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Liquid chromatographic method for extracellular Guanosine 5′-triphosphate and tetrahydrobiopterin pathway products analysis from cadaveric samples and human biofluids

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ABSTRACT

To gain a deep insight and to obtain a superior understanding about guanosine-based pathway, this paper reports an innovative approach to study this critical subject. Firstly, after an exhaustive analysis of literature with a focus in legal medicine and extracellular vesicles, it was understood that a new method is inevitable to follow, determine, and quantify these analytes (Guanosine monophosphate - GMP, guanosine diphosphate - GDP, guanosine triphosphate - GTP, Guanosine, Neopterin, and tetrahydrobiopterin - BH4).

Starting from a previously method, we implemented and validated a new HPLC-DAD method in gradient elution mode with these six target analytes fully resolved in 18 min. The HPLC-DAD method uses a stationary phase XTIMATE C18 (4.6 mm × 250 mm, 5 µm, Welch, Shanghai, China) and mobile phase's phosphate buffer (40 mM, pH 7) (A) and Acetonitrile (B). Good correlation goes from 0.05 to 10 µg/mL with a limit of detection equal to 0.02 µg/mL and a limit of quantification equal to 0.05 µg/mL ($R^2 \geq 0.9824$).

Method was tested on human extracellular vesicles, isolated from different human parts, like urine, saliva and muscle, giving interesting results as different quantification of analytes depending on the sample matrix used. Interesting to underline is that saliva was the poorest source of these analytes, if compared with growth medium and urine.

1. Introduction

The biology of extracellular vesicles (EVs) has redefined physiological cell system compositions and dynamics since the lipid membrane of EVs encapsulates and protects their contents as a source of physiological and pathological information. Thereby EVs are involved in the regulation of main routes of signaling [1] and can be extracted from any human biological fluid and tissue [1]. It is important to highlight that their analysis is not limited to living humans, but also to deceased organisms [2].

Starting from the evidence that several purines increased their concentration in biological fluids after physical bouts [3] and from the perspective of purines transported by EVs as a path for organs-brain crosstalk [4], our working group previously demonstrated the differential presence of purines and derivates in EVs isolated from human urine. Specifically, among adenosine and guanosine series, guanosine and to a lesser extent ATP were found, both in triathletes and physically inactive adults [5].

Within the signaling exerted by guanosine and metabolites, pterins have been interestingly defined as sensitive biomarkers of the tissue immune system status and of the impact of physical exercise intervention on inflammation [6]. It is well known that EVs can be used in clinical applications, as diagnosis and treatment in postmortem corps. For this reason, in this scenario the availability of a quantitative method could be useful in the autopsy [2]. However, their presence in EVs has not been defined, yet, also related to their important function not only

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during organogenesis but also in adult phase, acting as inductors and/or regulator in excitable tissues [7]. Purines are fundamental for all cells, specifically guanine-based purines are characterized by two fused linked rings containing five carbon and four nitrogen atoms, and their derivatives nucleotides have nitrogenous bases, ribose and phosphate and they can be mono, di or tri phosphorylated, consequently they are guanosine monophosphate (GMP), guanosine diphosphate (GDP) and guanosine triphosphate (GTP) [8]. This pathway, from GTP, continues obtaining Neopterin, as by product of tetrahydrobiopterin synthesis, and tetrahydrobiopterin (BH4), as reported in Fig. 1.

Neopterin is a sensitive marker for inflammatory response, and it has many biological effects in different inflammatory conditions. Instead, BH4 is a cofactor for some enzymatic conversions of some biomolecules, as nitric oxide, monoamine neurotransmitters, etc. BH4 cannot be considered only as enzymatic cofactor, it is a cytoprotective pathway [9].

During last years, many studies have reported how human cells are able to secrete exosomes, specially containing guanosine-based exosomes, and these possess an important role in different aspects, as skeletal muscle or central nervous system [4,10]. Therefore, guanosine-based exosomes can act as regulators and influencing positively the target system [4,10].

EVs are currently receiving increasing interest [1–4] in various application fields, even though at present there is no method that allows evaluating the guanosine-based molecules in the exosomal-vehiculated signaling. This is especially true both for studies in the physiological field and in the medical, legal and forensic field. With the aim of obtaining a more complete vision of Guanosine-based derivatives, we aimed to extend the field of purine signaling in humans by setting and testing the procedure of quantifying molecules of guanosine series stuffed in EVs isolated from several biological matrices. We also aimed to integrate previous evidence of purines as existing in human EVs by including neopterin and tetrahydrobiopterin within the analytical procedure. It is worth highlighting the possibility of applying this method to the analysis of forensic matrices in order to obtain more precise and reproducible information during the autopsy phase in the evaluation of the post-mortem interval (PMI). In fact, a critical element arises from the fact that today the PMI is a very complex parameter to evaluate, subject to the influences of many factors, which inevitably leads to having to provide a relatively wide range. The possibility of having reproducible analytical methods for the accurate (true and precise) quantitative analysis of specific markers that could be used in the assessment of PMI could certainly be an important factor in the future development of a more precise PMI predictive model.

2. Materials and methods

2.1. Chemicals and reagents

For HPLC analysis, tetrahydrobiopterin, Neopterin, GMP, GDP, GTP, and Guanosine were purchased from Sigma-Aldrich (St. Luis, MO, USA). Sodium phosphate monobasic anhydrous was obtained from ACROS ORGANICS (New Jersey, USA), meanwhile Sodium phosphate dibasic anhydrous from Carlo Erba Reagents (Milan, Italy). Acetonitrile (ACN) was purchased from VWR Chemicals (Pennsylvania, USA), instead Dimethyl sulfoxide (DMSO) from Honeywell (New Jersey, USA). The water was purified using Milli-Q Lab Water by Merck (Darmstadt, Germany).

2.2. Samples collection

Urine of a male healthy adult (age: 35 years) was collected in a sterile container as first morning specimens; the sample was centrifuged for 20 min at 2000×*g*, 4 °C, and stored at −80 °C; once defrosted, the sample was split in 3 mL (U3), 6 mL (U6), and 9 mL (U9) aliquots. Saliva was collected from the same participant using Salivette® collection devices

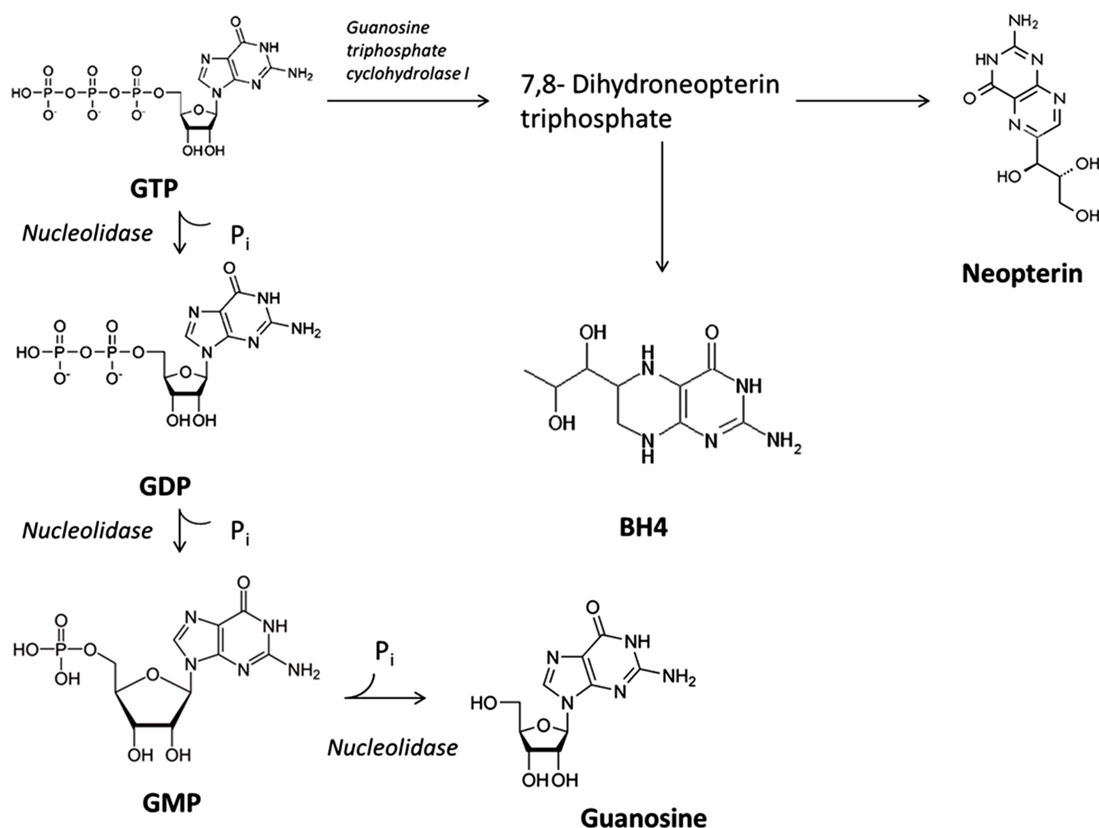

Fig. 1. A schematic and shortened representation of GTP and BH4 pathway.

(Sarstedt Nümbrecht). The sample was centrifuged for 2 min at 1000×*g* and stored at –80 °C. Once defrosted, the sample was split in 350 μL (S350) and 700 μL (S700) aliquots. One sample (CTR) remained as a null control, containing only water.

The cadaveric samples are referred to a wider project, i.e., "Satellite cells Postmortem Regeneration Ongoing and Usefulness for Thanatochronological estimation (SPROUT)", whose initial results are published elsewhere [11]. Skeletal muscle biopsies of approx. 1 cm^3 were sampled during judicial autopsies, after informed consent submission to the judicial authority, and according to the Ethic Committee approval (COET n 6065–04.03.2021). Three human cadavers, two males and one female of 40, 43 and 71 years old respectively were considered and two biopsies were obtained from each corpse. Inclusion criteria were a body mass index (BMI) between 18 and 25, known time and cause of death and age between 18 and 75 years old. Exclusion criteria were signs of recent significant muscular trauma, history of Chemo/radiotherapy in the last year, known muscular pathologies or diabetes or insulin resistance and death due to systemic infections. For each corpse, of the two biopsies analysed, one was sampled from a presomitic muscle (Thyrohyoid) and the other one from a somatic muscle (Iliopsoas muscles), through a small accessory cutaneous cut. Sampling on the Thyrohyoid muscle was carried out on the medial margin, in its point of insertion to the oblique line of the thyroid cartilage while Iliopsoas muscle sample was obtained from its lateral margin, 2 cm from its insertion on the lesser trochanter of the femur. After collecting the samples, muscles have been immediately immersed for 24 h in sterile solution containing HAM's F10 and gentamicin, then transferred in cryovials with FBS and DSMO solution and stored at −80 °C. Histopathological examination of tissue samples obtained during autopsies showed no signs of pathologies that could invalidate the value of further investigations. Frozen dissected muscle biopsies were thawed at 37 °C and washed with PBS before the treatment for explant formation. Satellite cells were isolated from muscle tissues using the explant procedure as previously described [11]. After detaching with trypsin-EDTA, the cells were counted, and the population doubling level was calculated at each passage with the following equation: log10(N/n)/ln2 with N as the number of cells at the time of the passage and n as the number of cells initially plated. At the first passage, the cell population was considered at 1 population doubling level (PDL). The proliferative state was maintained by feeding the hMPCs with a growth medium (GM) containing (% vol/vol): HAM's F10 (Euroclone), 0.1 gentamycin and 1 penicillin/streptomycin 100X (Euroclone), 20 FBS heat-inactivated (56 °C, 36 min) (Hyclone), and 1 L-Glutamax 100 × (Gibco). The growth medium was collected at three different PDL: 1 (C1), 2 (C2) and 6 (C6), whose corresponding volume was 9 mL, 4.3 mL and 8.1 mL, respectively. The samples were stored at −80 °C after collection.

2.3. EVs isolation

Frozen samples were thawed and EVs were isolated following our previous work on human EVs and purines [5], by using differential ultracentrifugation (UC) method. The first step consisted of 20 min of centrifugation at 2000×g, 4 °C. The resulting supernatant was centrifuged 30 min at 10,000×g, 4 °C. The following supernatant was then ultracentrifuged for 70 min at 100,000×*g*, 4 °C to obtain the pellet containing the small EVs remained. An additional step of ultracentrifugation for 60 min at 100,000×*g* was conducted to wash the small EVs, after resuspending in PBS 1X. Finally, the resulting pellet was resuspended in ≃100 µL of PBS 1X. The last three centrifugation steps were performed with the Optima XL-100 K ultracentrifuge, rotor SW 41 Ti Swinging-Bucket Rotor (Beckman Coulter, USA). Two different groups for assuring blindness carried out EVs isolation and analytes quantification. Fig. 2 summarized the entire process (sampling, sample treatment, and analysis.

2.4. Preparation of standard solutions

Stock solutions of BH4, GMP, GDP, GTP, and Guanosine were prepared weighing 1 mg of each analyte and solubilizing it in 1 mL of Water. About Neopterin, 1 mg was weighted and dissolved in 1 mL of DMSO, as reported on data sheet provided by producers. The working solutions concentration range was evaluated considering a 10-folds

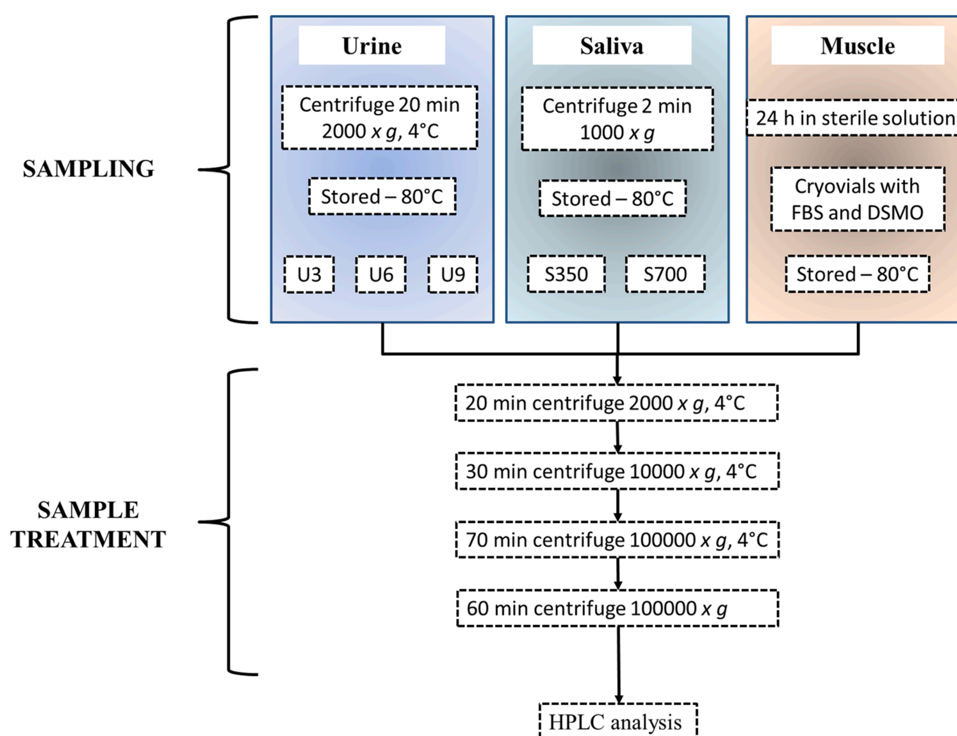

Fig. 2. Summary of the sampling, sample treatments, and analysis for the herein reported work.

matrix dilution (10 % matrix modification, as allowed for bioanalytical method validation). Linearity goes from 0.05 to 10 μg/mL and working solutions were prepared by diluting the stock solutions with water.

2.5. HPLC-DAD method

The analysis of the analytes of interest were conducted on Thermo Fisher Scientific liquid chromatography (model Spectra System P2000) equipped with a diode array detector (DAD, mod. Spectra System UV6000LP), a degasser from Lab Service, Analytica. Data acquisition and processing were performed with XCalibur Software (Thermo Fisher). The implementation of this method was started from an HPLC method validated in 2018 by Pietrangelo et al. [10]. The stationary phase used for the analysis was XTIMATE C18 (4.6 mm×250 mm, 5 μm, Welch, Shanghai, China) and the system was thermostated at 25 °C (±1 °C). Meanwhile mobile phases were 40 mM phosphate buffer pH 7 and ACN. It has been used a gradient course to permit at each analyte to separate in different times. The gradient is reported in *Supplementary Material, section S1*.

2.6. Method validation, AGREEprep and BAGI evaluation

The validation of the analytical method was obtained in compliance with the International Guidelines, regarding linearity, selectivity, precision and trueness intra- and inter-day [12–14]. During last decades, attention towards Green Analytical Chemistry has exponentially grown, and sample preparation plays a crucial role in analytical chemistry. For this reason, on the last paragraph, the herein validated method was also submitted to the AGREEprep evaluation. This tool allows checking how the procedure complies with the Green Sample Preparation (GSP) principles [15,16] specifically designed and tailored on the sample preparation steps. Furthermore, also a new index, Blue applicability grade index (BAGI) [17] was applied in order to deeper characterize the method.

3. Results and discussion

3.1. Method optimization and validation

Starting from a previously applied HPLC-DAD method [10], the procedure and the gradient were tested with the two new analytes to check the absence of interferences and the respective retention times. As observed, Neopterin and tetrahydrobiopterin show retention times that do not overlap with GMP, GDP, GTP, and Guanosine. As such, a mixture of the six analytes was injected to ensure that there was no cross-interferences between the analytes and their retention times. After verifying the applicability of the developed HPLC gradient, the entire procedure was submitted to the validation procedure to evaluate the retention times and maximum wavelength, linearity, LOD and LOQ, intra and interday accuracy (both in terms of precision and trueness). The chromatographic method results were shown in Table 1.

This method has shown limit of quantification (LOQ) of 0.05 μg/mL for each analyte (based on signal-to-noise S/N ratio of 10, and bias% values). The limit of detections (LOD) was 0.02 μg/mL (based on S/N ratio of 3). R^2 values are equal to or greater than 0.9824. Fig. 3 represents a typical chromatogram with the six analytes.

As reported in Table 2, intraday and interday precision and trueness were within the range of ±15 %, as advised by International Guidelines [12–14].

As seen in Fig. 3, from approx. 5.2 min, the baseline shows a change. This is related to the gradient elution (see *Supplementary Material S1*). During the single peak evaluation, this phenomenon was deeply reduced and as highlighted by the reproducibility of the procedure, it does not affect the result. The use of gradient elution allows to clean the system and avoid the carry over drawbacks, even if in this mode the total runtime is quite higher due to the column re-equilibration. Furthermore, in *Supplementary Material S2* the chromatograms were reported related to the specific maximum wavelengths for the quantitative analyses.

Table 1
Calibration parameters.

Analytes	Ret. times (min)*	Wavelenghts (nm)	LOD (μg/mL)	LOQ (μg/mL)	Linearity (μg/mL)	R^2
GTP	3.46 (± 0.03)	256	0.02	0.05	0.05 - 10	0.9862
GDP	3.64 (± 0.07)	256			0.05 - 10	0.9950
GMP	4.55 (± 0.07)	256			0.05 - 10	0.9960
Neopterin	5.24 (± 0.04)	347			0.05 - 10	0.9926
BH4	5.67 (± 0.06)	230			0.05 - 10	0.9946
Guanosine	6.1 (± 0.1)	256			0.05 - 10	0.9824

[*] in round brackets were reported the standard deviations ($n = 6$).

The different analytical figures of merit, in the absence of a blank matrix, were validated using the real matrix and the real matrix fortified at the different concentration levels. Through the blank subtraction procedure and evaluation of the back calculated concentration, it was possible to validate linearity, precision and trueness for all analytes even in the absence of real blank matrix. Furthermore, the trueness validation procedure also highlights the recovery method.

3.2. Real sample analysis

Starting from the oldest analytes confirming the method with four analytes (GTP, GDP, GMP, and guanosine), our study was then focused on the two new analytes (neopterin and BH4). As shown in Table 3, GTP and guanosine were found in all three biological sources. Saliva was the poorest source, with only those two analytes. Instead, growth medium of cadaver's muscle biopsy was the only one that contained BH4. As expected, the control sample contained no analytes.

The growth medium was the richest sample, as all the analytes were found. However, differences were found in the presence of each analyte within the same sample group. It should be noted that growth medium did not result in the post-treatment split in the three samples, being the latter rather the results of three independent culture, despite from the same muscle biopsy. Moreover, rather than the starting volume, different population doubling level (PDL) have resulted in different levels of analytes. Although speculatively, it is possible that the differences in guanosine, neopterin and BH4 among the three samples were the result of different biological processes across the growth processes. Outliers were found in urine samples, concerning both GMP, GDP, and neopterin, because results are more heterogeneous and could be related to different volumes used. Instead, results were stable in saliva samples, although saliva was the poorest source, as containing only GDP and guanosine. There was not an increasing trend of analytes concentration with starting volume, in both urine and saliva. Overall, the process of isolation and the scarce concentration of analytes in EVs samples may result in great differences across samples, even if from the same source.

3.3. Green profile and BAGI evaluation

AGREEprep Calculator [15,16] is a software that calculates the impact of sample preparations giving different weight on sample preparation place, hazardous materials, renewability of them, amount of waste, size economy of the sample, number of samples prepared in one hour, automation, energy consumption, type of instrument for analysis and operator safety. These ten points are directly linked to Green Chemistry (GC) and Green Analytical Chemistry (GAC), and the pictogram that the system generated indicates the greenness of the procedure.

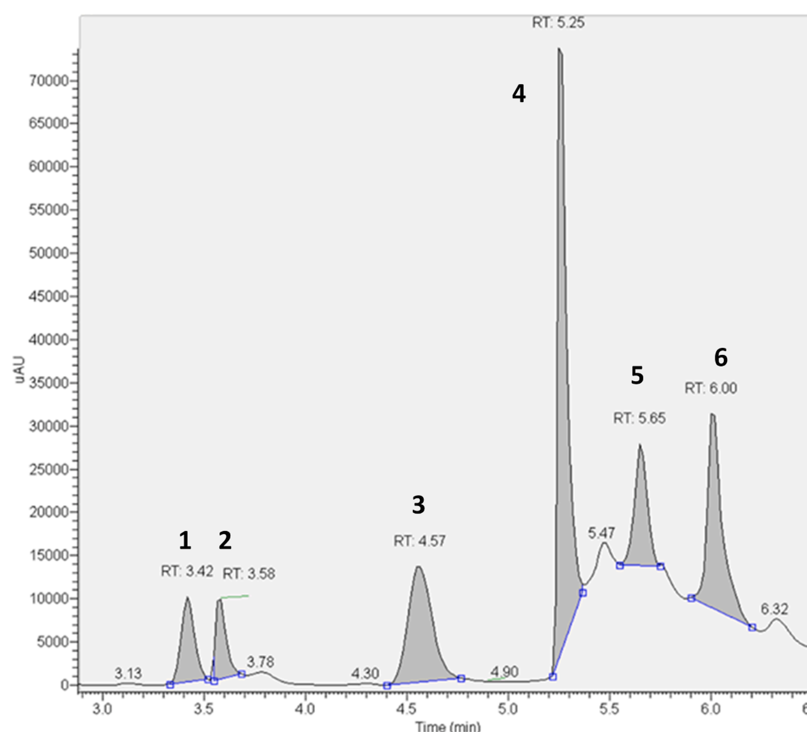

Fig. 3. An example of chromatogram of six analytes in standard solution at the concentration level of 5 mg/mL at 270 nm (wavelength were all the analytes were present). (1) GTP; (2) GDP; (3) GMP; (4) Neopterin; (5) BH4; (6) Guanosine.

Table 2
Intra-day and inter-day precision (RSD%) and trueness (Bias%) of the analytical method obtained.

			GTP	GDP	GMP	Neopterin	BH4	Guanosine
INTRADAY	**Precision**	**QC low**	3.09	7.11	7.76	7.54	1.75	9.30
		QC med	7.18	1.55	3.97	6.13	7.63	6.94
		QC high	5.59	2.60	4.23	3.54	2.57	2.43
	Trueness	**QC low**	5.56	7.67	−3.72	−1.61	6.94	−1.23
		QC med	−4.90	0.26	−11.0	8.02	5.05	0.23
		QC high	−2.45	8.36	−5.72	−6.56	7.66	−8.32
INTERDAY	**Precision**	**QC low**	1.16	2.59	5.76	4.87	2.98	12.9
		QC med	1.45	0.22	3.19	3.18	4.63	7.26
		QC high	1.97	1.28	0.07	0.92	2.84	2.88
	Trueness	**QC low**	4.50	−9.75	6.82	5.78	5.18	−2.39
		QC med	7.54	10.7	−1.39	−2.25	−3.98	3.05
		QC high	−2.06	−9.20	−14.4	−13.0	−8.61	−9.20

Fig. 4 reports the pictograms related to the sample preparation procedure divided into the different matrices herein considered (in *Supplementary material section S3* were reported the detailed criteria evaluation for each matrix).

In this evaluation, weights of each criterion were followed as default, thus, for example, the highest weight is given to second criterion (use safer solvents and reagents), because they have an important impact on the environment and it is better to avoid them or to use as less as possible. In addition, points 4 and 8 have a great weight, because they are about minimizing both waste and energy consumption, favour to simple procedures and less environmental impact.

About preparation step, in each case considered in this work sample preparation is *ex-situ*, cause of sample type used. The second point related to hazardous materials, they are used only for the muscle sample with the aim of avoiding rot and impossibility to use the sample. For the third and fourth criteria, we considered initial sample volume and solvents volume used for analysis, that during 18 min with a flow rate of 1 mL/min it consumes 18 mL, in this case, a hypothesis can be reducing the HPLC runtime, but anyway column needs time to re-establish after gradient. The fifth point is about size economy of the sample and the major volume used is 1 mL, corresponding at 1 cm^3 for muscle's sample, that can be certain reduced but, in this way, also the sensitivity of the quantitative procedure will be reduce.

Counting each step for preparation of one sample, it needs more than one hour, but several samples can be prepared at the same time, thanks to the presence of multiple allocations in the centrifuge/ultracentrifuge, so this can be an advantage. About the seventh criterion, sample preparation needs five steps, and this cannot change for the selected protocol. Energy consumption is due to using centrifuge/ultracentrifuge and the analysis is turned out on HPLC-DAD. For the last criterion considered, just for treatment of muscle, operator's safety is put at risk due to the use of only one hazardous solvent.

Blue applicability grade index (BAGI) is a new type of index with which it is possible to evaluate the practicality of an analytical method, and it can be combined with the most common about Green Chemistry. In this tool, ten parameters are evaluated, divided in analytical determination, sample preparation step or both [17]. Thus, type of analysis gives the better value, identify with dark blue, because it is a quantitative and confirmatory analysis. Following Green Analytical Chemistry principles, the second point, number of analytes, had good results thanks

Table 3
Results of analytes quantification by using HPLC-DAD.

Sample	GTP	GDP	GMP	Neopterin	BH4	Guanosine
Growth medium						
C1		1.1 ± 0.1	1.1 ± 0.1			
C2	0.57 ±0.06	0.52 ±0.05	0.92 ±0.08		0.46 ±0.05	
C6	0.30 ±0.03	0.56 ±0.06		2.4 ± 0.2		5.1 ± 0.5
Urine						
U3		2.4 ± 0.2				
U6	0.22 ±0.02	0.06 ±0.01		0.15 ±0.02		0.8 ± 0.1
U9	0.20 ±0.02	0.32 ±0.04	4.0 ± 0.4			0.7 ± 0.1
Saliva						
S350		0.15 ±0.02				0.40 ±0.04
S700		0.10 ±0.01				0.40 ±0.04
Control						
CTR						

Note: Values refer to back calculated concentration and are expressed in µg/mL (±standard deviation, $n = 3$).

to the multi-analyte chromatographic course, indeed we would add that the method was also implemented for these reasons. Being the method, validated on HPLC-DAD the third score was 7.5 for the simple instrumentation available. As we previously reported, sample preparation can take place simultaneously on different samples, based on number of allocations in centrifuge/ultracentrifuge. Sample preparation is easy to carry out, because it is a series of centrifuge/ultracentrifuge at different speeds and/ or time, so method obtained other 7.5 points. The analysis of a sample in HPLC-DAD with this validated method consists of 18 min, which is why 5 points were assigned to the method in relation to the sixth parameter. For both mobile phases and solvents used during sample preparation, types and cost of reagents and materials were easy to find on the market. 10 points were obtained for requirement of preconcentration, because method does not need. About penultimate point, manual treatment and analysis were used for the absence of common instruments. About amount of sample, the last parameter, in this pictogram we considered the worst situation that is 9 mL of urine used to extract EVs. The BAGI pictogram (with the single criteria selection) was reported in Fig. 5.

4. Conclusions

The validated method has permitted to increase and investigate in a more complete way the Guanosine-based pathway. Trueness, precision and linearity complied with International Guidelines and, additionally, allowed to quantify the above-mentioned analytes in complex matrix.

For the first time, a validated HPLC-DAD procedure was reported in literature that allows the simultaneous quantification of these six analytes that are fully involved in this specific pathway. The use of gradient elution certainly can limit its transferability to other instrument configurations (related to a different void volume), but due to its easy process, well-known instrumentation, fast analysis, this procedure can be a valuable tool available for EVs analysis.

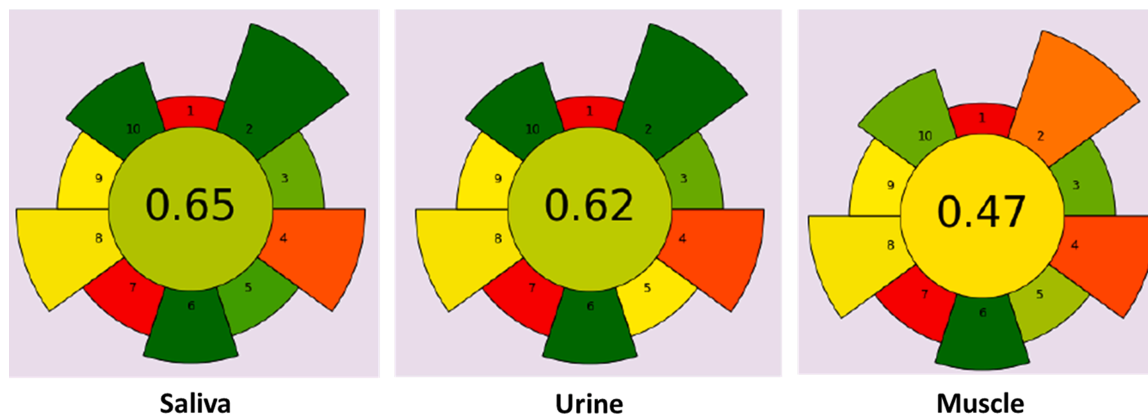

Fig. 4. Pictograms for the herein considered matrices obtained using AGREEprep tool.

1. Type of analysis	Quantitative and confirmatory
2. Multi- or single-element analysis	Multi-element analysis for 6-15 compounds of the same chemical class or 2-15 compounds of different chemical classes
3. Analytical technique	Simple instrumentation available in most labs (UV, HPLC-UV, HPLC-DAD, UHPLC, FAAS, ETAAS, ICP-OES, GC-FID etc.)
4. Simultaneous sample preparation	2-12
5. Sample preparation	Simple, low-cost sample preparation required (e.g. protein precipitation)
6. Samples per h	2-4
7. Reagents and materials	Common commercially available reagents (methanol, acetonitrile, HNO3, nitrogen or other common gasses, etc.)
8. Preconcentration	No preconcentration required. Required sensitivity and /or legislation criteria are met directly.
9. Degree of automation	Manual treatment and analysis
10. Amount of sample	>1000 µL (or mg) bioanalytical samples; >100 mL (or g) food/environmental

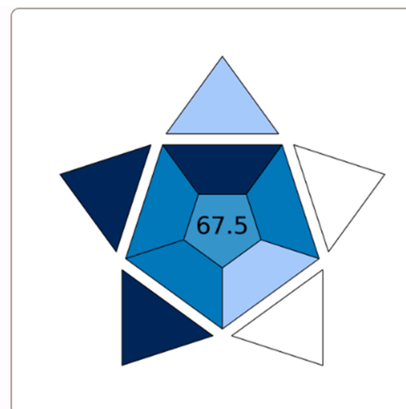

Fig. 5. Pictograms for the herein considered matrices obtained using BAGI tool. The selected volume is 9 mL (the worst condition, the analysis for all the other matrices consider a lower volume).

Our study addresses the need for precise determination of EVs' molecular composition by implementing a procedure for quantifying six analytes of the purines network. Similar studies will allow a clearer understanding and possibly a reassessment of extracellular vesicles' content [18], by including analytes other than proteins and nucleic acids.

From this preliminary evidence, it can be suggested to pay attention when using biological matrices in which even the same method inherently results in different quantities of elements (here EVs) obtained. Further studies may use our optimized method to quantify guanosine series molecules and compare different EVs' isolation and normalization methods, depending on the nature of the biological sample [19–22]. Studies aiming to evaluate differences between exosomes and other types of EVs [18,23] would integrate the analytes quantification with the origin and biogenesis of EVs.

CRediT authorship contribution statement

M. Perrucci: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. **C. Santangelo:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. **D. Bondi:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. **T. Pietrangelo:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. **F. Savini:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. **A.M. Catena:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. **M. Bonelli:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. **M. Locatelli:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. **C. D'Ovidio:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest

Data availability

Data will be made available on request.

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Supplementary materials

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