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Determination of phenolic compounds in human saliva after oral administration of red wine by high performance liquid chromatography

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ABSTRACT

Red wine is a relevant source of bioactive compounds, which contribute to its antioxidant activity and other beneficial advantages for human health. However, the bioavailability of phenols in humans is not well understood, and the inter-individual variability in the production of phenolic compounds has not been comprehensively assessed to date. The present work describes a new method for the extraction and analysis of phenolic compounds including gallic acid (Gal), vanillic acid (Van), caffeic acid (Caf), syringic acid (Sir); (−)-epicatechin (Epi); p-coumaric acid (Cum) and resveratrol (Rsv) in human saliva samples. The target analytes were extracted using Fabric Phase Sorptive Extraction (FPSE), and subsequently analysed by high-performance liquid chromatography (HPLC) coupled with photodiode array detector (PDA). Chromatographic separation was achieved using a Symmetry C18 RP column in gradient elution mode, with methanol and phosphate buffer as the mobile phases. The linearity (intercept, slope, and determination coefficient) was evaluated in the range from 1 to 50 μg/mL. The limit of quantification (LOQ) was 1 μg/mL (LLOQ ≥0.8 μg/mL), whereas limit of detection was 0.25 μg/mL. The intra and inter–day RSD% and BIAS% values were less than ± 15%. The analytical performances were further tested on human saliva collected from healthy volunteers after administering red wine. To the best of our knowledge, this is the first FPSE procedure for the analysis of phenols in saliva, using a non-invasive and easy to perform sample collection protocol. The proposed fast and inexpensive approach can be deployed as a reliable tool to study other biological matrices to proliferate understanding of these compounds distribution in human body.

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1. Introduction

Phenols are secondary metabolites widely distributed in the plant kingdom and plant-related substances, such as fruits, cereals, olive oil, and wine. Due to their beneficial properties on human health, they have attracted significant attention of the International Scientific Community in the last decades [1]. Consumption of foods and beverages containing phenolic compounds has been associated to several beneficial effects such as antioxidant activity, pressure reduction, antidiabetic activity, antithrombotic capacity (inhibition of lipoxygenase and platelet aggregation), anti-mutagenic properties (inhibition of squamous cells growth of many carcinomas), anti-inflammatory activity (prevention of leukocytes migration, histamine release and biosynthesis of prostaglandins) [2]. The food and agricultural industries produce significant amount of phenolic-rich by-products, which could be an important source of antioxidant compounds of natural origin. Wine, mostly red wine, represents a rich dietary source of phenols, which has been shown to be responsible for health benefits. Chemically, phenols are characterised by at least two phenyl rings and one or more hydroxyl groups as substituents. This shows the existence of a heterogeneous multitude of subclasses depending on substituents and/or the linker between benzene rings, and can be divided in two groups, flavonoids, and non-flavonoids. The common structure of flavonoids presents two phenolic rings (ring A and ring B) and one heterocyclic ring (ring C). Based on the different hydroxylation and oxidation state of the central ring, flavonoids can be classified into flavanols, anthocyanidins, anthocyanins, isoflavones,

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flavones, flavonols, flavanones and flavanonols. Non-flavonoids compounds include phenolic acid, stilbens, and lignans [3–5]. The general structure has been reported in Supplementary material Section S.1. In wine, primarily in red wine, most phenolic compounds are low molecular weight compounds possessing molar mass less than 3000 Da [1].

The health benefits of red wine (which presents about ten times the phenolic compounds of white wine) is also related to the synergic effect of the complex set of phenolic compounds and not only to the single classes, although flavonoids constitute the 85% of total red wine content [5]. Despite their powerful biological activities against atherosclerosis, cancer and inflammatory diseases demonstrated in vitro, there is considerable doubt whether the constituents present in red wine and other dietary components are effective in vivo. A large gap about bioavailability information is still present, and the right amount linked with valuable effects is yet to be understood. Some studies have highlighted that the molecules responsible for biological effects are probably the metabolites of flavonoids (mainly glucuronidated, sulphonated and methylated), which are the most present in the blood stream [6,7]. Indeed, after the consumption of red wine, its bioactive compounds must pass through different districts, including oral cavity, and gastrointestinal tract before exerting their effects. The oral cavity represents the first contact point between red wine bioactive components and the human body, and the interaction of these compounds with salivary proteins (SP) and oral microbiota could exercise a significant modification in their bioavailability. In Supplementary material Section S.2, the physicochemical characteristics and the chemical structure of gallic acid (Gal), vanillic acid (Van), caffeic acid (Caf), syringic acid (Sir); (−)-epicatechin (Epi); p-coumaric acid (Cum) and resveratrol (Rsv) were reported. These compounds have been chosen due to their relatively high content in red wine and their well-known biological activities.

Phenols are very heterogeneous compounds from the point of view of composition as well as their chemical structure. Discrimination of phenols is not an easy task and several methods are described in the literature [5]. Considering the selectivity and sensitivity required, sample preparation techniques are often necessary to pre-concentrate these target analytes. The most common extraction techniques used are liquid-liquid extraction (LLE), solid-phase extraction (SPE) and solid-phase microextraction (SPME), while the subsequent analysis are usually performed using HPLC-DAD, LC-MS/MS or GC-MS/MS [8]. However, the low selectivity associated with these traditional extraction techniques often involves the extraction of many matrix components, which could interfere with the subsequent analysis. In addition, the pretreatment steps are required and most of the analytical errors could be attributed to these steps; therefore, an ideal sample preparation technique should ensure that treatments on the original samples are reduced to a minimum.

On the basis of the foregoing, in this study an HPLC–PDA method was reported for the determination of gallic acid (Gal), vanillic acid (Van), caffeic acid (Caf), syringic acid (Sir); (−)-epicatechin (Epi); p–coumaric acid (Cum) and resveratrol (Rsv) in human saliva samples and the application of the validated method in real saliva samples. Thanks to an innovative extractive procedure, fabric phase sorptive extraction (FPSE), developed by Kabir and Furton [9], the sample preparation workflow, even in the case of saliva samples, have been substantially simplified, avoiding time-consuming preliminary steps. The advantages of this technique have already been demonstrated in many articles concerning the analysis of drugs in biological fluids [10–12] and environmental matrices [13–16], and other application fields, including food products [17–20]. This technique has substantially simplified the sample preparation, leading to a clean and interference-free sample that can be analyzed by chromatographic methods, reducing the consumption of hazardous and toxic organic solvents, and avoiding matrix modification [21].

In accordance with our previous investigations, which confirmed the advantages of this technique [10–12], the FPSE has been further applied here in human saliva sample, collected from healthy volunteers after consuming red wine. The procedure (Fig. 1) avoided the use of specific device to collect saliva, making the sampling step easy to perform. Moreover, due to the structural complexity and low molecular weight of these compounds, not many articles have been reported in the literature regarding their determination in human saliva [22]. In this work, human saliva was used as a matrix for quantitative analysis of these compounds, with the purpose to use a non–invasive and simple sampling procedure. The overall protocol avoided time-consuming sample preparation steps that are often needed prior to use of these analytical methods to reduce interferences related to the sample matrix. In addition, these methods may require the use of costly consumables, materials, and chemicals.

Furthermore, the availability of an extraction technique applicable to saliva for the determination of natural compounds opens the way to the possible development of new devices for the non-invasive sampling of natural molecules present in many illicit drugs and, consequently, to the possible applications in the pharmaco-toxicological and forensic fields.

2. Materials and methods

2.1. Chemicals, solvents, and devices

Reference standards of gallic acid (Gal), vanillic acid (Van), caffeic acid (Caf), syringic acid (Sir); (−)-epicatechin (Epi), p–coumaric acid (Cum), resveratrol (RSV) and sodium phosphate dibasic, sodium phosphate monobasic (>99% purity grade) and orthophosphoric acid were purchased from Sigma–Aldrich (Milan, Italy). Acetaminophen (IS) was obtained from Haoyuan Chemexpress Co. Ltd. (Shanghai, China). Acetonitrile and methanol (both HPLC–grade) were purchased from Honeywell (New Jersey, USA) and were used without further purification. Deionized water (18.2 MΩ-cm at 25 °C) was generated by a Millipore MilliQ Plus water (Millipore Bedford Corp., Bedford, MA, USA). The International Forensic Research Institute, Department of Chemistry and Biochemistry, Florida International University (Miami, FL, USA) provided all FPSE membranes tested in the present study (see Section 2.5).

2.2. Stock solution, calibration curves and quality control samples

Stock solutions of chemical standards were prepared in methanol (MeOH) at the concentration of 1 mg/mL and stored at −20 °C. Stock solution of the seven phenols and IS was made in methanol at the same concentration. The working solutions were prepared by dilution of a mixture stock solutions in methanol. All solutions were kept at 4 °C until analysis. The matrix-matched calibration curves were obtained using the blank saliva sample spiked with the working solutions in the concentration range 1–50 µg/mL. The analysis was replicated 6 times for each concentration. The quality control samples (QCs) used for the intra and inter-day precision and trueness evaluation were prepared in the blank matrix sample at three concentration levels of 2.5 (QC low), 15 (QC intermediate) and 40 (QC high) µg/mL and replicated for 6 times.

2.3. Human saliva samples collection and storage

Human saliva samples were collected from healthy volunteers, previously informed about the nature of the study. All the participants had no clinical condition that could potentially interfere with the analyses. Whole saliva samples (about 2.0 mL) were collected by spitting saliva into a graded tube at 15 time points: just before

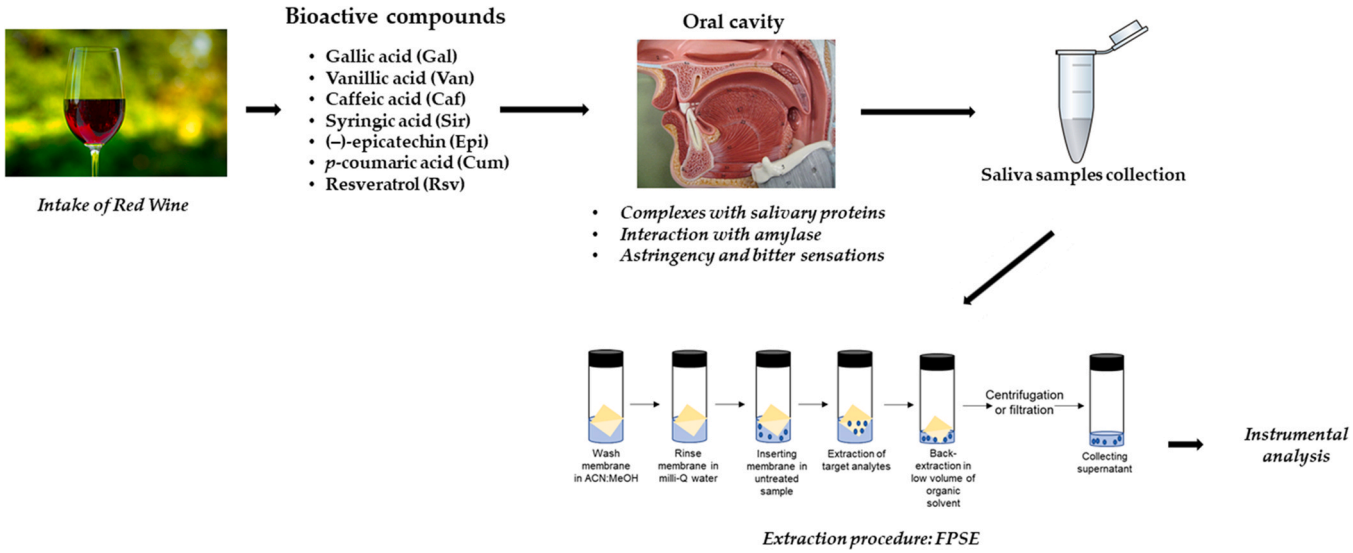

Fig. 1. Schematic presentation of the analytical procedure.

(baseline) and at 0, 1, 5, 10, 15, 20, 30, 60, 75, 90, 120, 180, 240 and 300 min after ingestion of 150 mL (single dose) of red wine (San Clemente, Montepulciano d'Abruzzo, Riserve, 2013, 14.5% vol. and Càstano, Merlot, 2019, 11% vol.). The samples preparation (for calibration and quality control) provides the following volumes: 450 µL of blank saliva, 25 µL of IS (50 µg/mL), and 25 µL of analytes working solution with increasing concentration. For the real sample analysis, the samples provide the following volumes: 475 µL of saliva sample and 25 µL of IS. In all cases, as indicated by the CDER guidelines [23], in the production of calibration and quality control (QC) samples, the entity of the solvent spike containing the analytes and internal standard does not exceed 15% in order not to significantly modify the biological matrix before proceeding to the FPSE procedure. In fact, the used volumes are at most 10% of the final volume of fortified sample. All samples were stored at −20 °C until further analysis.

2.4. Apparatus and chromatographic conditions

The chromatographic separation was carried out using Waters 600 HPLC system connected with Waters 2996 photodiode array detector (PDA). Mobile phases have been directly on–line degassed using Biotech 4CH DEGASI Compact (Onsala, Sweden). Symmetry C_{18} RP column (75 × 4.6 mm, 3.5 µm) was used to resolve the phenols and acetaminophen (IS). The column was thermostated at 26 °C (± 1 °C) using a Jetstream2 Plus column oven during the analysis. The chromatographic separation was conducted in gradient elution (Supplementary material Section S.3) using phosphate buffer (30 mM, pH=3) as solvent A and MeOH as solvent B. The flow rate was set at 1 mL/min. The injection volume was 5 µL. All the compounds were quantified at their maximum wavelengths, as reported in Supplementary material Section S.4 The run time was 30 min. Empower and GraphPad Prism v.4 software were used for data collection and elaboration.

2.5. FPSE membrane selection and preparation

Considering phenols' LogP (range from 0.70 for gallic acid to 3.10 for resveratrol) and pKa (range from 3.64 for caffeic acid to 9.00 for (−)–epicatechin) into consideration, the lipophilicity and acid–base properties were defined, helping to choose the best suitable FPSE membrane for the extraction process. Due to this broad polarity dispersion characteristics of the phenols, a logical selection would favour polar or medium polar FPSE sorbent to ensure a fast and uniform adsorption/desorption process for all the analytes. Another selection criterion should be the biocompatibility of FPSE device with the biological matrix. For this purpose, six polar and medium polar FPSE sorbents, synthetized following a previously reported procedure [24], were tested. The shortlisted FPSE sorbents tested were sol–gel polytetrahydrofuran (sol–gel PTHF, medium polar); sol–gel polyethylene glycol–polypropylene glycol–polyethylene glycol (sol-gel PEG–PPG–PEG, medium polar); sol–gel Carbowax® 20 M (sol–gel CW 20 M, polar); sol–gel octadecyl silane (sol–gel C18, medium polar); sol–gel polypropylene glycol–polyethylene glycol–polypropylene glycol (sol–gel PPG–PEG–PPG, medium polar); sol–gel polycaprolactone–polydimethylsiloxane–polycaprolactone (sol–gel PCAP–PDMS–PCAP, medium polar). The extraction procedures included different steps: i) cutting the membranes into circular disks (1 cm of diameter); ii) cleaning the membrane in a mixture of MeOH and ACN; iii) rinsing the membrane into milliQ water; iv) extraction of 100 µL of sample for 5 min; v) back-–extraction in 150 µL of MeOH for 5 min; vi) centrifugation and HPLC–PDA analysis by injecting 5 µL of sample.

2.6. Analytical method validation

The developed method was validated according to the International Guidelines for Bioanalytical Method Validation [23,25] with respect to selectivity, calibration curve, Limit of Quantification (LOQ), Limit of Detection (LOD), intra and inter-day precision and trueness.

3. Results and discussion

3.1. Selection of FPSE membrane chemistry and FPSE optimization

Monitoring the presence of compounds of interest in biological matrices requires an extensive sample preparation process to remove impurities that could interfere with target analytes. In the last decades, innovative micro(extraction) procedures have been introduced, also to minimize the use of toxic organic solvent consumption, in accordance with the principles of the Green Analytical Chemistry (GAC) [26]. In 2014, Kabir and Furton have developed a new sample preparation technique [9], that combines two mostly used traditional methods: solid–phase extraction (SPE) and solid–phase microextraction (SPME), eliminating the major limitations of traditional extraction techniques. The high selectivity of FPSE is due to three distinct

sources: the flexible fabric substrate (that can be hydrophilic or hydrophobic); the sol–gel precursor (generally methyl trimethoxysilane) that connects the fabric substrate with the organic/inorganic polymer/ligand and provides hydrogen bonding, dipole-dipole interaction and London dispersion type of interaction during the extraction; and the organic/inorganic polymer/ligand, that allows the fast adsorption/desorption of the analytes (Supplementary material Section S.5). The FPSE membrane synthesis steps foresee that the support (cellulose fabric) after having been previously cleaned and activated is subsequently immersed in a reaction bottle where the sol solution has been prepared. In this way, a 3D network of the sorbent is formed both on the surface of the support and in the porous cavities. After the reaction time (approx. 4 h), the coating process was completed [24].

Subsequently, the FPSE membranes were cut into round pieces by a puncher (internal diameter of 0.6 or 1 cm), allowing to get extraction devices with an identical surface area (device standardization). After that, the sol–gel sorbent coated FPSE membranes were cleaned and activated by immersing into 2 mL of ACN: MeOH (50:50, v:v) for 5 min, followed by washing for 2/3 times in 2 mL of MilliQ water, as general preliminary procedure [11], before further FPSE procedure optimization following the one-variable-at-time (OVAT) method. Before carrying out the optimization of each parameter of FPSE procedure in matrix, an injection of the standard mix (analytes and IS) was analysed to obtain a reference chromatogram. A standard solution at 20 µg/mL was used for the optimization process. The preliminary conditions tested are: i) 100 µL of sample, ii) extraction for 5 min, iii) MeOH as back extraction solvent, iv) 150 µL of back extraction solvent, and v) 5 min of back extraction time.

Six different FPSE membranes were evaluated: sol–gel CW 20 M (polar), sol–gel PTHF (medium polar), sol–gel PEG–PPG–PEG (medium polar), sol–gel C18 (medium polar), sol–gel PPG–PEG–PPG (medium polar) and sol–gel PCAP–PDMS–PCAP (medium polar). Two different diameters were tested, as membrane size: 0.6 cm (surface area of 0.2826 cm^2) and 1 cm (surface area of 0.785 cm^2). In these preliminary experiments, the best three FPSE membranes were sol–gel CW 20 M, sol–gel PTHF, sol–gel PEG–PPG–PEG. After further optimizations, sol–gel CW 20 M (1 cm of diameter) showed the best extraction sensitivity, as shown in Table 1. The enrichment factors were calculated as the percentage of peak area enhancement with respect to the area of reference standard solutions.

The preliminary conditions were subsequently tested to these back–extraction solvent volumes: 150 µL, 200 µL, 300 µL, 400 µL and 500 µL. Back extraction time was also optimized, testing 5 min, 10 min, 15 min and 20 min. The procedure was also tested with different sample volumes: 100 µL, 200 µL, 500 µL and 1000 µL. Moreover, the best extraction time was optimized keeping the sample under stirring (using roller DLAB MX-T6-S) for 5 min, 10 min, 15 min, 20 min, 30 min and 60 min. By plotting the area values of the chromatographic peaks of each analyte as a function of the extraction volumes and time, the optimal extraction was achieved with 100 µL of sample for 5 min. All the graphs related to the FPSE procedure optimization are shown in Supplementary material Section S.6. Generally, the pH of the solvent is also an important factor in the extraction process. In the present work, organic solvents as such (MeOH and ACN), a combination of them (MeOH: ACN, 50:50, v:v), but also a mixture of MeOH and phosphate buffer at pH 3 were evaluated as the back extraction solvent (5:95, v:v). From the obtained results, it can be observed that MeOH was found to be the best back-extraction solvent and that the presence of the buffer at pH 3 reduced the analytes recovery efficiency from the FPSE membrane, particularly for coumaric acid and resveratrol. The resulting final procedure that allowed the best analytes extraction, using the lowest amounts of solvent and sample was: (i) cut the FPSE sol–gel CW 20 M membrane into round disks of 1 cm diameter; (ii) activation in 2 mL of MeOH: ACN (50:50, v:v) for 5 min; (iii) rinsing in 2 mL of MilliQ water for 2/3 times; (iv) extraction of 100 µL of sample for 5 min; (v) back-extraction in 150 µL of MeOH for 5 min; (vi) centrifugation at 12,000 rpm for 5 min; (vii) withdrawal of 80/100 µL of supernatant and (viii) injection of 5 µL into HPLC system. The selected optimal conditions using standard solutions were further tested on biological samples (human saliva), which confirmed the previous obtained data.

3.2. Optimization of chromatographic separation

The main goal of the chromatographic separation was to achieve a good peak resolution in a relatively shorter time. To accomplish this, different parameters should be tested: column chemistry, mobile phases, elution mode, and temperature. Analysing polarity and LogP of each phenolic standard, Symmetry C_{18} RP (75 × 4.6 mm, 3.5 µm) column was tested. Mobile phase composition was subsequently optimized, starting with an isocratic elution, using MilliQ water and MeOH in different percentages (50:50; 40:60; 30:70; 20:80; 60:40; 70:30, v:v). Subsequently, first testing the retention time of resveratrol (the most lipophilic compound) and gallic acid (the most hydrophilic compound), different gradient elution methods were evaluated to obtain a better chromatographic resolution. The gradient was further optimized, previously acidifying the aqueous phase and then both phases with 0.5%, 2%, 3% and 5% of acetic acid. To optimize the chromatographic resolution and above all to maximize the stability and reproducibility of the separative system, the use of a phosphate buffer at different pH and ion strength was also evaluated. Following these tests, it was decided to use a phosphate buffer, acidified with orthophosphoric acid (30 mM,

Table 1
Enrichment factors (%) for sol–gel CW 20 M, sol–gel PTHF, sol–gel PEG–PPG–PEG achieved in a) MeOH, b) ACN, c) MeOH: ACN (50:50) and d) PBS: MeOH (95:5).

a)		PTHF	PEG-PPG-PEG	CW20
	GAL	12.32	15.19	21.38
	IS	11.72	15.41	19.22
	VAN	10.35	13.24	16.94
	CAF	13.11	19.17	15.49
	SIR	12.28	15.44	19.12
	EPI	12.65	16.55	22.46
	CUM	12.14	15.97	16.62
	RSV	12.71	16.37	21.05

b)		PTHF	PEG-PPG-PEG	CW20
	GAL	2.58	1.70	3.36
	IS	14.08	12.25	15.27
	VAN	11.40	9.38	15.47
	CAF	11.11	4.72	15.58
	SIR	11.57	12.14	14.38
	EPI	9.52	10.79	14.41
	CUM	13.41	14.13	16.39
	RSV	13.12	14.34	17.38

c)		PTHF	PEG-PPG-PEG	CW20
	GAL	14.54	13.10	18.36
	IS	15.71	14.04	18.47
	VAN	15.41	12.42	17.32
	CAF	17.10	15.97	20.53
	SIR	14.29	12.60	16.60
	EPI	16.05	16.42	20.33
	CUM	15.41	13.77	17.72
	RSV	15.02	12.69	16.57

d)		PTHF	PEG-PPG-PEG	CW20
	GAL	19.55	15.74	19.32
	IS	11.66	11.44	18.03
	VAN	9.38	7.81	11.60
	CAF	13.67	11.55	16.56
	SIR	9.30	8.36	12.38
	EPI	12.12	11.62	16.31
	CUM	8.69	7.37	10.04
	RSV	2.84	1.99	2.64

pH=3) as solvent A and MeOH as solvent B. While testing these conditions, three different sample volumes were injected (5, 10 and 20 µL), preferring to use 5 µL, because with higher volumes there was the fronting phenomenon. Flow rate was also optimized (from 0.7 mL/min to 1.2 mL/min), trying to reduce the total run time. Best separation conditions for the phenolic compounds and the Internal Standard were achieved with Symmetry C_{18} RP (75 × 4.6 mm, 3.5 µm), using phosphate buffer (30 mM, pH=3) as solvent A and MeOH as solvent B in gradient elution as mobile phases, flow rate 1 mL/min, and injection volume 5 µL. When optimizing the separation process, temperature plays an important role. For this reason, three temperature values were tested starting from 30 °C (temperature used in [27] for the resolution of 22 phenolic compounds in matrices of natural origin on stationary phase C18), 26 °C and 34 °C. The best performances were observed at 26 °C (± 1 °C) and this value was maintained in the method validation process. The analytes were eluted within 23 min in the following order: gallic acid, IS, vanillic acid, caffeic acid, syringic acid, epicatechin, coumaric acid, and resveratrol (Supplementary material Section S.7). Retention times and maximum wavelength for all analytes (without IS) are collected in Supplementary material Section S4.

3.3. FPSE-HPLC-PDA method validation

The method validation was carried out according to the International Guidelines for Bioanalytical Method Validation, with respect to selectivity, linearity, precision, and trueness (both intra and interday). The whole validation protocol was performed in blank spiked matrix with analytes and internal standard accordingly to the procedure in the paragraphs 2.2 and 2.3.

The linearity (intercept, slope, determination coefficient) was evaluated in the range from 1 to 50 µg/mL, by plotting the analyte/IS ratio area on the ordinate (y-axis) and the concentration of each standard solution on the abscissas (x-axis). The curves showed a linear correlation in the tested range and the determination coefficients $r^2 \geq 0.9805$. The curves were plotted using a weighting factor of $1/x^2$. All the data regarding the method validation are reported in Supplementary materials S.8, S.9, S.10, and S.11.

The LOD and LOQ values were validated on the basis of what is reported by the International Guidelines [23,25] and in particular for the LODs a signal/noise ratio (S/N) equal to 3 was evaluated, while for the values of LOQ an S/N ratio of 10, as well as having precision and trueness values at this level within ± 20%. Based on these criteria, the limit of quantification (LOQ) was 1 µg/mL for each analyte in saliva (LLOQ 0.8 µg/mL) whereas limit of determination (LOD) was 0.25 µg/mL.

The values of intra and inter–day RDS% and BIAS% were less than ± 15%, according to current guidelines.

For selectivity, as indicated by the Guidelines [23], the present method was tested and applied to six blank matrices of saliva coming from as many different donors. The absence of interfering signals was observed for each analyte (at the respective maximum wavelengths used for quantitative analysis) and for each white matrix, even at the LLOQ.

Recovery was already evaluated by the validation of the trueness (both intra and inter-day). No significant decrease of analytes concentrations or changes in the chromatographic profiles were observed under the specified conditions (−20 °C) during the analysis period.

3.4. Comparison with existing methods published in the literature

As already described above, discrimination and identification of phenols are not easy procedures, due to their structural diversity. In Table 2 have been reported different analytical methods for the analysis of phenolic compounds, comparing the used human and/or animal biological fluids, pre–treatment procedure/extraction technique, retention times and linearity range. An overview of the works reported in the literature showed that there is not a single method able to simultaneously analyse these compounds in human saliva sample; moreover, these compounds are often evaluated using hyphenated and sophisticated instrumentation not available in all laboratories (the most present components in red wine are characterized only by UHPLC–ESI–MS/MS). Furthermore, human saliva was not considered as biological fluids, despite oral cavity represents the first contact between compounds and human body. To probe clinical investigations, a suitable and representative biological fluid from the body must be analysed. Human saliva fits many of the criteria for this quantitative analysis for many reasons. Oral exposure of compounds passes through the mouth before being transferred into the rest of the body. In addition, sampling of human saliva is one of the simplest and least invasive routes for biomonitoring compared with the fluids collection such as blood and urine, among others.

The validated method herein reported shows as a "limiting" element the fact that it provides a gradient elution of the analytes. This element implies that, if the method is transferred to other instrumentation with different dead volumes from those present on the instrument in our laboratory, it may involve the need for small changes in the elution profile (in order to maintain the same chromatographic resolution and avoid peaks overlapping) with the consequent need to partially revalidate the method before being able to apply it.

3.5. Application to real saliva samples and analysis

The new FPSE-HPLC-PDA method was applied to human saliva samples collected from four adult and healthy volunteers, ranging from 25 to 41 years of age (Supplementary materials **S.12**). All volunteers were informed about the study, and they signed a letter of consent before their enrolment. None of the participants was following any pharmacological treatments or taking dietary supplements. The volunteers were required to follow some conditions the days just before the experiments in order to standardize the sampling procedure: i) avoid drinking alcoholic beverages; ii) avoid consuming phenol-rich foods or beverages at least twelve hours (washout time) before saliva collection; iii) avoid brushing teeth using toothpaste before saliva collection; iv) not consume food and drinks during samples collection. Volunteers came to the laboratory at 8.00 am and, after consuming a light breakfast (40 g of whole bread and 125 mL of milk), they drunk 150 mL (single dose) of red wine (San Clemente, Montepulciano d'Abruzzo, Riserve, 2013, 14.5% vol. and Càstano, Merlot, 2019, 11% vol.). The saliva collection started just before (baseline) the consumption of the wine single dose, and at time 0, 1, 5, 10, 15, 20, 30, 60, 75, 90, 120, 180, 240 and 300 min. After collection, the samples were extracted using optimized FPSE protocol and 5 µL of supernatant were analysed in HPLC system. Before starting the study, wine samples (after centrifugation at 14,000 rpm for 10 min) were analysed, to verify the presence of phenols quantitatively and qualitatively (Supplementary materials S.13), in order to evaluate the dose. Data provided quantities in µg of gallic acid, coumaric acid, epicatechin and resveratrol (Supplementary materials S.14). The data obtained from human saliva samples were shown in Fig. 2 (in the figure were considered merely the values ≥LOD).

The results were compared for both the wines, claiming that the highest concentration of all the analytes was obtained at time 1 min. The quantitative data support the validity of the herein reported FPSE-HPLC-PDA method to simultaneously monitoring the phenolics of red wine in human saliva.

Table 2
Various analytical methods reported in the literature for the analysis of phenolic compounds in different biological and natural matrices.

Sample/Matrices	Analytes	Extraction procedure	Instrument configuration	Retention time (min)	Linearity Range	Reference
Human urine and plasma	Gallic acid	SPE	UHPLC–ESI–QqQ–MS/MS	1.48	–	[7]
	Catechin			3.35		
	Epicatechin			3.65		
	Galloyl glucose			2.22		
	Quercetin rhamnoside			4.41		
	Quercetin-3-O-glucoside			4.17		
	Quercetin-3-O-glucuronide			4.13		
	Myricetin hexoside			3.84		
	Syringetin hexoside			4.45		
	Quercetin rutinoside			4.00		
	Procyanidin dimer B-type			3.04		
	Procyanidin dimer B-type			3.20		
	Procyanidin B2			3.42		
	Procyanidin dimer gallate B-type			3.73		
	Procyanidin trimer B-type			3.62		
	Procyanidin trimer B-type			3.26		
	Procyanidin trimer B-type			2.07		
	Cyanidin-3-O-glucoside			4.50		
	Delphinidin-3-O-glucoside			3.24		
	Petunidin-3-O-glucoside			3.46		
	Malvidin-3-O-glucoside			3.67		
	Malvidin-3-O-acetylglucoside			4.20		
	Petunidin-3-p-coumaroylglucoside			4.44		
	Malvidin-3-p-coumaroylglucoside			4.68		
	Malvidin-diglucoside			4.39		
Rat plasma	Gallic acid	LLE	UPLC–MS/MS	3.91	5.135–1027 ng/mL	[28]
	p-hydroxybenzoic acid			4.70	4.108–822 ng/mL	
	Syringic acid			4.86	8.07–1614 ng/mL	
	Gentisic acid			4.94	2.014–402.8 ng/mL	
	Ethyl gallatep-coumaric acid			5.33	4.016–803 ng/mL	
	Ferulic acid			5.39	10.07–2014 ng/mL	
	Salicylic acid			5.54	2.006–401.2 ng/mL	
				6.62	4.004–801 ng/mL	
Whole blood	Quercetin and Resveratrol	LLE	HPLC–UV	–	0.15–25 μM	[29]
Human plasma Human urine	67 (poly)phenol metabolites	μ–SPE	UHPLC Q–TOF MS	–	0.04–86 nM 0.01–136 nM	[30]
Rat plasma	Syringic acid	LLE	UHPLC–ESI–MS/MS	–	1.050–1050 ng/mL	[31]
	Ferulic acid				0.8320–832.0 ng/mL	
	Caffeic acid				0.8800–880.0 ng/mL	
	Vanillic acid				0.3264–326.4 ng/mL	
	p–coumaric acid				0.8440–844.0 ng/mL	
	3,4–dihydroxybenzoic acid				0.8080–808.0 ng/mL	
	4–hydroxybenzoic acid				0.8560–856.0 ng/mL	
Human plasmaHuman urine	Cyanidin–3–O–glucoside	SPE	UPLC–ESI–MS/MS	–	0.00018–4.18 μM	[32]
	Malvidin–3–O–glucoside				0.005–41.8 μM	
	p–hydroxybenzoic acid					
	Gallic acid					
	Protocatechuic acid					
	Caffeic acid					
	p–coumaric acid					
	Ferulic acid					
	Syringic acid					
	Catechin					
	Epicatechin					
	Resveratrol					
Rat plasma	Gallic acid	LLE	UHPLC–ESI–MS/MS	6.50	0.03–3.00 μg/mL	[33]
	Protocatechuic acid			8.64	01–1.00 μg/mL	
Human urine	Urinary metabolities	Centrifugation	UHPLC–TOF–MS	–	–	[34]
Human saliva	Gallic acid	FPSE	HPLC–DAD	2.94	1–50 μg/mL	Current study
	Vanillic acid			15.97		
	Caffeic acid			18.18		
	Syringic acid			20.61		
	(-)-epicatechin			21.39		
	p-coumaric acid			21.68		
	Resveratrol			22.29		

3.6. Green analytical procedure index (GAPI)

Nowadays, analytical laboratories try to operate in environmentally friendly conditions to avoid pollutants in water, soil, etc. On the other hand, many solvents and reagents are required in the extraction procedures and sample analysis. The great challenge is thus to reach the best compromise between analytical results and operation in a healthy and safe environmental conditions, following the rules of so-called Green Analytical Chemistry (GAC). To better understand the "greenness" of analytical procedure, in 2018 Płotka-Wasylka [35] has introduced a new tool, called Green Analytical Procedure Index, or GAPI.

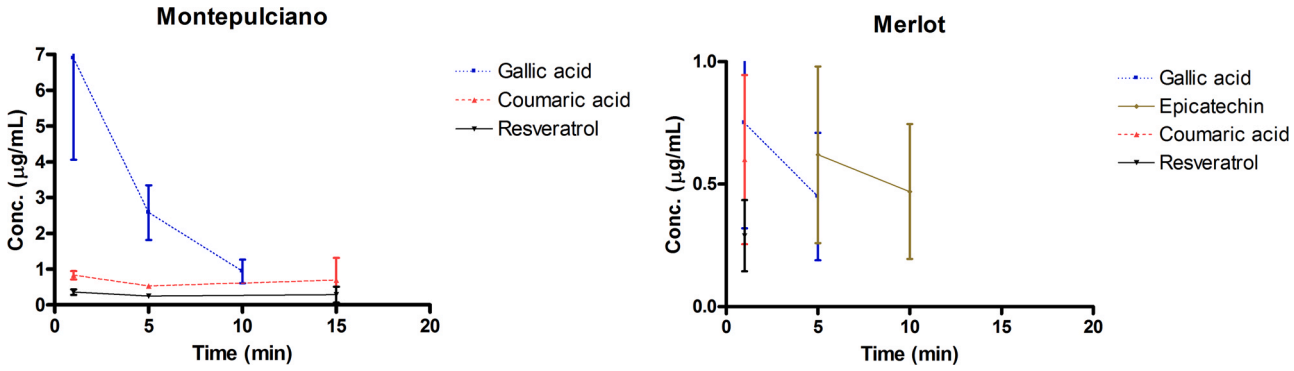

Fig. 2. Quantitative data obtained in saliva real samples analysis: San Clemente, Montepulciano d'Abruzzo, Riserve, 2013, 14.5% vol. (left) and Càstano, Merlot, 2019, 11% vol. (right).

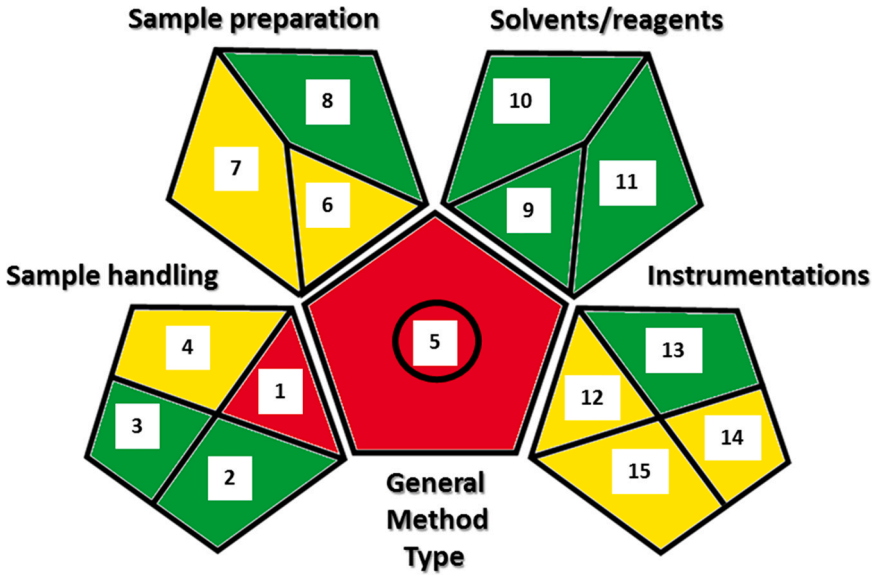

Fig. 3. GAPI pictogram for the reported innovative procedure.

This innovative tool allows researchers to make the own evaluation of the entire analytical methodology, from sample collection to instrumental determination, including solvents and reagents used. GAPI tools included different pentagrams, related to sample handling, sample preparation, solvents/reagents, and instrumentation, that were used to evaluate the environmental impact of the procedure using different colours, from green (low environmental impact), through yellow (medium environmental impact), to red (high environmental impact). Fig. 3 shows the pictogram related to the reported method, built according to all the parameters included in the Green Analytical Procedure Index (see Supplementary Material S.15).

4. Conclusions

The reported study aimed at expanding the knowledge on the fate of phenolic compounds contained in wine, including data in human saliva. The study confirmed the innovation and applicability of fabric phase sorptive extraction on biological samples, allowing to reduce costs, time, and waste. At the end, in addition to confirming FPSE advantages, for the first time we developed a new multi-analytes FPSE-HPLC-PDA method to research more phenolic compounds of wine simultaneously by a non-invasive sampling. This method appeared to be simple, rapid, cheap, easy to reproduce, sensible, and avoiding pre-treatment steps. The new strategy can be easily adopted for the analysis of numerous chemical compounds in oral fluids for clinical, pharmaceutical, toxicological, and forensic applications. The current study demonstrates that low-end laboratory instrument such as HPLC-PDA can easily provide comparable analytical data typically obtained from expensive instrument such as LC-MS/MS that often require trained personnel, high maintenance costs and a deep knowledge of analytical problems, imposing a challenging burden to the analytical/bioanalytical laboratories. In the future, the method should be applied to studies in others biological matrices (plasma, urine, whole blood), to better understand the bioavailability of phenolic compounds.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2021.114486.

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