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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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Corresponding Author:	Marcello Locatelli, Ph.D. Gabriele d'Annunzio University of Chieti and Pescara Chieti, ITALY
First Author:	Miryam Perrucci
Order of Authors:	Miryam Perrucci
	Carmen Santangelo
	Danilo Bondi
	Tiziana Pietrangelo
	Fabio Savini
	Antonio Maria Catena
	Martina Bonelli
	Marcello Locatelli, Ph.D.
	Cristian D'Ovidio
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 ($R2 \geq 0.9824$). 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.

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Prof. Marcello Locatelli
University "G. d'Annunzio" of Chieti-Pescara,
Dept. of Pharmacy, Build B level 2, Via dei Vestini 31, 66100 Chieti (CH), Italy
E-mail: marcello.locatelli@unich.it

Dear Prof. Salvatore Fanali,

Following Your invitation mail (21 Jul 2023), please find enclosed the revised manuscript titled "*Liquid chromatographic method for extracellular Guanosine 5'-Triphosphate and Tetrahydrobiopterin pathway products analysis from cadaveric samples and human biofluids*" submitted to **Virtual Special Issue: "EDITOR'S CHOICE" of Journal of Chromatography Open**.

All revisions and suggestions were accepted and reported in the current version. We also would thank the Reviewers for their very valuable suggestions for the improvement of the paper quality.

We hope that the revised manuscript will receive favorable peer reviews and subsequent publication in your esteemed journal.

Sincerely,

Marcello Locatelli, PhD
Associate Professor, Analytical and Bioanalytical Chemistry
Dept. of Pharmacy; "*G. d'Annunzio*" University of Chieti – Pescara
E-mail: marcello.locatelli@unich.it

Proposed Reviewers:

1. **Prof. Dr. Victoria Samanidou**; Aristotle University of Thessaloniki, Department of Chemistry, Laboratory of Analytical Chemistry, Greece; samanidu@chem.auth.gr
2. **Prof. Dr. Halil I. Ulusoy**; Department of Analytical Chemistry, Faculty of Pharmacy, Cumhuriyet University, Sivas, Turkey; hiulusoy@yahoo.com
3. **Prof. Dr. Sibel A. Ozkan**; Ankara University, Faculty of Pharmacy, Ankara, Turkey; ozkan@pharmacy.ankara.edu.tr
4. **Prof. Dr. Dora Melucci**; University of Bologna, Department of Chemistry "G. Ciamician", Bologna, Italy; dora.melucci@unibo.it
5. **Prof. Dr. Donato Cosco**; Dipartimento di Scienze della Salute, Università Magna Graecia di Catanzaro; donatocosco@unicz.it
6. **Prof. Dr. Agnese Giacomino**; University of Turin, Turin, Italy; agnese.giacomino@unito.it
7. **Prof. Dr. Laura Mercolini**; University of Bologna, Bologna, Italy; laura.mercolini@unibo.it

Prof. Marcello Locatelli
University "G. d'Annunzio" of Chieti-Pescara,
Dept. of Pharmacy, Build B level 2, Via dei Vestini 31, 66100
Chieti (CH), Italy
E-mail: marcello.locatelli@unich.it

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Associate Professor, Analytical and Bioanalytical Chemistry
Dept. of Pharmacy; "*G. d'Annunzio*" University of Chieti – Pescara
E-mail: marcello.locatelli@unich.it

Editor

Dear Marcello Glad to inform you that the manuscript can be considered for the publication in J. Chromatogr. Open, however a minor revision is necessary.

Dear Editor Prof. Fanali, we are very pleasured that the manuscript can be considered for publication and we would like to thank you for your suggestions and for the Reviewers comments very valuable to improve the paper quality.

In addition to the enclosed remarks, please

1) Modify the title, abbreviations are not allowed

As suggested, the title has been changed in "Liquid chromatographic method for extracellular Guanosine 5'-Triphosphate and Tetrahydrobiopterin pathway products analysis from cadaveric samples and human biofluids".

2) Use the format of JCO see instructions, e.g., authors' name abbreviated first and family name; TrAC is TrAC-Trends Anal. Chem.

All the references have been corrected according to Guidelines, as also the authors' name.

3) As recommended by reviewer #3, revise the English.

Dear Editor, the English was deeply revised by Prof. Kabir of Florida International University (USA).

Reviewer #1

The paper "HPLC-DAD method for extracellular Guanosine 5'-Triphosphate and Tetrahydrobiopterin pathway products analysis from cadaveric samples and human biofluids" is a good work. It reports all figure of merits related to the validation procedure and additionally, real samples analysis were reported not only for clinical applications, but also in forensic field. The paper was well structured and organized. I suggest the acceptance pending revisions:

Dear Reviewer, thanks for the good evaluation. All your suggestions were accepted and reported in the revised version

- Explain why, if the last analyte was eluted at 6 min, the total runtime is 18 min.

Accordingly to this point, in section 3.1 was added the reason.

- Improve the discussion on AGREEprep

Accordingly to this suggestion, the discussion on AGREEprep was improved.

- Add a paragraph related to the application of BAGI (see Green Chem. 25 (2023) 7598- 7604. 10.1039/D3GC02347H)

Accordingly, in section 3.3 was added a paragraph related to BAGI index.

- Check the paper for some typos and grammar errors (e.g. "x g" need to be in italics)

Revised

- Line 179-182 can be deleted. These info are reported into the Guidelines

Deleted

- In supplementary materials add the single chromatograms related to the maximum wavelength for each analyte

Accordingly, these info were added in supplementary material

Reviewer #2

Good paper. Useful for many applications. Minor revision for trivial editing. Revise the English.

Accordingly, the English was deeply revised by Prof. Kabir of Florida International University (USA).

Line 44: ERRATA: "linearity" CORRIGE: "correlation". High correlation is not equivalent to linearity.

Revised

Line 60: ERRATA: "In" CORRIGE: "It is"

Revised

Line 59 and Line 91: ERRATA: "signalling" CORRIGE: "signaling", like on line 68

Revised

Line 70: ERRATA: "has been yet to be defined" CORRIGE: "has not been defined, yet"

Revised

Line 77: ERRATA: "continue" CORRIGE: "continues"

Revised

Line 128: ERRATA: "hrs" CORRIGE: "h"

Revised

Line 153: ERRATA: "eVs" CORRIGE: "EVs"

Revised

Table 1. The result "6.10 (Â± 0.11)" is differently formatted with respect to the other results. For the sake of coherence, it should be written "6.1 (Â± 0.1)" (one significant digit for the error).

Revised

Line 198: ERRATA: "showed" CORRIGE: "shown"

Revised

Line 216: ERRATA: "highlight" CORRIGE: "highlighted"

Revised

Table 3. Same comment as Table 1: I recommend to use a homogeneous way of formatting errors. I prefer always one significant digit for the error, but also always two significant digits for errors is acceptable.

Revised

Line 228: ERRATA: "were found differences" CORRIGE: "differences were found". I recommend to pay attention to this syntax error, which is recurrent in the text. Please, check all-over the text.

Revised

Line 235: "Outliers were found": please, describe the criterion used to find outliers.

Revised

Line 243: ERRATA: "calculate" CORRIGE: "calculates"

Revised

Line 258: ERRATA: "minutes" CORRIGE: "min"

Revised

Line 258: ERRATA: "flow" CORRIGE: "flow rate"

Revised

Line 274: ERRATA: "allow" CORRIGE: "allows"

Revised

Line 283: ERRATA: "can be suggested" CORRIGE: "it can be suggested". Also this grammar error (lack of subject) is frequent in the text. Please, check all-over the text.

Revised the entire text

Reviewer #3

The manuscript proposed herein by M. Perrucci et al. deals with the implementation of an analytical method based on HPLC-DAD for the analysis of guanosine monophosphate, guanosine diphosphate, guanosine triphosphate, guanosine, neopterin and tetrahydrobiopterin as the main representative compounds of guanosine-based pathways. The method has been developed for

application to human extracellular vesicles isolated from different biological samples, namely urine, saliva and muscle. The proposed methodology represents an interesting implementation of potential interest for the bioanalytical community as a tool that can be exploited in the context of different types of research, whenever quali-quantitative analytical investigation on an expanded set of target analytes implicated in guanosine-based pathways is needed, especially within approaches involving the isolation and analysis of extracellular vesicles obtained from varied sets of biological matrices.

Dear Reviewer, thanks for the good evaluation. All your suggestions were accepted and reported in the revised version

However, this Reviewer believes that some improvements are advisable in order to strengthen the validity of this study and improve its overall quality before publication:

-In the Introduction section, the Authors should better contextualize the frameworks in which this type of analysis is placed. It would be appropriate to better specify the implications and advantages that this methodology would bring to these fields, also for comparison with the current methods present in the literature aimed at the analysis of guanosine-derived compounds. For example, the fact that the Authors take into consideration a diverse set of biological samples for method development and evaluation is certainly a valuable aspect. However, it may not be clear to the audience which implications and which contexts may make it necessary to analyse target compounds in extracellular vesicles isolated from urine, oral fluid, and post-mortem muscular tissue. As an example, the reference to forensic application also reported in the manuscript keywords may not be clear to the reader, and therefore a more in-depth discussion of the potential application of this method to such field would certainly make the purpose of the work clearer.

Dear Reviewer, following your interesting suggestion, the introduction was improved, highlighting this point

-For some sections relating to sample collection and sample pretreatment aimed at the isolation of extracellular vesicles from the matrices under examination, the Authors refer to protocols previously developed and published elsewhere. Although this Reviewer agrees with the summary of information already published focusing the descriptions on the novelty aspects of this work, a synthetic and schematic but complete description of all the applied protocols would be advisable. This would allow for the reader a smoother and more effective consultation of the specific protocols applied to the biological tissues considered in this work.

Accordingly to this suggestion, the specific protocols were added in the revised version as new Figure 2 in order to summarize the general steps required in this study.

-In the Method validation section, the Authors do not refer to extraction yield and matrix effect assays. This Reviewer believes that this is plausibly due to the intrinsic nature of the analytes representing ubiquitous markers which therefore preclude obtaining devoid of analyte matrices to

be appropriately fortified. The Authors should implement discussion paragraphs delving into this aspect and the choice not to include extraction yield and matrix effect parameters in the method validation of the method. Alternatively, the Authors could consider implementing the external addition approach of analytes in order to include an evaluation of these parameters.

Regarding the Reviewer's comment, it should be emphasized that trueness evaluation is often used to validate method recovery.

Regarding the matrix effect phenomena, they were previously evaluated in the first reported publication [10] for four of the molecules of interest. For the two new analytes the same tests were performed for both recovery and matrix effect.

Specifically, in the absence of a white matrix, we operate using a real matrix (white) and a real matrix fortified at the three 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.

These info were added in section 3.1 in order to reply to the correct Reviewer's suggestion.

-The authors report an interesting paragraph relating to the Green profile evaluation. However, this information is reported exclusively in the Results and Discussion section. It would be appropriate and interesting for readers if a similar paragraph were implemented in the Materials and methods section, outlining the principles of this evaluation approach.

As correctly suggested, a paragraph in section 2.6 was added related to AGREEprep index and BAGI index with the corresponding references

-The entire manuscript requires in-depth revision and polishing of the English language, as there are numerous grammatical and syntax errors, which in some cases would also require the rewriting of entire paragraphs to improve understanding by the audience. This Reviewer would recommend a thorough review by a native English speaker.

Accordingly also with the other Reviewers, the English was deeply revised by Prof. Kabir of Florida International University (USA).

The present Reviewer believes that the manuscript proposed herein represents an analytical approach of potential interest to the bioanalytical community, and a versatile strategy to be applied in different frameworks where a complete evaluation of guanosine-derived compounds in extracellular vesicles is required. Based on the gaps and shortcomings highlighted above, this Reviewer recommends a moderate revision aimed at completing the manuscript with some crucial aspects, and at further improving its overall clarity and readability before publication.

Dear Reviewer, thanks again for the good evaluation. All your suggestions were accepted and reported in the revised version

Highlights

1. Guanosine-based pathway evaluation in different matrices
2. Simultaneous HPLC-DAD quantification of guanosine and pterins molecules
3. Human extracellular vesicles isolated from different human parts

Liquid chromatographic method for extracellular Guanosine 5'-Triphosphate and Tetrahydrobiopterin pathway products analysis from cadaveric samples and human biofluids

M. Perrucci[1], C. Santangelo[2], D. Bondi[2], T. Pietrangelo[2], F. Savini[3], A.M. Catena[4], M. Bonelli[5], M. Locatelli[1,*], C. D'Ovidio[5]

[1] Department of Pharmacy, University of Chieti–Pescara "G. d'Annunzio", Via dei Vestini 31, Chieti 66100, Italy, marcello.locatelli@unich.it, miryamper97@gmail.com
[2] Department of Neurosciences, Imaging and Clinical Sciences, University of Chieti–Pescara "G. d'Annunzio", Via dei Vestini 31, Chieti 66100, Italy, tiziana.pietrangelo@unich.it, danilo.bondi@unich.it, carmen.santangelo@unich.it
[3] Pharmatoxicology Laboratory—Hospital "Santo Spirito", Via Fonte Romana 8, Pescara 65124, Italy, fabio.savini@ausl.pe.it
[4] Institute of Legal Medicine, University of Rome 2 "Tor Vergata", 00133 Rome, Italy; medicinalegale@uniroma2.it
[5] Department of Medicine and Aging Sciences, Section of Legal Medicine, University of Chieti–Pescara "G. d'Annunzio", Chieti 66100, Italy, cristian.dovidio@unich.it, martina.bonelli@unich.it

*** Corresponding Author:**
Prof. Marcello Locatelli
Department of Pharmacy, University of Chieti–Pescara "G. d'Annunzio", Via dei Vestini 31, Chieti 66100, Italy marcello.locatelli@unich.it, Tel.: +39-0871-3554590

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.

Keywords

HPLC-PDA; extracellular vesicles; guanosine and pterins; cadaveric sample; liquid biopsy; forensic application.

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 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 **Figure 1**.

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

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 (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-100K ultracentrifuge, rotor SW 41 Ti Swinging-Bucket Rotor (Beckman Coulter, USA). Two different groups for assuring blindness carried out EVs isolation and analytes quantification. **Figure 2** summarized the entire process (sampling, sample treatment, and analysis.

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

2.4 Preparation of standard solutions

Stock solutions of BH4, GMP, GDP, GTP, and Guanosine were prepared weighing 1mg 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 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 and AGREEprep 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**.

Table 1. Calibration parameters

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. **Figure 3** represents a typical chromatogram with the six analytes.

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

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

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

As seen in **Figure 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.

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.

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

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 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. **Figure 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).

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

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 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 **Figure 5**.

Figure 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)

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.

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.

Declaration of Competing Interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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~~HPLC-DAD~~Liquid chromatographic method for extracellular Guanosine 5'-Triphosphate and Tetrahydrobiopterin pathway products analysis from cadaveric samples and human biofluids

~~Miryam~~M. Perrucci[1], ~~Carmen~~C. Santangelo[2], ~~Danilo~~D. Bondi[2], ~~Tiziana~~T. Pietrangelo[2], ~~Fabio~~F. Savini[3], ~~Antonio Maria~~A.M. Catena[4], ~~Martina~~M. Bonelli[5], ~~Marcello~~M. Locatelli[1,*], ~~Cristian~~C. D'Ovidio[5]

[1] Department of Pharmacy, University of Chieti–Pescara "G. d'Annunzio", Via dei Vestini 31, Chieti 66100, Italy, marcello.locatelli@unich.it, miryamper97@gmail.com
[2] Department of Neurosciences, Imaging and Clinical Sciences, University of Chieti–Pescara "G. d'Annunzio", Via dei Vestini 31, Chieti 66100, Italy, tiziana.pietrangelo@unich.it, danilo.bondi@unich.it, carmen.santangelo@unich.it
[3] Pharmatoxicology Laboratory—Hospital "Santo Spirito", Via Fonte Romana 8, Pescara 65124, Italy, fabio.savini@ausl.pe.it
[4] Institute of Legal Medicine, University of Rome 2 "Tor Vergata", 00133 Rome, Italy; medicinalegale@uniroma2.it
[5] Department of Medicine and Aging Sciences, Section of Legal Medicine, University of Chieti–Pescara "G. d'Annunzio", Chieti 66100, Italy, cristian.dovidio@unich.it, martina.bonelli@unich.it

*** Corresponding Author:**
Prof. Marcello Locatelli
Department of Pharmacy, University of Chieti–Pescara "G. d'Annunzio", Via dei Vestini 31, Chieti 66100, Italy marcello.locatelli@unich.it, Tel.: +39-0871-3554590

Abstract

~~In order to~~To gain a deep ~~evaluate~~insight and ~~have a major complete view~~to obtain a superior understanding about guanosine-based pathway, this paper reports an innovative approach to study this critical subject. Firstly, after ~~a long~~an exhaustive analysis of literature ~~and~~with a ~~focusing about~~focus in legal medicine and extracellular vesicles, it was understood that ~~is necessary~~ 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~~x~~ × 250 mm, 5 µm, Welch, Shanghai, China) and mobile phase's phosphate buffer (40 mM, pH 7) (A) and Acetonitrile (B). ~~Linearity~~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~~. Good linearity is shown also by~~ ($R^2 \geq 0.9824$~~.~~).

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.

Keywords

HPLC-PDA; extracellular vesicles; guanosine and pterins; cadaveric sample; liquid biopsy; forensic application.

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 ~~signalling~~signaling [1~~],~~] and can be extracted from any human biological fluid and tissue [1]. ~~In~~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 ~~vehicled~~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. ~~In particular~~Specifically, among adenosine and guanosine series~~ was found,~~ 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 ~~yet to be~~ defined, yet, also related to their important function not only 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 ~~triphosphorylated~~tri phosphorylated, consequently they are guanosine monophosphate (GMP), guanosine diphosphate (GDP) and guanosine triphosphate (GTP) [8]. This pathway, from GTP, ~~continue~~continues obtaining Neopterin, as by product of Tetrahydrobiopterin synthesis, and Tetrahydrobiopterin (BH4), as reported in **Figure 1**.

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

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 ~~have~~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 ~~derivates~~derivatives, we aimed to extend the field of purine ~~signalling~~signaling in humans by setting and testing the procedure of quantifying molecules of guanosine series stuffed in EVs isolated from several biological ~~matrixes~~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 ~~Dimethylsulfoxide~~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 (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 ~~in~~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 ~~has~~have been immediately immersed for 24 ~~hrs~~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.~~3mL~~3 mL and 8.~~1mL~~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-100K ultracentrifuge, rotor SW 41 Ti Swinging-Bucket Rotor (Beckman Coulter, USA). Two different groups for assuring blindness carried out ~~eVs~~EVs isolation and analytes quantification. **Figure 2** summarized the entire process (sampling, sample treatment, and analysis.

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

2.4 Preparation of standard solutions

Stock solutions of BH4, GMP, GDP, GTP, and Guanosine were prepared weighing 1mg 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 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 and AGREEprep evaluation

The validation of the analytical method was obtained ~~according~~in compliance with the International Guidelines, regarding linearity, selectivity, precision and trueness intra- and inter-day [12-14]. ~~Specifically, as required by the Guidelines, the mean value for inter- and intra-day trueness (bias%) should be within 15% of the real value except at LOQ, where it should not deviate by >20%. Similarly, the mean value for inter- and intra-day precision (relative standard deviation, RSD%) should be within 15% except at LOQ, where it should not deviate by >20 %.~~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], ~~was tested~~ the procedure and the gradient were tested with the two new analytes ~~in order~~ 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. ~~Thus, it was injected~~ As such, a ~~mix~~mixture of the six analytes~~, in order~~ was injected to ~~avoid~~ensure that there was no cross-interferences between the analytes and their retention times. ~~Verified~~After verifying the applicability of the developed HPLC gradient, the entire procedure was submitted to the validation procedure ~~in order~~ 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 ~~show~~shown in **Table 1**.

Table 1. Calibration parameters

This method has ~~showed~~shown limit of quantification (LOQ) of 0.05 µg/mL for each ~~analytes~~analyte (based on signal-to-noise S/N ratio of 10, and bias% values). The limit of detections

227 (LOD) was 0.02 µg/mL (based on S/N ratio of 3). R^2 values are equal to or greater than 0.9824. ~~In~~
228 **Figure ~~2 was reported~~3** represents a typical chromatogram with the six analytes.
229
230
231 **Figure ~~2~~3**. An example of chromatogram of six analytes in standard solution at the concentration
232 level of 5 µg/mL at 270 nm (wavelength were all the analytes were present). (1) GTP; (2) GDP; (3)
233 GMP; (4) Neopterin; (5) BH4; (6) Guanosine.
234
235 As reported in **Table 2**, intraday and interday precision and trueness were ~~included in~~within
236 the range of ±15%, as ~~requested~~advised by International Guidelines [12-14].
237
238 **Table 2**. Intra-day and inter-day precision (RSD%) and trueness (Bias%) of the analytical method
239 obtained.
240
241 As ~~reported~~seen in **Figure ~~2~~3**, from approx. 5.2 min, the baseline shows a change. This is
242 related to the gradient elution (see *Supplementary Material S1*). During the single peak evaluation,
243 this phenomenon was deeply reduced and~~,~~ as ~~highlight~~highlighted by the reproducibility of the
244 procedure, it does not affect the result. The use of gradient elution allows to clean the system and
245 avoid the carry over drawbacks, even if in this mode the total runtime is quite higher due to the column
246 re-equilibration. Furthermore, in *Supplementary Material S2* the chromatograms were reported
247 related to the specific maximum wavelengths for the quantitative analyses.
248 The different analytical figures of merit, in the absence of a blank matrix, were validated using
249 the real matrix and the real matrix fortified at the different concentration levels. Through the blank
250 subtraction procedure and evaluation of the back calculated concentration, it was possible to validate
251 linearity, precision and trueness for all analytes even in the absence of real blank matrix. Furthermore,
252 the trueness validation procedure also highlights the recovery method.
253
254 **3.2. Real sample analysis**
255 Starting from the oldest analytes confirming the method with four analytes (GTP, GDP, GMP,
256 and guanosine), our study was then focused on the two new analytes (neopterin and BH4). As shown
257 in **Table 3**, GTP and guanosine were found in ~~the entire~~all three biological ~~source~~sources. Saliva was
258 the poorest source, with only those two analytes. Instead, growth medium of cadaver's muscle biopsy
259 was the only one that contained BH4. As expected, the control sample contained no analytes.
260

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

The growth medium was the richest sample, as all the analytes were found. However, ~~were found~~ 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, ~~the~~ 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 ~~process~~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 evaluation

AGREEprep Calculator [15, 16] is a software that ~~calculate~~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 ~~sample~~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. ~~In~~ **Figure** ~~**3** were reported~~**4** reports the pictograms related to the sample preparation procedure divided into the different matrices herein considered (in *Supplementary material section* ~~*S2*~~*S3* were reported the detailed criteria evaluation for each matrix).

Figure ~~**3**~~**4**. Pictograms for the herein considered matrices obtained using AGREEprep tool.

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 ~~minutes~~min with a flow rate of 1 mL/min it consumes 18 mL, in this case ~~an~~, 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 ~~can be reduced~~, 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 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 **Figure 5**.

Figure 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)

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 ~~allow~~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.

Our study addresses the need ~~of~~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 [~~17~~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 [~~18-21~~19-22]. Studies aiming to evaluate differences between exosomes and other types of EVs [~~17, 22~~18, 23] would integrate the analytes quantification with the origin and biogenesis of EVs.

Declaration of Competing Interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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Author Contributions

All of the authors contributed equally to the writing of the present review article. All authors have approved the final version of the manuscript.

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Figure 1

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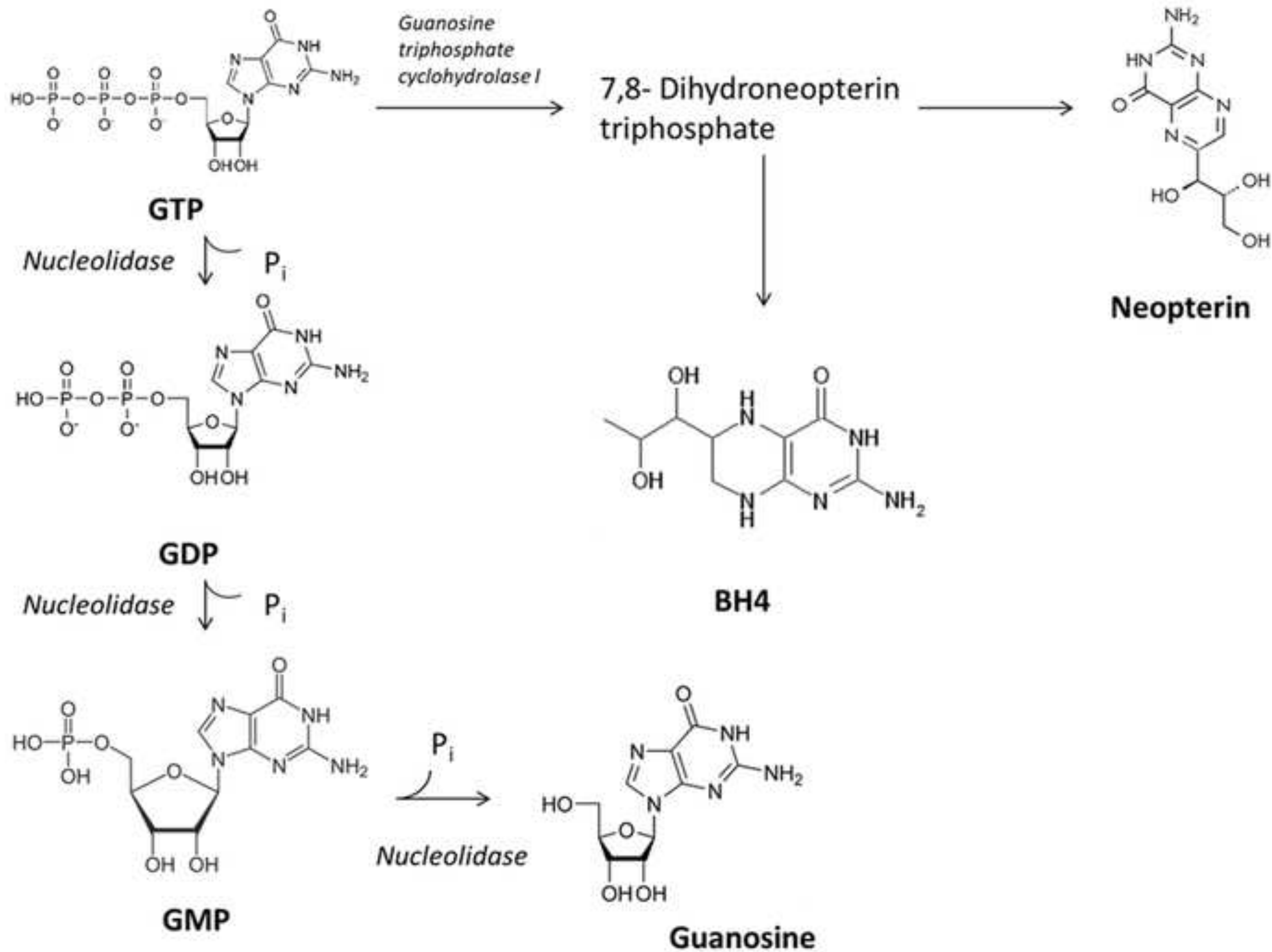

Figure 2

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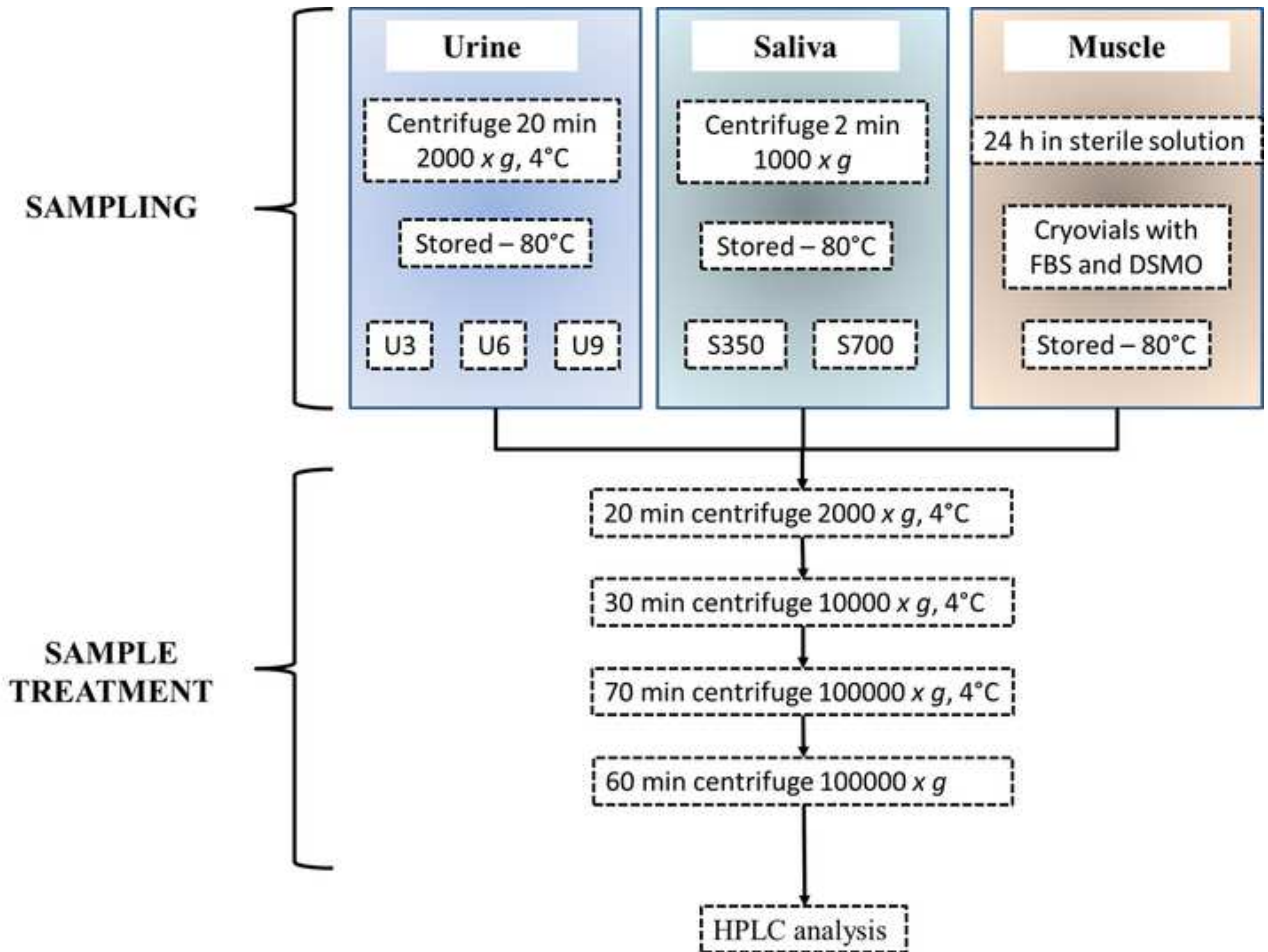

Figure 3

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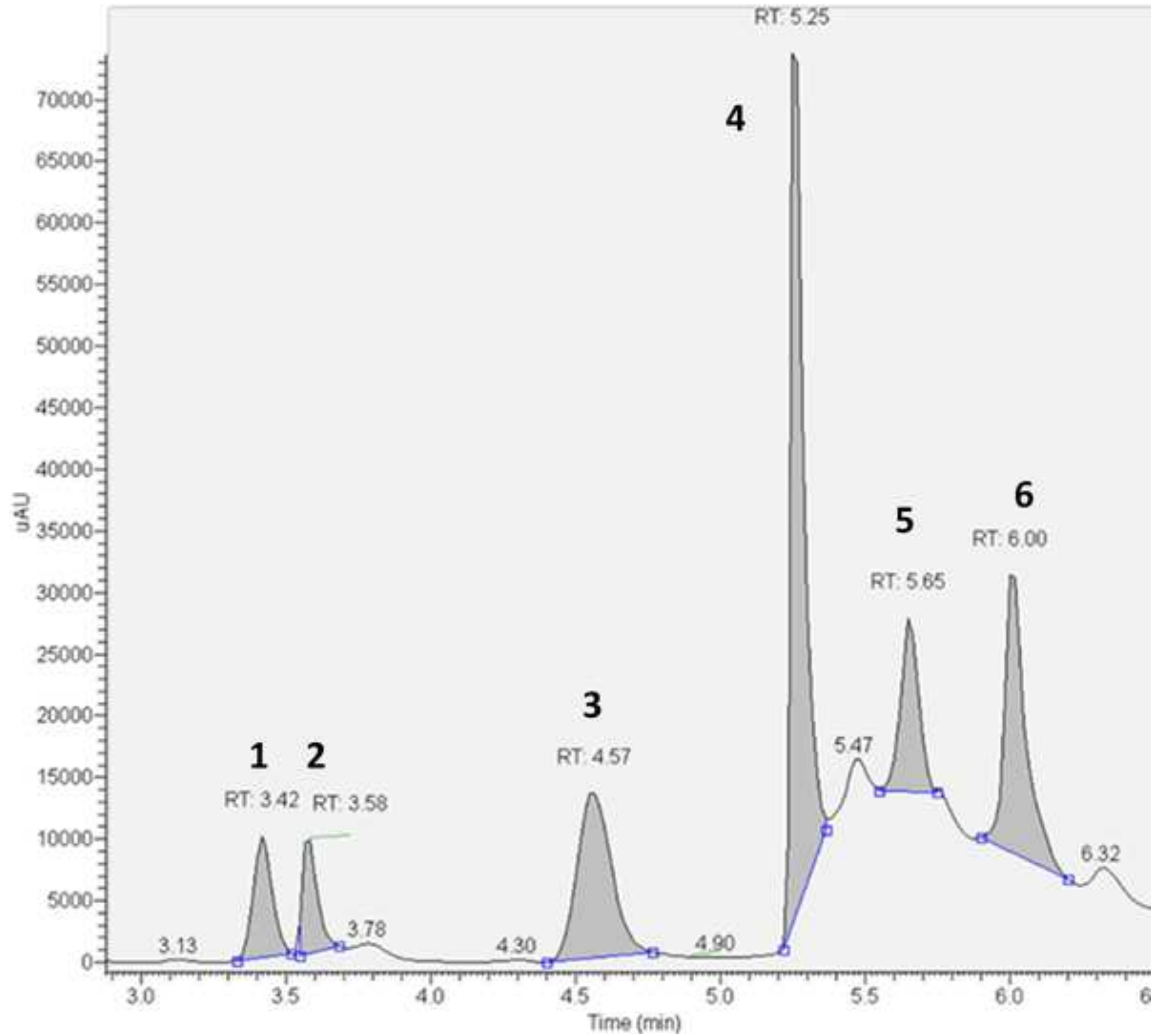

Figure 4

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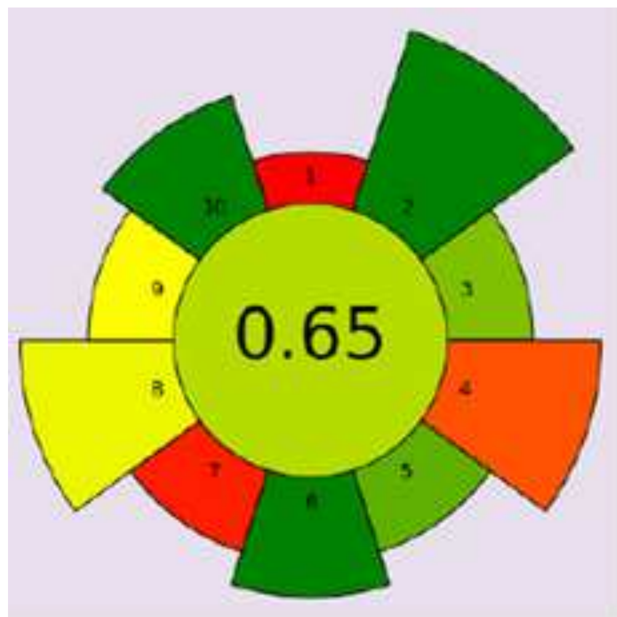
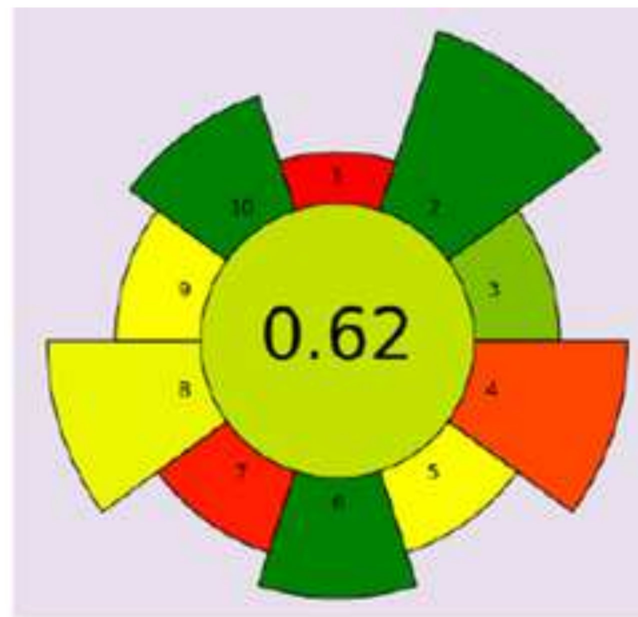


Saliva



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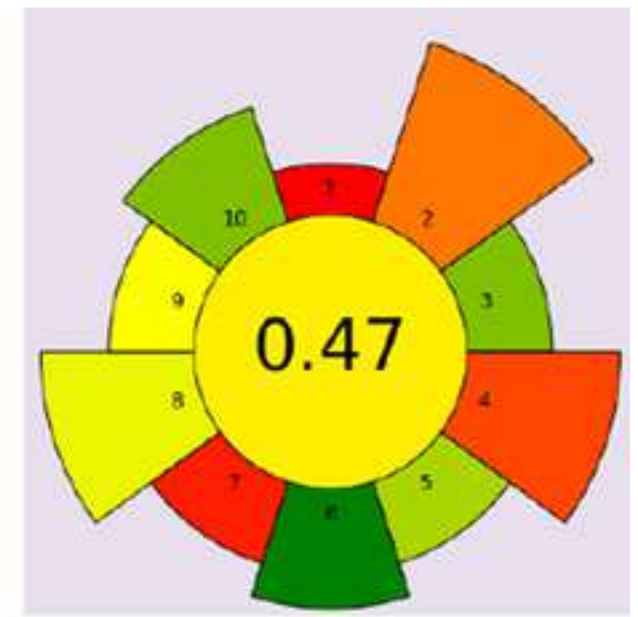

Muscle

Figure 5

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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, HNO_3, nitrogen or other common gases, 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

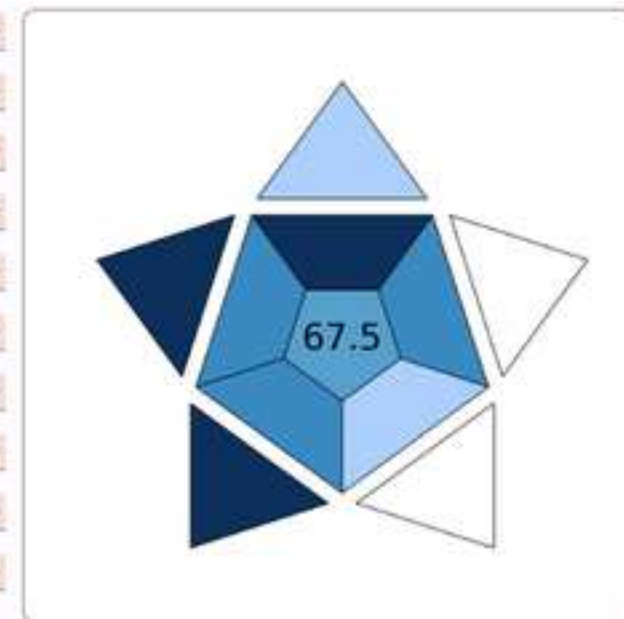

Table 1 Click here to access/download;Table (Editable version);Perrucci et al - BH4 and neopterin Table 1 - R1.docx

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)

Table 1 track changes

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Guanosine	6.~~10~~1 (± 0.~~11~~1)	256			0.05 - 10	0.9824

* in round brackets were reported the standard deviations (n=6)

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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

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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)

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

Sample	GTP	GDP	GMP	Neopterin	BH4	Guanosine
Growth medium						
C1		1.~~06~~1±0.~~12~~1	1.~~08~~1±0.~~11~~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.~~40~~4±0.~~23~~2		5.~~05~~1±0.~~51~~5
Urine						
U3		2.~~37~~4±0.~~22~~2				
U6	0.22±0.02	0.06±0.01		0.15±0.02		0.~~78~~8±0.~~08~~1
U9	0.20±0.02	0.32±0.04	4.~~01±~~0~~.41~~±0.4			0.~~73~~7±0.~~07~~1
Saliva						
S350		0.15±0.02				0.40±0.04
S700		0.10±0.01				0.~~38~~40±0.04
Control						
CTR						

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

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Conflicts of Interest

The authors declare no conflict of interest

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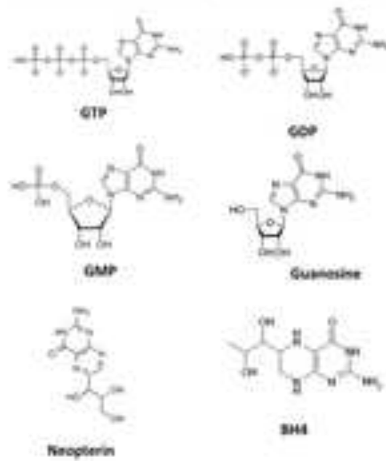
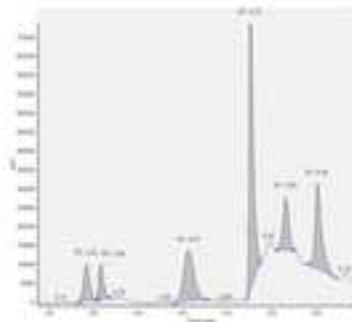
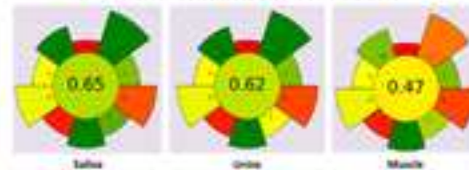
Guanosine and pterins analytes



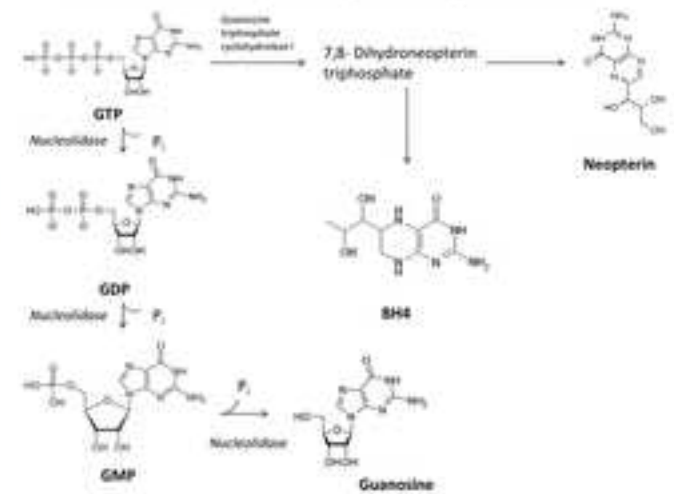
HPLC-DAD method validation



Green profile evaluation



Guanosine and pterins pathway evaluation



Invitation letter

VIRTUAL SPECIAL ISSUE: "EDITOR'S CHOICE"

Prof. Marcello Locatelli
Chieti University, Chieti, Italy

July 21th , 2023.

Dear Marcello,

I have a pleasure to invite you to contribute a paper to a virtual special issue of *JC-O "Editor's Choice".* This special issue will consist of review articles, short communications, and full-length research papers in the field of chromatography and related techniques, all of which will be subject to the journals' strict international peer review policy.

This special issue will provide intensive visibility of your work to experts on a worldwide basis via the online platform Science Direct.

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We would be delighted if could respond to confirm your contribution, and the proposed title by July 31th, 2023. The deadline for manuscript submission is **October 15th, 2023** and detailed instructions for on-line submission are on the next page. Please closely follow the Guide to Authors which can be found on https://www.editorialmanager.com/jc-o

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With best regards,

Salvatore Fanali

(Editor-in-Chief)

salvatore.fanali@cnr.it

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