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Virtual Mobility grant

Systematization of methods for measuring RONS in various types of the treated sample

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Introduction

Creation of reactive oxygen and nitrogen species (RONS) is among the most important phenomena occuring during the interaction of cold plasma and liquid. Detection of these species in treated targets is generally an integral part of studying the effects of plasma treatment on aqueous solutions and involves the use of standard colorimetric and/or spectrophotometric methods. However, the treatments of targets with different physico-chemical properties are carried out in different applications. Identification and quantification of the aqueous reactive long-lived species such as nitrate, nitrite, and hydrogen peroxide generated in plasma-activated liquids could therefore help to shed light on the mechanisms of action of plasma-activated liquids. Therefore, it is important to choose an appropriate method that will be able to identify and accurately measure the concentration of RONS.

The purpose of this project was to create a report that systematizes the methods of RONS measurement available in the literature according to the type and properties of the treated target, which is important for plasma agriculture. Thus, researchers would have a quick insight into the most commonly used methods, their advantages and disadvantages, which would make it easier to choose the correct method.

Description of the work carried out during the VM

The first part of working plan was to select the most important reactive species in plasma-activated liquids for desired effects in the treatment of biological samples for application in agriculture. The literature was analyzed and it was discussed with participants from the working groups in order to define reactive species produced until now by different plasma treatment conditions. This part was finalized the parameters that influence the production of different reactive species in plasma-activated liquids.

The second part was to make a literature survey to establish the methods and protocols for measurements of different reactive species in plasma-activated liquid. This analysis of the literature gave results from research that is published in the last 20 years. With relevant keywords (plasma-activated liquid, plasma-activated water, plasma-activated medium diagnostics, H2O2, NO3- and NO2- colorimetric methods and procedures, cell culture medium, plant growth medium, wastewater, cold plasma treatment), results were selected according to an available number of articles. Based on the obtained data the standard methods for measurements of reactive species and problems which researchers face when they use them were sorted.

Plasma-activated liquids are a cocktail of reactive species so, during the measurement, at the same moment, there is an influence and reaction of reactive species in the same solution. This situation disturbs standard methods for measurement which has to be overcome in order to know the concentration of reactive species in plasma-activated liquids.

The research was focused on long-lived species (NO_2^-, NO_3^-, H_2O_2) that can be detected in the treated samples after exposure to plasma.

Searching the literature included: research papers on the investigation of the effect of plasma on different types of liquids and plant growth media, and review articles dealing with RONS measurement techniques. Based on the previously published works in plasma agriculture field the most commonly used:

- Plasma sources
- Types of treated samples
- Spectrophotometric and colorimetric techniques for RONS determination

were established.

Virtual collaboration and activities

The Virtual Mobility involved two PhD students from the Institute of Physics Belgrade (Serbia, ITC Country) who are members of the Laboratory for Non-Equilibrium Processes and Application of Plasma, Olivera Jovanović and Anđelija Petrović. Communication with WG participants dealing with PAW diagnostics was done through written email correspondence about possible solutions for future measurements of RONS in the treatment of liquids. A questionnaire (Google Form) about the hands-on experience of researchers was made for participants of the CA19110 dealing with the diagnostics of plasma-treated liquid samples. Their problems and the ways they overcame them are part of this report.

GDPR statement of the questionnaire: The processing of your personal data, undertaken in the questionnaire, will take place in compliance with the EU General Data Protection Regulation 2016/679 (the'GDPR'), as well as any applicable national data protection legislation. These personal data will not be communicated further by any means or used in presentatino of the grant results. They serve only for informing participants of the survey on the results.

Results

Literature review

In the available literature, one can find various papers dealing with chemical reactions in the gas phase of the discharge, plasma-liquid interactions, review articles on plasma devices for the treatment of a wide range of targets, but a small number of papers dealing with measurement techniques and protocols.

A comprehensive diagnostic analysis of both short-lived and long-lived species in PAW can be found in [Hu et al. 2021] This review provides a complete description of the established and promising detection methods for RONS in PAW, particularly derived from the food sterilization system. In the field of plasma biomedicine, some authors have examined the possibility of applying colorimetric methods in the analysis of non-colorless media and stated their limitations [Veronico et al. 2021]. Protocol for measuring nitrate, nitrite and hydrogen peroxide in a liquid medium can be found in [Tornin et al. 2021] Advantages and limitations of some of the most commonly used detection techniques are given in [Bruggeman et al. 2016].

The following sub-chapter presents an overview of photometric methods used in the field of plasma agriculture. The review was made on the basis of previously published literature and the answers of the participants of the working groups who filled out the questionnaire.

Methods for measuring RONS

H_2O_2

1. The titanium oxysulfate (TiOSO4) assay

The method is based on absorbance measurement of yellow colored pertitanic acid formed in reaction of hydrogen peroxide and titanyl ions in acidic solution (titanium (IV) oxysulfate solution):

$$Ti^{4+} + H_2O_2 + 2H_2O = H_2TiO_4 + 4H^+$$

The absorption peak value at 407nm can be measured by spectrophotometer or colorimeter.

This is a standard method, reproducible and pecise, quite simple to use and it is not time consuming, but requires making calibration curves. It has linear response up to mM concentration of H_2O_2 [Bruggeman et al. 2016].

It was used in research with plasma-treated distilled water [Lu et al. 2017], water with immersed biofilm [Zhou et al. 2019], saline solution and phosphate buffer solutions [Machala et al. 2013], but is not applicable fot the all food examples and non-colorless media.

The limitations of this methods are:

- not applicable for all treated liquids
- low sensitivity
- working with dangerous sulfuric acid used for preparing detection reagent
- Ti sulphate reagent precipitates in presence of strong buffered solutions (>20mM buffer) [Veronico et al. 2021].

Note: If the plasma-treated samples, besides H_2O_2, contain NO_2^-, then H_2O_2 solution should be fixed by sodium azide to prevent hydrogen peroxide decomposition in reaction with nitrites [Lukes et al. 2014]. Adding sodium azide under acidic condition reduces nitrites into molecular nitrogen [Machala et al. 2013].

2. Amplex Red Hydrogen Peroxide Assay Kit- chemiluminescence method

Amplex red reagent in presence of horse radish peroxidase (HRP) reacts with hydrogen peroxide to produce red fluorescent resorufin found to be proportional with H_2O_2.The product concetration is detected by using microplate reader. As with other colorimetric methods, it is necessary to make calibration curves.

The method has high sensitivity to H_2O_2 with low detection limit (~10 pM) and linear response up to μM concentration of H_2O_2 [Bruggeman et al. 2016].

Amplex red reagent can be used to analyze both treated pure water samples [Laurita et al 2021] and treated gelatin samples [Labay et al. 2020], agarose gels [Omran et al 2020] or cell culture media [Tornin et al. 2021].

The limitations of this methods are:

- not applicable for all treated liquids
- poorly reproducible in many cases
- poor specificity - there are many sources of interference (thiols, peroxynitrite [Bruggeman et al. 2016, Veronico et al. 2021]
- air sensitve
- peroxidase is light sensitive
- cost

3. The copper-phenanthroline assay

In this method, hydrogen peroxide in the presence of phenanthroline derivative (2,9-dimethyl-1,10-phenanthroline) reduces copper (II) ions to copper (I) ions resulting in the formation of an yellow-orange-coloured complex. The absorbance is measured photometrically at peak wavelength of 455nm.

The assay is fast one-step process that works at neutral pH, but requires care to be properly applied and high-quality reagents. H2O2 in the reaction with the detection reagent behaves as a reducer rather than an oxidant. Therefore, oxidative species from plasma-treated samples do not interfere in this method. [Veronico et al 2021].

The limitations of this methods are:

- low sensitivity
- H2O2 may decompose the yellow copper(I) complex and dillution is required.

Two more methods for hydrogen peroxide measurements can be found in the literature, but the surveyed researchers did not mention their advantages and limitations.

4. **The ammonium metavanadate (NH_4VO_3) method** – that was used for H_2O_2 measurements in both treated clean and wastewater [Park et al. 2017, Matra et al. 2020],
5. **The potassium iodide (KI) method** for analysis in biological samples [Aleksieva et al. 2001, Junglee et al. 2014]

$\mathbf{NO_2^-}$, $\mathbf{NO_3^-}$

1. Griess assay

The most commonly used method for determining the concentration of nitrite.

In acidic solution NO_2^- reacts with Griess reagent to produce red-violet azo dye with absorption maximum at 540nm/525nm, which can be determinated photometrically. The calibration curves are made with sodium nitrite solution.

Griess reagent: sulfanilamide + N-(1-naphthyl)-ethylene diamine dihydrochloride + phosphoric acid in water [Tornin et al. 2021, Bruggeman et al. 2016].

The method is very specific and precise, with good reproducibility and sensitivity. It is also good for measurements of low concentrations. It was emplyed for the analysis of a wide range of treated targets: distilled water, tap water [Kučerová et al. 2021], buffered solution

[Machala et al. 2018], gelatin solution [Labay et al. 2020], cell culture media [Tornin et al. 2021].

The limitations of this methods are:

- applicability in complex media matrixes
- interferences from many biomolecules, in some cases need of baseline correction
- Interferences with H_2O_2 in acidic conditions
- Toxic
- *pH dependence*

This method is also used for determination of nitrates - after previus reduction of nitrates to nitrites. Reducing agents are used for conversation of NO_3^- to NO_2^-:

- vanadium (III) chloride [García-Robledo et al. 2014
- nitrate reductase enzyme [Machala et al. 2019]
- Cd-reduction

The limitations or this multistep method are:

- reducing agents can interact with other RONS from solution or with buffer
- V and Cd are toxic
- time consuming

2. 2,6-xyleon assay

This is direct method for NO_3^- detection, which is based on nitration of 2,6 dimethylphenol (also known as 2,6 xyleon) to 4-nitro-2,6 dimethylphenol with absorption maximum at 337nm.

The method is used for nitrate detection in different liquids: distilled water [Luet al. 2017], river water and wastewater [Montgomery et al. 1963], cell culture media [Tornin et al. 2021].

The limitations or this multistep method are:

- It works in extremely acidic conditions that convert all NO_2^- to NO_3^-. In that case NO_2^- measurements are needed and NO_2^- concentration must be subtracted.
- It works for higher NO_3^- concentrations
- interferences with some components of cell culture media
- poor precision in some cases

Methods used in the detection of different species

1. UV-VIS spectroscopy

The method where the concentrations of multiple reactive species in the liquid are measured from direct absorption of each species and calibration curves previosly made for each measured species. Beer–Lambert's law is used to determine the concentration:

$A_\lambda = \varepsilon l c$

where A_λ is absorbance and ε is the molar absorptivity of the chemical species at a certain wavelength, l is the optical path length, and c is the concentration of certain species.

One important advantage of this method is possibility to measure in situ while plasma running with no waste of the sample.

The disadvantages of this methods are:

- use of chemicals, expensive reagents
- time consuming
- direct absorption only for high concentrations of NO_3^-
- not applicable for some types of treated liquids (food examples)
- insufficiently precise when many different RONS species are present in the liquid

A detailed description of the method for measuring RONS is given in [Liu et al. 2019, Oh et al. 2015, Oh et al. 2017, Girard et al. 2016].

2. Semi-quantitative test strips

The semi-quantitative method is the simplest and fastest colorimetric method that allows the simultaneous measurement of different species. If refractometers (test strip readers) or digital colorimetry are used instead of the color chart, quantitative analysis and higher accuracy are possible [Hu et al. 2021].

3. Digital colorimetry

Digital colorimetry is a method for analyzing data measured by some other technique. The method is based on the use of smart devices that scan the colored field and software that analyzes color using a color model [Woolf et al. 2021].

Survey description and summary

A questionnaire (Google Form) about the hands-on experience of researchers was made for participants of the CA19110 dealing with the diagnostics of plasma-treated liquid samples. Their problems and the ways they overcame them are part of this report.

The form consisted of 20 questions and was divided into four sections. In the first one, data was collected about researchers* and the subject of their investigation (types of plasma sources and treated targets). The remaining sections were related to one of the three reactive species: NO_2^-, NO_3^-, H_2O_2. They included questions about measurement methods, their advantages and disadvantages according to the properties of the treated samples, availability and cost-effectiveness. The participants cited publications in which they applied some of the techniques or which dealt with this topic.

The chart in Figure 1 shows the answers on question "*What samples do you expose to plasma in experiments*?" The offered answers could be selected and/or another answer could be added. Water is the most common choice of target, but in a significant number of invastigations more complex solutions such as growth media, cell culture media, agar, and waste water are also studied.

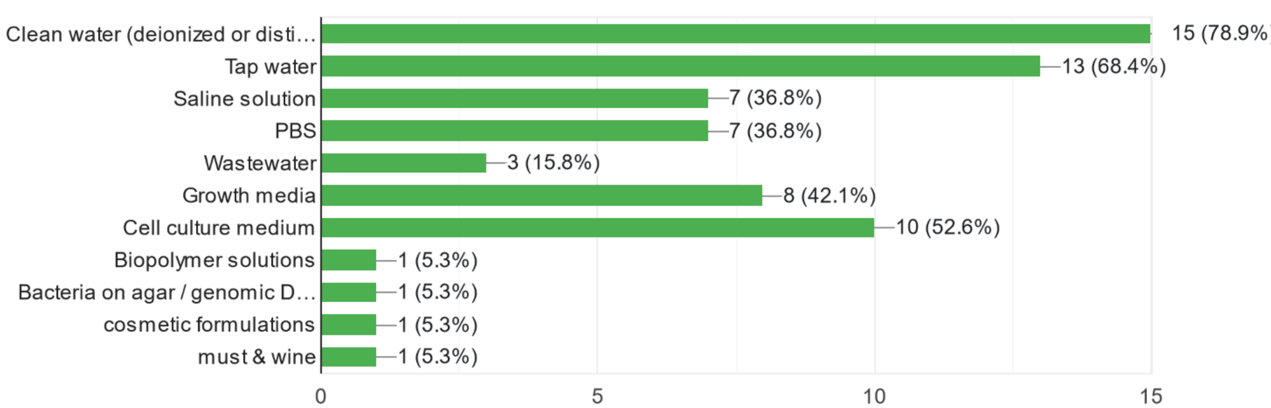


Figure 1. Plasma treated liquids. The first answer in the chart is Clean water (deionized or distilled water), PBS – phosphate-buffered saline, the nineth answer is Bacteria on agar/ genomic DNA in liquide.

Plasma sources and type of discharges used in research in the field of plasma agriculture are:

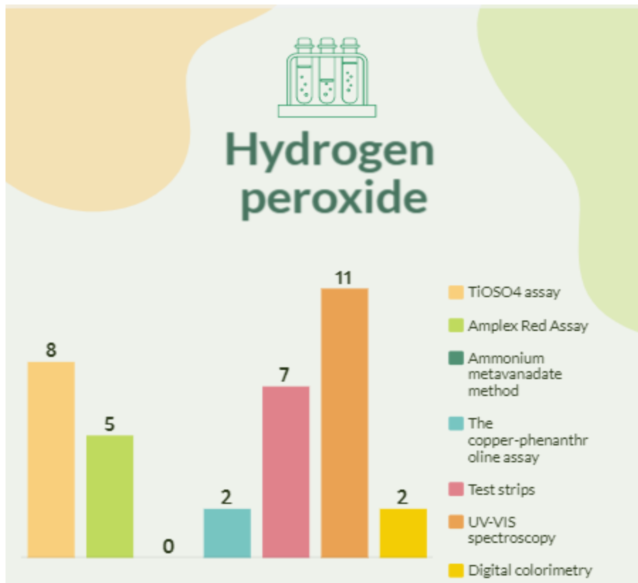
- Atmospheric pressure plasma jet
- Streamer corona discharge
- Surface dielectric barrier discharge
- DC pulsed plasma discharge
- RF plasma
- RF plasma + droplets
- Plasma-aerosol reactors
- Volume barrier dielectric discharge
- Transient spark systems
- Gliding arc discharges
- Low pressure plasma

UV-VIS spectroscopy, Griess assay and semi-quantitative test strips are the three most used measurement techniques.

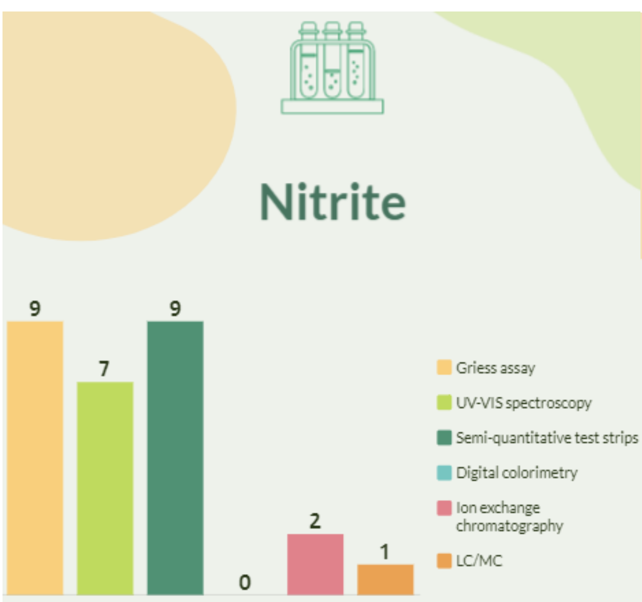
Figure 2 shows the answers on questions "What measurement technique do you use to detect hydrogen peroxide/nitrate/nitrire in plasma treated samples?".

In the 3 sections of the questionnaire related to the detection of RONS, the respondents stated the reasons why they chose certain methods. They also mentioned the problems they encountered during the measurement. Tables 1-4 show the advantages and disadvantages of each method. The answers are divided into four groups named: general (Table 1), measurement (Table 2), target (Table 3), and cost (Table 4).

a)



b)



c)

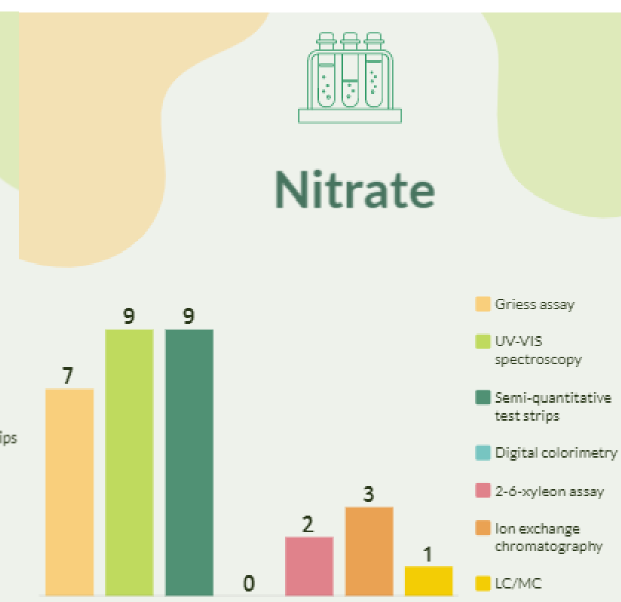


Figure 2. The most used measurements techniques for a) H_2O_2, b) nitrite, and c) nitrate detection, based on the number of respondents.

Table 1. Pros and cons of eight methods related to general user experience.

	Pros and cons	$TiOSO_4$ assay	Amplex Red assay	Cu-phenanthroline assay	Griess assay	2,6-xyleon assay	Test strips	UV-VIS	Digital colorimetry
GENERAL	fast	☑	☑	☑	☑		☑	☑	☑
	well documented	☑	☑		☑		☑		
	easy to use /simple	☑	☑	☑	☑	☑	☑	☑	☑
	standard technique	☑			☑				
	use of (dangerous) chemicals	X			X			X	
	not knowing how do they work chemically						X		
	time consuming	X	X		X			X	
	Calibration curves to be done	X	X		X			X	
	requires care to be properly applied			X				X	
	multistep method				X				

Table 2. Pros and cons related to general user experience and detection limit of the methods.

Pros and cons		$TiOSO_4$ assay	Amplex Red assay	Cu-phenanthroline assay	Griess assay	2,6-xyleon assay	Test strips	UV-VIS	Digital colorimetry
MEASUREMENT	reproducible	☑		☑	☑	☑			
	selectivity	☑							
	minimum interferences	☑							
	precise	☑	☑		☑			☑	
	accurate							☑	
	good sensitivity		☑		☑				
	low sensitivity	X		X			X		
	not too accurate	X					X		
	insufficiently precise				X*	X	X	X	X
	detection limit					X	X		
	poorly reproducible		X						
	non-selective				X				

Table 3. Pros and cons of the methods according to the applicability in the analysis of different treated liquids.

Pros and cons		$TiOSO_4$ assay	Amplex Red assay	Cu-phenanthroline assay	Griess assay	2,6-xyleon assay	Test strips	UV-VIS	Digital colorimetry
TARGET	work in acid pH	☑	☑			☑	☑	☑	
	work in neutral pH		☑	☑	☑			☑	
	not applicable for all treated liquids	X	X	X		X	X	X	X
	precipitates in presence of strong buffered solutions	X							
	absorption overlaps in complex media matrixes	X		X	X	X	X		
	many sources of interference			X		X			
	pH dependence					X	X	X	

Table 3. Pros and cons of the methods according to cost of reagents or equipment.

Pros and cons		$TiOSO_4$ assay	Amplex Red assay	Cu-phenanthroline assay	Griess assay	2,6-xyleon assay	Test strips	UV-VIS	Digital colorimetry
COST	not expensive	☑					☑	☑	
	no expensive equipment is required	☑			☑		☑	☑	☑
	expensive	X	X						
	high-quality reagents			X				X	

Conclusion and plans for future collaborations

The analysis of the available literature complemented with the direct experiences of the researchers collected through a questionnaire provided a comprehensive data set of all problems and obstacles in the measurements of reactive species in plasma-activated liquids. The report represents a starting point for someone who wants to use low-temperature atmospheric pressure plasma for the treatment of liquids for further desired effects on biological samples. Also, in future collaborations and treatment of different liquids, this report will systematically present problems, solutions, and challenges in this field to help the wide research population.

The questionnaire that was filled out by more than 20 participants from 13 countries (see Figure 3) who are members of different working groups (WG1 -WG5). The form allowed the research community to submit their published work to a centralized location and also the report will allow them to search through past work. It is the important first step in generating the research directions, provide useful information that can be exploited for review paper(s), and that will provide information for deliverables of the COST action:

- WG3 - Low-temperature plasma treatment of plants

    T3.1. PAW Treatment of plants and soils.

•	WG4 - Plasma treatment of agricultural wastewater, growth media, manure and production of PAW

T4.1. Wastewater treatments and decontamination of water by atmospheric pressure low-temperature plasma (LTP).

T4.2. Plasma treatment of water for the creation of PAW.

T4.3. Plasma-assisted treatment of the plant growth media (soil, water) and manure/organic waste.

Figure 3. The questionnaire was filled out by members from following countries: Ireland, UK, Estonia, Spain, France, Italy, Slovenia, Croatia, Germany, Czechia, Slovakia, Romania and Serbia.

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