Neuroprotective properties of RSV have also been observed. CR in elderly humans may improve memory (53) and activation of SIRT1 could possibly counteract neurodegenerative disease (54). However, data on RSV improving neurodegeneration in mouse brain is not consistent (55).

Changes in phenotype, protein and mRNA levels in CR compared to RSV-supplemented mice

On April 15th, 2020, we searched PUBMED (pubmed.ncbi.nlm.nih.gov) for original research articles using the search terms ‘restriction AND (mouse OR mice) AND resveratrol’. We screened the articles retrieved for studies that compared *ad-libitum* fed non-supplemented animals to a RSV-supplemented group and mice on CR that were describing aging- or obesity-related parameters beyond changes in BW. We excluded genetic disease models and studies focusing on the brain. Since outcomes from studies on RSV often yield contradicting data, we concentrated on parameters that had been analyzed by at least 2 of these studies **(Figure 3)**. This led to finding 7 studies that had evaluated BW, body composition, insulin sensitivity, serum hormone and/or lipid levels, activation of known molecular targets of CR and/or changes in gene transcription (**Table 1**). Five studies used C57BL/6 strains and the other 2 studies employed the F1 generation obtained from crossing C57BL/6 x C3H/He mice. All but one study, which included male and female rodents, studied male mice (56-62). Diets provided 10 - 60% calories from fat. The RSV supplementation dose and duration varied from 18.6 mg to 4 g/kg diet and 8 weeks to 16 months. In the majority of these studies, RSV application showed little to no effect on mouse phenotype. In contrast to CR, RSV did not decrease BW and only slightly blunted adipose tissue mass increase in one out of 3 studies. Analyzed feed intake was not changed by RSV application. Cholesterol was measured in 3 studies, of which 2 showed reduction by CR. However, RSV did not affect cholesterol levels (58, 59, 62). In one study, RSV, but not CR, increased TG levels (62). However, in the mice from studies by Günther et al. (58) and Pallauf et al. (59), TG levels remained unchanged by RSV as well as CR (63). Fasting glucose levels were monitored in 6 studies and decreased by most CR interventions. RSV tended to decrease glucose levels in genetically heterogeneous but increased them in C57BL/6 mice on a low-dose RSV-supplemented standard diet (56, 62). The other 4 studies found no significant changes in blood glucose by RSV (56, 58-60, 63). Fasted insulin levels were measured in 5 studies, 2 of which showed a reduction by RSV as well as CR (58, 60). Two trials revealed no influence by CR or RSV, and one showed an increase by RSV supplementation but not by CR on fasting insulin (56, 57, 62). Insulin levels after glucose application as indicator of insulin sensitivity were decreased and thus improved

in the CR groups of both studies that measured challenged insulin (58, 59). However, RSV only improved insulin sensitivity in one of these 2 studies (58). CR lowered IGF1 levels in one out of 2 studies, while RSV did not affect the growth factor (57, 62). In 30-month-old mice, CR and RSV decreased muscular SIRT1 (57), while in 4-5-month-old mice, CR increased SIRT1 levels (61). Two g/kg RSV had no effect and 4 g/kg RSV, similar to CR in the same study, up-regulated SIRT1 protein in the muscle (61) (Table 1).

Supplementation of RSV was shown attenuate the damage of a HFD on mouse heart (43), possibly by somewhat mimicking CR (56). Since CR induced phosphorylation of the putative RSV target AMPK (64), we analyzed p-AMPK levels in the heart of mice described in (58). While RSV-fed mice showed high variations of AMPK phosphorylation within the group, *ad-libitum* control mice, CR mice and RSV mice did not differ from each other in cardiac levels of activated AMPK (unpublished results (63)).

Various studies explored changes in gene expression induced by aging and whether CR and/ or RSV could partly attenuate these changes. In the heart of 30-month-old C57BL/6xC3H/He F1 hybrid mice, after 16 months of dietary intervention, CR as well as RSV (50 mg/kg AIN-93M diet or 4.9 mg / (kg mouse BW · d)) opposed the majority of age-related changes in gene transcription. Differential gene expression because of aging was observed for 1029 genes (57). However, in younger (5-month-old) mice supplemented with 1.25 mg RSV / (kg mouse BW · d), transcription of less than 10% of the 304 genes differentially transcribed in corresponding CR mice was changed towards a more CR-like pattern (56). Park et al. (65) studied the variations in gene expression in 5- and 25-month-old mice of seven inbred strains. They found that age changed 6-15 % of more than 22000 gene transcripts measured in the heart of the mice. However, which genes were affected by age depended greatly on the strain. Of the differentially regulated genes, only 20 genes were altered consistently over at least 6 out of 7 strains. Interestingly, in a study with Balb/C mice at 4 and 28 months of age, individual variation in hepatic gene transcription increased significantly with age. In studies by Park et al. (65) and White et al. (66), gene ontology terms obtained by functional annotation analysis of the transcriptome indicated that immune response was up-regulated in aged mice. Analyzing

cardiac gene transcription, in the majority of the strains studied, RSV at 50 mg/kg diet was as effective as CR in inhibiting age-dependent gene up-regulation when applied from 15-30 months of age. Yet in the cerebellum, measuring the transcription of 5 genes that were analyzed as markers for dietary interventions attenuating age-related changes in mice, RSV supplementation only affected the transcription of complement C1q subcomponent subunit A that codes for a protein involved in the complement system (65). Moreover, PCR data from mouse liver showed CR-mediated upregulation of *Ppargca1, Sirt1* and phosphoenolpyruvate carboxykinase 1 (*Pck1,* a gene that codes for a central protein in gluconeogenesis), while RSV-supplementation left mRNA levels of these genes unaffected (Table 1 (58, 59)). Of interest, Svensson et al. (50) found decreased *Ppargca1 mRNA* levels in the livers of RSV-supplemented mice (4 g/kg diet for 4 and 13 weeks) implying that RSV may even act contrarily to CR on *Ppargca1* gene regulation. In the heart, *Ppargca1* transcription showed no changes by CR in old, genetically heterogeneous mice on a standard diet (56) while in young C57BL/6 on a HFD (40% energy from fat), *Ppargca1* was transcriptionally up-regulated by CR (Table 1 (58, 63)). However, RSV supplementation did not influence *Ppargca1* transcription in young or old mouse heart (56, 57, 63). The pyruvate dehydrogenase lipoamide kinase isozyme 4 (*Pdk4*), which inhibits the use of glucose for metabolism, and mitochondrial uncoupling protein 3 (*Ucp3*) were transcriptionally up-regulated in the heart of old and young genetically heterogeneous mice under CR. Young but not old RSV-supplemented mice also showed increased cardiac mRNA levels of *Pdk4* and *Ucp3 (56, 57).* However, in young C57BL/6 on a HFD, neither CR nor RSV affected cardiac *Pdk4* mRNA levels (63). These findings indicate that, similar to CR-induced transcriptional changes, RSV supplementation effects may also depend on mouse strain, age, diet and dose.

In C57BL/6NIA mice on an AIN-93G diet supplemented with 0.4 g RSV / kg diet, Pearson et al. (47) detected transcriptional changes in liver, heart, muscle and WAT of mice supplemented from 12-18 and 12-27 months of age. They found that RSV, similar to EOD, counteracted age-related changes in the liver. However, they did not detect such anti-aging transcriptional alterations by RSV or EOD in the heart. In muscle, RSV but not EOD slowed down age-related

changes. Contrarily, in adipose tissue, RSV and EOD (67) enhanced age-related changes. Yet, RSV improved parameters that decline with age and obesity. In old mice, supplementation of the stilbene increased bone strength (tissue mineral density in the distal femur) and improved cataract (30-month-old mice), prolonged the time before falling off a rotarod (21- and 24-month-old mice) and, at doses of 0.24%, enhanced endothelial function measured as acetylcholine relaxation and reduced oxidative stress (18-month-old-mice). However, in a leptin-deficient background, RSV supplementation could not improve murine motor functions and possibly decreased endurance as was shown in a treadmill test (68).

Similar to findings from mouse studies comparing CR with RSV application, RSV-supplementation in humans renders inconclusive data

Future and ongoing studies could possibly show a moderate impact of RSV on human health, yet discouraging findings from studies in rodents are somewhat reflected in humans. A PUBMED search on August 30th, 2020, for recent (published 2014 or later) meta-analyses of human trials supplementing RSV and using glucose, insulin, triglyceride, cholesterol, body weight or inflammatory markers as endpoints retrieved 19 publications (search terms 'resveratrol AND (glucose OR insulin OR lipid OR inflammation OR body weight)'). Of these 19 publications, 2 were on preclinical models and thus not considered. Total cholesterol levels were analyzed by 8 articles. Four reports found no influence of RSV supplementation (69-72), 2 found a reduction (73, 74) and 2 publications focusing on obese and non-alcoholic fatty liver disease (NFALD) patients concluded that RSV could increase cholesterol levels (75, 76). Of 7 meta-analyses examining the impact of RSV supplementation on TG levels, only one study discovered reliable evidence for a reduction after treatments longer than 6 months in diabetic patients (70-74, 76, 77). Waist circumference (WC) and / or BW were investigated by 4 studies (69, 77-79). While 3 of these found a reduction of WC by RSV supplementation, 2 studies also included fat mass in their analyses. Although both reports detected reduced BW, only Tabrizi et al. (79) furthermore observed decreased fat mass. In contrast, Elgebaly et al. (77) found unchanged BW upon RSV supplementation. Seven meta-analyses covered data on glucose

296 levels after RSV supplementation. Of these, 3 reported no influence on glucose levels after
297 stilbene application (71, 72, 77) and 3 analyses concluded that RSV could decrease blood
298 glucose (69, 74, 80). However, Liu et al. (80) observed only diabetic patients to be responsive.
299 Intriguingly, the authors of a meta-analysis from 2020 in patients with T2DM, stated that data
300 were insufficient for the evaluation of health benefits, since after excluding trials with
301 incomparable interventions and controls, solely 3 studies remained for their assessment (81).
302 Further glucose homeostasis-related parameters such as insulin levels, HOMA-index or
303 glycated hemoglobin HbA1c, were analyzed by 4 publications (71, 74, 77, 80). Interestingly,
304 the marker for average glucose levels during the last three months, HbA1c, appeared to be
305 responsive towards stilbene treatment. While measuring insulin levels and determining the
306 HOMA-index yielded little promising results, all three meta-analyses evaluating HbA1
307 concluded that patients may benefit from RSV supplementation (71, 74, 80).

308 During aging, low-grade, chronic inflammation occurs (22). By counteracting expression of
309 inflammatory cytokines such as TNF, IL6 and acute-phase protein CRP (C-reactive protein),
310 RSV could possibly counteract development of age-related illnesses (82). Of the meta-
311 analyses retrieved by our PUBMED search, 6 studied inflammation markers after RSV
312 supplementation. While 4 out of 6 studies found reduced CRP levels in RSV-supplemented
313 human subjects (72, 74, 83-86), all 4 studies evaluating IL6 found no difference between
314 stilbene and non-supplemented individuals (83-86). Levels of TNF were decreased by RSV in
315 2 out of 3 analyses (84-86).

316 The contradicting data on RSV benefits in human trials may indicate that, while there are some
317 well-controlled and well-designed studies, many trials lack appropriate controls and are difficult
318 to compare with each other. Furthermore, studying different patients (gender, health status,
319 age) and types of interventions (dose, time point, duration) might have contributed to these
320 controversial outcomes.

321 In a small number of original research articles, laborious muscle biopsies and analysis of
322 AMPK, SIRT1 and PPARGCA1 (molecular targets that may mediate putative CR-like

properties of RSV as mentioned in section ‘Resveratrol and putative lifespan extension’) were conducted (87-89). Two of these trials with 10-11 obese and/or men suffering from T2DM showed CR-like effects by RSV-supplementation with elevated activation of AMPK and SIRT1 in the muscle (87), increased mitochondrial function, improved insulin sensitivity and lowered plasma levels of pro-inflammatory cytokines and triglycerides (88). In contrast, with a larger number (n=45) of non-obese women, none of these CR-like outcomes were observed after RSV supplementation (89).

Of interest, RSV could also affect patients negatively. In human subjects suffering from NAFLD, 3g RSV per day increased hepatic stress (90). High doses may even lead to increased levels of TNF, as did a single dose of 5g RSV in healthy men (91). Furthermore, RSV supplementation may blunt the positive effects from health-improving interventions such as exercise (92). Negative impact of supplements on exercise-induced benefits has been reported before for antioxidant vitamins (93). Of interest, application of vitamin C and vitamin E at high doses has also been discussed as putatively favoring healthspan. However, lifespan studies with these vitamins have yielded discouraging data (94, 95).

Another factor possibly contributing to controversial outcomes in human RSV supplementation trials could be their duration, since weeks or months may not suffice to adequately monitor metabolic, hormonal and physiological changes caused by an intervention and evaluate how these changes may affect aging during years or decades. While studies in laboratory animals can render endpoints such as lifespan and easy access to organ tissues, in human trials, choosing the correct biomarker can be challenging (96). The Targeting Aging with Metformin (TAME) trial evaluating if metformin may also benefit non-diabetic patients (97) has studied various possible biomarkers for aging research. They emphasize that a suitable biomarker needs to show a measurable change with age and be age-dependently associated with all-mortality risk. Furthermore, it should be robust across data sets and populations as well as reliable and reproducible across labs. Of interest, blood IGF1 levels show U-shaped concentration patterns for mortality risk, since high and low levels of IGF1 are associated with increased risk of cancer and cardiovascular mortality (98). Candidate biomarkers for

inflammation IL2, IL1B, IFNG or TNF may be unstable during storage or present at very low levels (97, 99). Here, IL6, CRP and TNF receptor II seem more suitable for measuring inflammation. Fasting insulin and IGF1 levels respond to changes in nutrient signaling, and HbA1c appears to be useful for monitoring metabolic aging (97). A recent 6-month-supplementation trial in overweight adults found that, while insulin sensitivity was not improved, HbA1c levels were decreased by 150 mg RSV per day (67). Interestingly, non-enzymatically glycated tissue proteins (advanced glycation end products, AGEs) also reflect increased glucose levels over time. AGEs can induce inflammation and increase oxidative stress. RSV supplementation may lower AGE toxicity (100). Since RSV may possibly, similar to CR, affect numerous pathways implicated in aging, analyzing the serum metabolome as an –omics approach in human subjects could also produce relevant and feasibly reproducible data (101).

Conclusion

RSV supplementation studies in humans yield less promising data for aging-related benefits than epidemiological data on consumption of RSV-rich food (102, 103). Studies in model organisms comparing CR to RSV and studying age- and obesity-related biomarkers find small, non-existent and even contrary effects of RSV on CR targets. Based on our literature review, we consider that RSV CR-mimicking properties are rather moderate or only applicable under certain circumstances. Therefore, we conclude that RSV supplementation cannot replace restricting dietary intake and may not be suitable for the prevention of age- or obesity-related diseases. With diet-derived molecules, hopes were that they might have less side effects than pharmaceutical drugs. However, non-plant-derived small molecules such as metformin may be superior to polyphenols when counteracting age-related disease (104, 105).

In lifespan studies with RSV in mice, genotype or diet may influence the experimental outcome (42, 43, 47). Possibly, responsiveness towards RSV supplementation in humans may depend on the genome and dietary matrix. Genome-wide-association studies (GWAS) searching for longevity genes and analyzing gene-diet interactions may identify different alleles of genes coding for proteins in nutrient-sensing cascades that are affected by RSV supplementation. To further evaluate whether RSV promotes healthy aging in human trials, long term studies with high enough numbers of participants to detect possible subgroups benefitting or not benefitting (106), using adequate biomarkers (96) and applying doses that have been regarded as safe (8) are needed. Possibly, supplementation of a single polyphenol compared to consumption of polyphenol-rich food may be less effective or even abolish positive effects because of side effects such as toxicity issues as well as nutrient-drug interactions (107). Moreover, health benefits from consuming a plant-based diet appear to not solely rely on secondary plant metabolite uptake but on other factors such as fiber consumption (108, 109). Therefore, further research on these other components found in plant-derived food and how they may positively affect aging is warranted. To date, to prolong healthspan, it seems that trying to increase the compliance for known lifespan-prolonging interventions such dietary changes and exercise may yield more successful than supplementation of single dietary factors such as RSV.

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Author contributions

KP designed the article, collected the data and wrote the manuscript. GR designed the article and wrote the manuscript. DC, IG, GK and SPT contributed experimental data. All authors revised the manuscript.

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Table 1: Studies comparing resveratrol supplementation to CR and an *ad-libitum* control in mice[1].

Mice and diet	*male 14-mo-old C57BL/6xC3H/He F1 hybrid* on AIN-*93M (10% energy from fat)* (57)		*male 2-mo-old C57BL/6xC3H/He F1 hybrid on AIN-93M (10% energy from fat)* (56)		*male 7-wk-old C57BL/6J on 60% energy from fat (CR, RSV and controls)* (61)				*female & male 6-mo-old C57Bl/6J on a standard diet* (62)		*male 1-y-old C57BL/6Rj on 40% energy from fat* (59)		*male 18-wk-old C57BL/6Rj on 40% energy from fat* (58)		*male 4-mo-old C57BL/6Rj on AIN-93G (17% energy from fat)* (60)	
duration	16 mo		3 mo		12 wk				6 mo		8 wk		8 wk		8 mo	
treatment	**CR**[2]	**RSV**	**CR**	**RSV**	**LFD**	**CR**	**RSV**		**CR**	**RSV**	**CR**	**RSV**	**CR**	**RSV**	**CR**	**RSV**
dose	75%[3]	0.05 g/kg diet	75%[3]	0.05 g/kg diet		70% HFD	2 g/kg HFD diet	4 g/kg HFD diet	58% +8%[4]	18.6 mg/kg diet	60%	i.p. 24 mg/kg BW[5]	60%	0.35 g/kg diet[6]	60%	0.1 g/kg diet
phenotype																
BW	n.a.	n.a.	**0.7**[7]	≈	**0.8**[7]	**0.6**[7]	≈	≈	**0.9**[7]	*1.1*[7]	**0.6**	≈	**0.6**	≈	n.a.	n.a.
ATM	n.a.	n.a.	n.a.	n.a.	**0.5**	**0.3**	≈	≈	n.a.	n.a.	**0.2**	≈	**0.2**	**0.8**	n.a.	n.a.
energy intake	CR	n.a.	CR	n.a.	≈	CR	≈	≈	n.a.	n.a.	CR	≈	CR	≈	CR	n.a.
cholesterol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	≈	≈	**0.5**	≈	**0.7**	≈	n.a.	n.a.
glucose[8]	*0.8*[7,9]	*0.9*[7,9]	*0.8*[7]	≈	n.a.	n.a.	n.a.	n.a.	≈	**1.3**[7]	**0.7**[10]	≈[10]	≈[10]	≈[10]	**0.8**[7]	≈
insulin	≈[8,9]	≈[8,9]	≈[8]	≈[8]	n.a.	n.a.	n.a.	n.a.	≈[8]	**1.2**[7,8]	**0.5**[11]	≈[11]	**0.3**[8,10] **0.2**[11]	**0.5**[8,10] **0.5**[11]	**0.2**[7,8]	**0.4**[7,8]
IGF1	**0.7**[7,9]	≈[9]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	≈	≈	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
mRNA levels																

liver																
Ppargc1a	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	**3.6**	≈	**2.7**	≈	n.a.	n.a.
Sirt1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	**3.2**	≈	**1.9**	≈	n.a.	n.a.
muscle																
Ppargc1a	**3.4**[7]	≈	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	≈	*1.4*	n.a.	n.a.
heart																
Ppargc1a	≈	≈	≈	≈	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	**1.4**[10]	≈[10]	n.a.	n.a.
protein levels																
muscle																
SIRT1	**0.7**[7]	**0.6**[7]	n.a.	n.a.	≈	**1.3**[7]	≈	**1.4**[7]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
liver																
SIRT1	≈	≈	n.a.	n.a.	**1.5**[7]	**1.6**[7]	≈	≈	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

[1] endpoints with assumed relevance to aging measured in more than one study are listed; values are given related to the controls and therefore do not show units, values < 1 show decrease and values > 1 show increase by the treatment; bold print values show statistically significant differences to the controls with $p < 0.05$, when values are given as italic font, p-values are <0.1; since many values were derived from graphs rather than the original values, they are approximations and no SDs or SEMs can be shown; n.a. – not analysed or value not given; ≈ – not statistically significant different compared to non-supplemented / non-CR animals.

[2] abbreviations: ATM – adipose tissue mass; BW - body weight; CR - caloric restriction, the amount of feed consumed by the CR group is given as percentage of feed consumed by the controls; IGF1 – insulin-like growth factor 1; LFD - low fat diet with 10% energy from fat; mo - months; *Ppargc1a* - peroxisome proliferator-activated receptor gamma coactivator 1-alpha; RSV – resveratrol; *Sirt1* – silent information regulator 1; wk - weeks; y-years.

[3] compared to intake of the control group, control was -10% *ad-libitum*

[4] the diet for the CR group consisted of 58% control diet + 34% dietary fibre + 8% soy

[5] 24 mg/kg BW injected 3 times per week

[6] with increasing BW of the mice, RSV concentration in the diet was increased from 0.3- 0.4 g/kg diet halfway through the trial

[7] approximated from figure

[8] measured in fasted mice

[9] measured in 5-month-old mice after 3 months of supplementation

[10] previously unpublished data obtained by using methods and mice described in (58, 59)

[11] measured in glucose-challenged mice (after application of a glucose bolus)

Figure 1: Desired properties of a caloric restriction mimetic that would lead to health- and lifespan increase.

Figure 2: Molecular targets and cellular processes identified as being affected directly or indirectly by resveratrol. Contoured block arrows depict activation, the color-filled blunt arrow shows inhibition. AMPK – adenosine-monophosphate-activated kinase, IGF1 - insulin-like growth factor 1, IGF1R – IGF1 receptor, MTOR - mechanistic target of rapamycin, NFKB1 – nuclear factor kappa B, PPARGC1A - peroxisome proliferator-activated receptor gamma coactivator 1-alpha, SIRT1 – sirtuin 1, RSV - resveratrol

Figure 3: Selection of studies on resveratrol application compared to caloric restriction in mice.

Editor's comments:

Although authors have demonstrated that drying technology on the main polyphenolic compounds present in Ipomoea batatas has not been reported so far in lines 70-72, in other part (lines 101-103), they described that several publications have reported the effect of drying conditions on the hydroxycinnamic acid derivatives or anthocyanins content of Ipomoea batatas. Both statements are inconsistent each other. Authors should make clear the novelty of the present study.

We agree with the Editor that in the original manuscript the objective was not clearly presented, and therefore the novelty of this work was not correctly highlighted. In the revised version, the objectives have been clarified (L01-110).

In this sense, in the previous version, L70-72 we referred to that the specific effect of "spray drying" on the main polyphenolic compounds present in ipomoea has not been reported. Previous studies reported the negative effect of temperature on polyphenols of Ipomoea when different cooking conditions were compared. However, the specific effect of spray drying process, that also requires the use of high temperatures in the process, on the polyphenolic compounds had not yet been studied. This has now been clarified in the introduction in the revised version (L68-73).

On other hand, in L101-103, we referred to the absence of works studying the effect of temperature on the different groups of polyphenols present in ipomoea simultaneously. This has been clarified in the revised version (L105-107)

In addition, authors have demonstrated that objective of the present study is to characterize the polyphenolic compounds present in the root of a purple-fleshed sweet potato variety of Ipomoea batatas native from Peru (lines 104-105); however, there have been many studies on the phenolic composition of sweet potato. There will be a little novelty in the analysis of polyphenols of the sweet potato cultivated in Peru. The major focus of the present study should be the effect of drying procedure on changes in the polyphenol contents.

We are grateful for your remarks. Accordingly, and as it was also pointed out by reviewers 1 and 4, the objectives were not clearly presented in the original manuscript, and have now been reviewed in this new version.

We agree that the main novelty in this paper is the study of the effect of the spray drying process on the main polyphenolic compounds present in *Ipomoea batatas* roots which was not previously studied. Accordingly, the objective has been rewritten (L101-110), the title has been changed (L1-2), the abstract (L14-15) and the discussion related with the effect of spray drying on cinnamoylquinic acids (section 3.1.4, L354-376) and anthocyanins (section 3.2.4, L449-471) and conclusion (L544-554) have been more deeply discussed in the revised version.

Additionally, we consider that another novelty in this study is the exhaustive characterization of cinnamoylquinic acids made that has resulted in the identification of an important number of new structures containing feruloyl moieties not previously reported in Ipomoea. In this sense, although the presence of (mono and di) caffeoylquinic acid have been widely described in

orange varieties, the presence of feruloylquinic acids has been rarely documented. Additionally, in the specific case of purple varieties, the characterization of phenolic compounds have been focused on anthocyanins, and few studies have characterized both anthocyanins and cinamoylquinic acid simultaneously (this has been now farther clarified in the objectives section (L105-107).

Consequently, in addition to the novelty of the study of spray drying, we also consider appropriated to stress the characterization of these new structures. Accordingly, this has been highlighted in the revised version in the objective (L105-107), and the findings related with the identification of these new structures more emphasized in the discussion (in L238-243; L289-294 and L325-335, for mono, di and tri CQAs respectively), and in the conclusion (L535-541) and abstract (L20-22). Finally, the fact that the presence of some of these structures (mono FQA) seemed to confer a better stability than others during the process of spray drying (see L346-348, L548-549) is also an interesting results that, we think, provides novelty to our results

Reviewer #1: This study investigated phenolic compounds, which includes CiQAs, anthocyanins, and flavonols, in purple sweet potato.

The objective of this study is ambiguous, characterization or effect of thermal processing?

We thank the reviewer for his/her remarks. In fact, the objective of this study was double, to study in deep the polyphenolic composition of this variety of purple *Ipomoea batatas* and to stablish the effect of spray drying on its composition. This has been more clearly presented in the revised version (L101-110).

Despite the potential of spray drying in converting sweet potatoes into functional ingredients or foods the impact of such technology on the different polyphenolic compounds present in *Ipomoea batatas* roots remained unknown. Thus, in this study we firstly made an exhaustive characterization of the different polyphenolic groups present in this purple variety of Ipomoea batatas. Among them and, in addition to the anthocyanin, we have delved into the study of the cinnamoylquinic acids which are rarely reported in purple varieties. This has been now clarified in the objective (L101-105).

if so, the NMR data is necessary to differenticate 27 CiQAs.

Both NMR and mass spectrometry have been successfully used for CiQAs characterization, each one presenting their advantages and disadvantages. NMR provide more complete information about the structure (unambiguous identification) buy also present a low sensitivity requiring most abundant and pure samples. By contrast, MS is more sensitive and specific, and the information provided by LC-MS-qtof and MS/MS fragmentation along with the hierarchical keys provided by Clifford and colleagues, provide enough level of confidence to identify and differentiate the various CiQAs structures and isomers. These authors established hierarchical keys for CiQAs identification based on fragmentation patterns, relative hydrophobicity and bonding strength to quinic acid. This information is indicated in the manuscript in lines (L181-197) and the description and steps followed for their identification have been explained in their respective sections. In the particular case of tricinamoylquinic acids, where our results and

available information don't allows us to identify the compound with sufficient confidence, this has also been mentioned in the text (see L331-L335)

The difference in phenolic compounds content between freeze-dried and spray dried samples can be already expected. please add the reason why…

This remark was already commented in the manuscript (L74-77)

To generalize the effect of drying method, more samples should be included.

In our case and considering the great difference observed among samples dried by lyophilisation and spray dried, we do not think that an increase of the sample size would increase the power analysis. However, and according to our results, it would be necessary to optimize the conditions employed during the process and pre-process in order to better preserve the different bioactive substances. Accordingly an additional sentence has been included (L552-554)

Reviewer #2: This is an interesting and well written submission reporting novel information appropriate to the journal.

The characterisation of the acyl-quinic acids seems to be sound but there are a couple of minor oddities which could usefully be mentioned in the discussion. The putative 3,4,5-triCQA and 3F4CQA elute surprisingly early. Conceivably the putative 3,4,5-triCQA might be a diCQA-glycoside in which case a fragment at one or more of m/z 497, 341 or 323 might have been observed. If these were not observed, it is worth commenting on the early elution and adding this information to the discussion.

We are grateful to the reviewer for their positive and constructive remarks.

Following the suggestion made by the referee we checked the fragmentation pattern that was initially assigned to 3,4,5 TriCQA, and, we found two additional weak MSMS fragments at m/z 497 and 341 (323 was missing) that, as was pointed by the referee, might suggest the presence of a dicaffeoylquinic glucoside. This compound and their fragmentation behaviour was described in Chrysanthemum (Clifford et al. 2007). Indeed, the loss of a m/z 162 fragment may correspond to the loss of a caffeic acid or a hexose, being the latter more hydrophilic and therefore eluting earlier. Thus, we agree with the reviewer remarks and this compound has been reassigned to dicaffeoyl glucoside instead of tricaffeoylquinic acid. This has been explained in the revised version (L280-288) and their respective modification has also been included in tables 1 and 2 and figure 1.

Also, this referee has never encountered a cis-isomer that is not accompanied by, and indeed dominated by, the corresponding trans-isomer. The apparent absence of 5-FQA and dominance of 4-FQA is also a little unusual, and while these assignments seem to be correct it would be useful to add some appropriate comments to the discussion.

Following your suggestion a comment was added in L223-229. We agree with the reviewer that this is unusual. However, we were not able to find the trans isomer.

If the authors are able to pursue these items using UV-irradiation to generate cis-isomers, and using an extract of a green robusta coffee as a surrogate standard (again with UV-irradiation), then there is clear potential for a follow-up publication.

We thank for this interesting contribution that will be considered in future studies.

This referee is not so knowledgeable regarding anthocyanins but has not found anything of concern and recommends that the paper be accepted subject to the minor additions to the discussion suggested above. Thanks

reviewer 3: The manuscript provides additional information on phenolic compounds especially the cinnamoylquinic acids in the root of a purple-fleshed sweet potato variety from Peru. The effects of spray drying on the polyphenolic compounds were among the objectives of the study, but the methodology on sample preparation for spray drying was not well presented. I have the following specific comments for a revision before the manuscript can be accepted for publishing in Food Chemistry:

We thank the reviewer for their constructive remarks and thorough revision. We agree that the description made for the spray drying process in the original manuscript was not precise enough and has been more clearly detailed in this revised version (L123-132). Additionally, the discussion related with the effect of spray drying on cinnamoylquinic acids (section 3.1.4, L354-376) and anthocyanins (section 3.2.4, L449-465) has also been clarified and more extensively discussed in the revised version.

1. Title: a typo, it should be "cinnamoylquinic" not "cinnamolyquinic"

Sorry for the mistake. In this new version the tittle has been changed and the error corrected.

2. Introduction: Page 3, Line 64: "spray drying is … less expensive procedures"; it may not be true! Are there any references to support this statement?

This sentence has been rephrased (L76-77) and a reference included (Ratti 2001) in L673-674.

Line 65: replace "…will be" by "…was"

I am afraid that the reviewer missed the line, since we were not able to find "was" in L65.

3. Materials and Methods: Page 5, Section 2.2. Sample preparation and dehydration processes.
Line 120: What were the freeze-drying conditions such as temperature and time?

Temperature and time have been added (L124-125)

For spray drying, provide more details on the preparation of sweet potato roots. As described in Lines 122-124, "After washing roots were grinded, sieved and introduced in the atomizer with a carrier…"(lines 122-124), does it mean that cooking/pureeing steps were not included in the sample preparation prior to atomizing for spray drying? The authors may refer to the cited references about sample preparation for spray drying of sweet potatoes such as Peng, Z., Li, J., Guan, Y., & Zhao, G. (2013).

We appreciate this comment, although in the original manuscript in the discussion (L346-348) we pointed that the initial pre-processing could contribute to the degradation observed in the polyphenols, the description we made in material methods was not clear enough. Indeed, samples were pureeing (without cooking) before being introduced in the spray dryer. A more clear description has now been included in the revised version L126-129. Additionally our results have been more extensively discussed with the few studies reporting the use of spray drying in Ipomoea L355-376 , L449-465, and L544-548

Page 7, Line 160: "…MSMS with data provided by relevant literature references or databases…", cite key references and information on database?

This information, provided in tables 1 and 3, is now indicated also in the text (L168)

4. Results and Discussion: Page 8, Line 192-193: For clarity, it would be better to indicate that the …compound identified in "freeze-dried" purple sweet potatoes…

Thanks, It has been added in L200-201

Page 8, Line 194: 5FQA, spell out FQA for the first time mentioned in the text.

Thanks, It has been included in L202-203

5. Page 14, Lines 342-343: As mentioned above, were the uncooked/grounded sweet potatoes subjected to spray drying without cooking/pureeing? How about the degradation of polyphenolic compounds if these steps were involved prior to spray drying?

We agree with the referee, in fact, in the original version we pointed to this (L365-368), but was not enough described. As was previously commented (in point 3) samples were mashed (without cooking) before being introduced in the spray dryer. In the reviewed manuscript, we have clarified this pre-process in the material and methods (L126-129) and the contribution of this pre-process to the degradation of the polyphenolic compounds more discussed (L370-376 L456-465 and L546-548)

Page 16, Line 470: Replace "sweet potation o tubers" by "sweet potato roots", as correctly indicated throughout the manuscript.
Changed in L518

About free amino acids in various sweet potato varieties, there are recent articles published in 2020, the authors may want to update the cited references.

Reference has been updated (Qiu et al. 2020) in L-519 and L669.

6. Figure 1 - Among the two captions, which one will be presented in the final version?

Apologies for the mistake, the second one is the correct it has been corrected in the revised version and in L742-753

Reviewer #4: The paper reports the phenolic profile of sweet potato purple type. The paper is largely confirmation of previous studies reported decades ago. A few new compounds were identified. I am concerned about the novelty.

We thank the reviewer for their comments reflecting that, in the original manuscript, we were not clear enough describing the objectives and presenting the novelty of the study. The manuscript has been thought revised and modified taking this into account.

We agree that the main novelty is the effect of the spray drying process on the main polyphenolic compounds. Additionally, we consider that the exhaustive characterization of cinnamoylquinic acids made in this study, resulting in the identification of an important number of new structures containing feruloyl moieties, is also another novelty that should be considered and included.

In this sense, although, the presence of (mono and di) caffeoylquinic acid have been widely described in orange varieties, the presence of feruloylquinic acids has been less documented both in orange and purple varieties of sweet potato . Additionally, in the specific case of purple varieties, the characterization of phenolic compounds have been focused on anthocyanins, and few studies have characterized both anthocyanins and cinamoylquinic acid simultaneously. In this purple variety we have identified and quantified new cinnamoylquinic acids containing feruloyl moieties, rarely or not previously identified in Ipomoea. Among them, 3 isomers of FQAs, and 7 isomers of caffeoyl-O-feruloylquinic acid which were rarely documented in Ipomoea, accounted for a relevant amount of the total cinnamoylquinic acid content in this variety.

This has been now clarified in the introduction and objective (L01-110), and the findings related with the identification of these new structures emphasized in the discussion (in L238-243; L289-294 and L325-335, for mono, di and tri CQAs respectively), in the conclusion (L535-543) and abstract (L20-22). Additionally, the fact that the presence of feruloyl moieties (as FQA) confer more stability than others during the process of spray drying is also an interesting results that provides novelty to our results (see L548-549).

What kinds of compounds the phenolics degraded into during the spray drying process? I think this is more interesting than just reporting the confirmation of the old reports.

The information concerning the effect of spray drying has been improved throughout the manuscript. In the revised version the title has been changed (L1-2) and the objective rewritten (L101-110). In the particular case of the effect of spray drying the different phenolic groups, both sections have been farther discussed (L354-376 for cinnamoylquinic acids and L449-481 for anthocyanins), and also it is indicated in conclusion (L544-554).

Likewise, in order to clarify and highlight the findings related to the degradation of compounds during the spray drying, some information related with the quantification of the polyphenolic compounds has been moved and discussed in other sections (L380-385, L426-428)