Universidade de Aveiro Departamento de Química
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Margarida Menezes Amador Sacramento

Implementação e Validação do Método de Determinação do Carbono Orgânico Total em Matrizes Líquidas da Indústria de Pasta e Papel

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Relatório de estágio apresentado à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizado sob a orientação científica do Doutor João Oliveira, Professor Associado do Departamento de Química da Universidade de Aveiro e da Doutora Micaela Soares, Técnica Superior de Laboratório do Instituto de Investigação da Floresta e do Papel (RAIZ).

Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive.

(Fernando Pessoa)

o júri

presidente

Doutora Mara Guadalupe Freire Martins
Investigadora Coordenadora em Regime Laboral da Universidade de Aveiro

Doutora Ivone Valente de Oliveira
Técnica Superior do RAIZ – Instituto de Investigação da Floresta e Papel

Professor Doutor João António Baptista Pereira de Oliveira
Professor Associado da Universidade de Aveiro

agradecimentos

Agradeço à direção do RAIZ e à *The Navigator Company* por ter possibilitado a oportunidade de realização deste estágio, que tanto me fez evoluir, tanto a nível académico como pessoal e por fornecer todo o equipamento necessário à sua realização.
Ao meu orientador, Professor João Oliveira, pelo conhecimento científico, sugestões e enorme disponibilidade e simpatia que demonstrou para comigo ao longo deste trabalho.
À minha orientadora, Doutora Micaela Soares, pelos ensinamentos transmitidos, orientação, sugestões e paciência no esclarecimento das minhas muitas dúvidas ao longo de todo este percurso.
Aos analistas do RAIZ, em especial à Teresa, ao Licínio e ao Sr. Vale, por todo o apoio que me deram e por todos os momentos de boa disposição no laboratório.
Aos meus colegas de gabinete durante a realização deste estágio, Marta, Joana, e João, pela ajuda, boa disposição e por me fazerem sentir em casa.
Aos colaboradores do RAIZ com que fiz amizade, Célia, Margarida, Sandra, Isabel, Flávia e Filipe, pelos divertidos momentos de descontração e por tantas vezes tornarem mais leves os meus dias de trabalho.
À Liliana, que como eu desenvolveu a sua tese de mestrado no RAIZ, pela ajuda, companheirismo e momentos de partilha mútua dos nossos problemas quando estes pareciam maiores do que as soluções.
À minha família, em especial ao meu pai, mãe e avó, pelas palavras de motivação e apoio, e por serem os responsáveis por tudo o que sou hoje.
Aos meus amigos, em especial à Margarida e à Inês, pela disponibilidade para me ouvirem e apoiarem em todos os momentos de dificuldade.
Sem todos os aqui referidos e muitos outros não teria sido possível a realização deste trabalho. A todos o meu sincero obrigada!

palavras-chave Validação, carbono organico total (COT), relação CQO/COT, efluentes, indústria da pasta e papel (P&P)

resumo

A indústria da pasta e do papel (P&P) é uma das mais importantes do mundo e também uma das maiores consumidoras de água. Um elevado consumo de água traduz-se na produção de um elevado volume de efluentes, sendo uma das suas características a elevada concentração de compostos orgânicos, que podem ter um impacto ambiental negativo nos sistemas aquáticos recetores, devido à diminuição da concentração de oxigénio. A carência química de oxigênio (CQO) é usada para monitorizar a matéria orgânica presente nas águas residuais, mas devido à produção de resíduos perigosos durante a análise, tem aumentado o interesse na sua substituição pelo carbono orgânico total (COT).

Após a aquisição de um analisador de COT pelo laboratório do RAIZ, o presente estudo consistiu em validar o método direto (NPOC) para análise de COT. Os parâmetros avaliados foram a gama de trabalho, linearidade, sensibilidade, limite de quantificação (LQ) e precisão. A gama de trabalho da fábrica, [0,3–3,0] µg foi testada, tendo-se confirmado a condição de homocedasticidade. A curva de calibração, da área do pico de NPOC em função da massa de NPOC, apresentou boa linearidade, comprovada estatisticamente e pelo coeficiente de correlação (r = 0,99989). A sensibilidade, avaliada pelo declive da curva de calibração, foi satisfatória. Além disso, o desvio médio do declive (4,9 %) relativamente ao de fábrica ficou dentro do critério de aceitação estabelecido, i.e., 5 %. O limite de quantificação (LQ - 0,24 µg) mostrou-se adequado para o método.

A repetibilidade e a precisão intermediária (PI) foram avaliadas para as matrizes de efluente não tratado e tratado. A primeira foi aceitável para ambas as matrizes, com ambos os valores de CVr inferiores a 10 %, 4,3 % para efluente não tratado, e 8,9 % para efluente tratado. Os limites de repetibilidade foram também definidos para os efluentes não tratados (Δr=0,093 µg) e tratados (Δr=0,071 µg). A PI foi avaliada por cartas de controlo de amplitudes que mostraram que o processo estava sob controlo, para ambos os tipos de amostras. A reprodutibilidade (CV_R=8,2%) foi avaliada através da participação num ensaio interlaboratorial. O valor consensual de TOC da amostra de água residual foi de 91,9 mg/L e um score-Z de 0,12 foi atribuído ao RAIZ. Além disso, a incerteza expandida (U) do método NPOC foi calculada para efluente não tratado (U=28,3 %) e efluente tratado (U=22,3 %).

Foi realizado um estudo para estabelecer fatores de correlação COD:NPOC, para as matrizes de efluente não tratado e tratado, da indústria de P&P. O modelo do tipo y=bx+a foi usado para estabelecer equações para ambas as matrizes, tendo sido obtidos os valores de b de 3,02 e 2,36, respetivamente.

A validação do método da diferença (DM) para análise de COT também foi iniciada no presente trabalho. No entanto, a calibração de fábrica provou não estar em vigor e foi realizada uma nova calibração que foi devidamente validada pela análise de um material de referência certificado.

Finalmente, devido à capacidade de análise de azoto total ligado (TNb) do analisador de COT do RAIZ, o equipamento foi calibrado para a análise da matriz águas superficiais.

keywords

Validation, total organic carbon (TOC), COD/TOC relationship, effluents, pulp and paper (P&P) industry

abstract

The pulp and paper (P&P) industry is one of the most important industries in the world and also one of the largest consumers of water. High water consumption implies the production of a high volume of effluents, being one of its characteristics the high concentration of organic compounds, which can have an environmental impact in the receiving aquatic systems, due to the decrease in the oxygen concentration. Chemical oxygen demand (COD) is widely used for monitoring the organic matter present in wastewater but, due to the production of hazardous wastes during the analysis, has increased the interest in replacing this method by total organic carbon (TOC).

Following the acquisition of a TOC analyser by the RAIZ laboratory, the present study consisted in validating the direct method (NPOC) for TOC analysis. The evaluated parameters were the working range, linearity, sensitivity, limit of quantification (LQ) and precision. The factory working range, [0.3–3.0] µg was tested, having been the homoscedasticity condition of the data confirmed. The calibration curve, of the NPOC peak area as a function of NPOC weight, showed good linearity, proven statistically and by the correlation coefficient (r=0.99989). Sensitivity, assessed by the slope of the calibration curve, proved to be satisfactory. Also, the average slope deviation (4.9 %) from the factory slope was within the established acceptance criteria, i.e., 5 %. The quantification limit (LQ – 0.24 µg) proved to be appropriate for the method.

The repeatability and intermediate precision (IP) were assessed for untreated and treated effluent. The former was acceptable for both matrices, with both CVr values below 10 %, 4.3 %, for untreated influent, and 8.9 %, for treated effluent. The repeatability limits were also defined for untreated (Δr=0.093 µg) and treated (Δr=0.071 µg) effluents. The IP was evaluated by range control charts, which showed that the process was under control for both types of samples. Reproducibility (CV_R=8.2 %) was assessed through the participation in an interlaboratory test. The TOC consensus value of the wastewater sample was 91.9 mg/L and a Z-score of 0.12 was attributed to RAIZ. The uncertainty of the NPOC method was also calculated for both untreated (U=28.3 %) and treated effluent (U=22.3 %) matrices.

Also, a study was carried out to establish COD:NPOC correlation factors for untreated and treated effluents from the P&P industry. The y=bx+a model was used to establish equations for both matrices, and b values of 3.02 and 2.36 were obtained, respectively.

The validation of the difference method (DM) for TOC analysis was also started. However, the factory calibration proved not to be in effect and a new calibration was performed and properly validated by the analysis of a certified reference material.

Finally, due to the total nitrogen (TNb) analysis capability of the TOC analyser purchased by RAIZ, the equipment was also calibrated for the surface waters matrix analysis.

Contents

Abbreviations

a	Coefficient of the calibration function
Abs	Absorbance
Adt	Air dry tonne (of cellulose pulp)
AOX	Adsorbable organic halogens
ASTM	American Society for Testing and Materials
b	Coefficient of the calibration function
b'_i	Recovery deviation
BOD	Biochemical oxygen demand
b_{rms}	The square root of the average deviations of the recovery tests
c	Coefficient of the calibration function
CC	Control chart
CL	Central limit of CCs
COD	Chemical oxygen demand
CRM	Certified reference material
CS	Control standard
CV*r*	Coefficient of variation of repeatability
$\mathbf{CV}_R$	Coefficient of variation of reproducibility
DF	Degrees of freedom
DM	Difference method
DOC	Dissolved organic carbon
$\mathbf{DS}^2$	Difference of variances
EC	Electrochemical cell
ECF	Elemental chlorine-free (bleaching)
En	Standard error
Er	Relative error
f	Dilution factor
F_{exp}	The calculated value for the *F*-test
F_{critical}	Tabled value from Fisher distribution
GHG	Greenhouse gas (effect)
HTC	High-Temperature Combustion (method)
i	Subscript of the concentration levels (i = 1, 2, ..., N)

IP	Intermediate precision
IPAC	Portuguese Accreditation Institute
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
k	Expansion factor
L_{CL}	Lower control limit of CCs
LD	Limit of detection
LQ	Limit of quantification
m_1/m_2	Weight of the first and second duplicates
MPE	Maximum permissible error
m_{sample}	NPOC weight of the sample
$m_{spiked\ sample}$	Carbon mass of the spiked sample present in the injection volume
Mt	Million tonnes
n	Number of recovery trials, replicates and size of a subgroup in a CC;
N	Number of calibration standards; the number of tested samples
N_m	Mean value of the recovery trials (%)
NDIR	Nondispersive infrared (detector)
NPOC	Non-purgeable organic carbon
ODR	Orthogonal distance regression
p	Number of participating laboratories
PCI	Creative Science Park
P&P	Pulp and paper (industry)
PG	Test value calculated for the Mandel's test and *F*-test
POC	Purgeable organic carbon
r	Correlation coefficient
Δr	Repeatability limit
R	Reproducibility limit; range
RAIZ	Forest and Paper Research Institute
REC	Individual sample recovery value
R_m	Moving range
r_w	Correlation coefficient of the WLR
s	Standard deviation

s^2	Variance
$Si_{( )}$	Standard deviation of the IP
s_i^{-2}	Inverse of variance
S^2_{Li}	Interlaboratory variance
$s_{precision}$	Standard deviation of precision
S_{ri}	Standard deviation of repeatability
S_{Ri}	Standard deviation of reproducibility
S^2_{ri}	Repeatability variance
S^2_{Ri}	Reproducibility variance
S^2_{wi}	Variance associated with the results of each laboratory
S_{xlab}	Standard deviation associated to X_{lab}
S_{y2}	Residual standard deviation obtained by second order regression calculation
$S_{y/x}$	Residual standard deviation obtained by first order regression calculation
$S_{(y/x)w}$	Residual standard deviation of the WLR
t	Number of samples analysed in duplicate
t_{exp}	Experimental *t*-value
$t_{critical}$	Tabled *t*-value
TC	Total carbon
TCF	Total chlorine-free (bleaching)
TIC	Total inorganic carbon
TNb	Total bound Nitrogen
TOC	Total organic carbon
U	Expanded uncertainty
u'_{add}	Uncertainty associated with the concentration of the added analyte
u_{atomic}	Standard atomic uncertainty
u_c	Combined standard uncertainty
U_{CL}	Upper control limit of CCs
u'_{conc}	Relative standard uncertainty associated with the spiking solution concentration
U_{lab}	uncertainty associated to X_{lab}
u_m	Uncertainty associated with a weighing
u'_m	Standard uncertainty of the total mass (tare and reagent mass)
$u_{precision}$	Standard uncertainty associated with precision

$u_{preci,\ CS}$	Standard uncertainty of precision associated with control standards
$u_{preci,\ samp}$	Standard precision uncertainty associated with sample replicates
$u_{\mathbf{pur}}$	Standard uncertainty associated with purity
U_{ref}	Uncertainty associated with the X_v
$u_{\mathbf{tare}}$	Standard uncertainty associated to the tare of the container
$u_{\mathbf{trueness}}$	Standard uncertainty associated to trueness
u_v	Relative standard uncertainty associated with volume
$u_{v,\ rep}$	Random uncertainty component of the volume
$u_{v,\ sys}$	Systematic uncertainty component of the volume
$\mathbf{V_{injection}}$	Injection volume
$\overline{x}$	Mean of *x* values
$\widetilde{X}$	Median
$\overline{X}_0$	Mean of a series of independently prepared blanks or trace standards
x_i	Individual values of the standards concentration (i=1, …, N)
X_{lab}	Experimental value obtained in the CRM analysis or interlaboratory test
X_v	True value of a CRM or interlaboratory sample
w_i	Weighting factor
WLR	Weighted linear regression
WLSLR	Weighted least squares linear regression model
y	Value of a measurand
$\overline{y}$	Mean of *y* values
y_i	Individual values of the instrumental signal of each concentration standard x_i
$\hat{y}_i$	Estimated value from y_i results of the corresponding x_i
$(y_{j1} - y_{j2})$	Difference between the duplicates of the sample j
σ_0	Standard deviation associated to $\bar{X}_0$

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1. Introduction

1.1. Background and motivation

The Pulp and paper (P&P) industry is very water-dependent, once water is an essential component of the P&P process because it is used in almost all process stages. Consumption of water used to vary between 200 m^3/ton and 1000 m^3/ton of paper. However, due to an increase in environmental concerns and social responsibility, efforts have been made by this industry in order to reduce water consumption.

High water consumption necessarily results in the production of a high volume of effluents. One of the main characteristics of these effluents is the high concentration of organic compounds, resulting from the contribution of all the P&P process stages. The problem with organic compounds in water is the depletion effect they exert on the receiving aquatic systems due to the deviation of the oxygen for its degradation, decreasing the dissolved oxygen concentration and affecting the living organisms. Although organic compounds are naturally present in water, the P&P industry contributes to the release of effluents with a high chemical oxygen demand (COD) value. Consequently, process and wastewaters must be monitored, with the purpose of minimizing the discharge of hazardous compounds into the environment.

COD is a measuring method that represents a quick and simple way to determine the organic matter concentration in water, being a good indicator of the pollution by these compounds. However, COD assay generates hazardous chemicals so, in order to overcome this disadvantage, new analytical techniques are arising in order to control these compounds in the water. Total organic carbon (TOC) is a more fast and precise technique, besides being the most direct representation of the total concentration of organic carbon in water, since it does not take into account other organic and inorganic bound elements. Additionally, it surpasses the hazardous reagents usage problem verified in the COD test, representing an alternative to the latter.

Despite the existing tendency of replacing COD by TOC, most of the established values in the legislation are represented as COD values, which implies that the use of the TOC technique must be accompanied by the establishment of a correlation between these two parameters.

The Forest and Paper Research Institute (RAIZ) is an accredited laboratory according to the normative reference NP EN ISO/IEC 17025:2018, whose main activity consists of the improvement of the competitiveness of the national forestry industry, having as main associate *The Navigator company.* One of the activities of RAIZ comprises the control of effluents from this industry, namely organic matter values. In this way and following the acquisition of a TOC equipment by this laboratory, the present work comprises the validation and implementation of the TOC analysis

method, as well as the establishment of the COD/TOC correlation in some of the effluents from the P&P industry.

1.2.The Forest and Paper Research Institute - RAIZ

The Forest and Paper Research Institute or RAIZ as it is generally named, is a non-profit research and Technology Transfer Center, being one of the entities of the National Scientific and Technological System. It is associated with *The Navigator company* and the Universities of Aveiro, of Coimbra and the High Institute of Agronomy of the University of Lisbon.

The main activities of RAIZ are:

- Investigation and Forest Consulting;
- Research and Technology Consulting;
- Technological Vigilance;
- Demonstration, Scale-Up and New Business;
- Formation.

In order to accomplish these activities, RAIZ comprises three research centers, two in Aveiro, *Quinta de São Francisco* and a facility in the Creative Science Park (PCI), and the other one in *Herdade de Espirra*, situated in Pegões.

The main goal of RAIZ is to improve the competitiveness of the national forestry industry and, as a consequence, the competitiveness of the Navigator company, in order to transform scientific knowledge in services, technology and products in the fields of forest, pulp, paper and forest-based biorefineries, always from the sustainable point of view.

The Central Laboratory of RAIZ, located in *Quinta de São Francisco*, is by the Portuguese Accreditation Institute (IPAC) according to the normative reference NP EN ISO/IEC 17025:2018 and its research methods meet the international standards, according to with the American Society for Testing and Materials (ASTM), the International Organization for Standardization (ISO), etc. It provides specific services for the industry, research and development, namely studies and analyses on wood, pulp, paper, water, effluents, solid waste, sludge, soils and plant material matrices. In addition, RAIZ is able to simulate, on a laboratory scale, the pulp and paper manufacturing process of the Navigator company, allowing the improvement of the various stages of the process.

Moreover, studies on eucalyptus pests and diseases are being developed in RAIZ, in the Forest Protection Laboratory. This RAIZ section is responsible for biological control with the major goal of controlling the main pest of the eucalyptus in the Iberian Peninsula, *Gonipterus platensis.*

The Research, Development and Production Nursery, as well as the Biotechnology laboratory, are located in Espirra. The former carries out research trials testing factors susceptible to influence the cloning process and also gather information about the rooting capacity of several materials. The

latter produces clonal and seminal plants for trials and clonal plants for The Navigator Company's Nurseries large-scale production.

The Forest and Paper research institute is an integral part of national and international research projects in order to be at the forefront of knowledge in the areas as forestry, pulp, paper and biorefineries (1).

1.3. Pulp and paper industry and production

P&P industry is one of the largest and most important industries in the world. As it can be observed in **Figure 1**, paper demand and consequently, paper production will tendentially increase, which makes the P&P industry a growing market. This growth represents, between 2006 and 2050, an increase from 365 million tonnes (Mt) to 700 Mt - 900 Mt (2).

In the lead as major exporters and importers are the United States and Germany. Regarding Portugal, this industry is responsible for the creation of 92,000 jobs, represents 2 % of the gross domestic product and 10 % of exports (3,4).

In terms of the environmental impact, this industry is one of the largest contributors to total energy and water consumption, and greenhouse gas (GHG) emissions worldwide. Actually, the P&P industry is responsible for near 5.7 % of global energy end uses, occupying the fourth place among industries (2), produces about 40 % of the worldwide industrial wastewater and originates 9 % of GHG emissions of manufacturing industries. Taking this into account, great research efforts are being made in order to reduce the environmental impact of this industry (5,6).

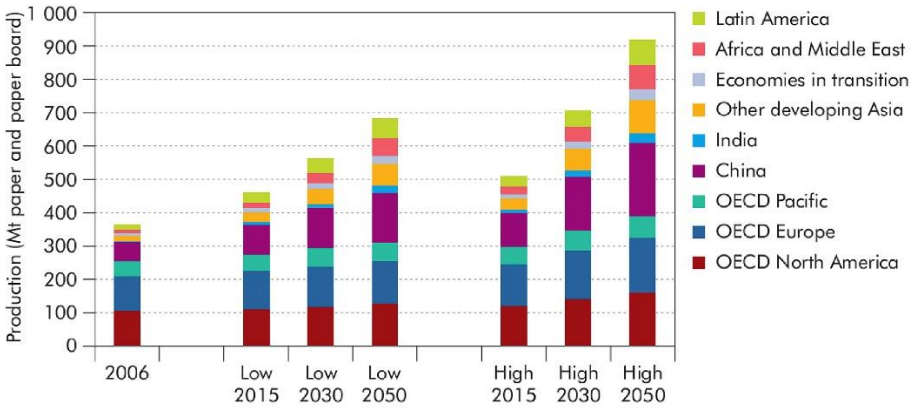

Figure 1 – Paper and paperboard production worldwide. OECD – Organization for Economic Co-operation and Development (2).

In the P&P process, several wood species have been the predominant raw material used. In the eastern European countries, which is the Portuguese case, *Eucalyptus globulus* is the main raw material used (**Figure 2**). This type of wood allows the kraft process to have the highest yield and the best pulp when it comes to quality. However, in recent years, several non-wood fibers, such as

straw from canola, rice and wheat, residual vines hoots, sunflowers talks, and sugarcane bagasse, have been used as raw material to produce cellulose. The choice of raw material is an important step because it has consequences in the waste generation and in cellulose production during the process (7).

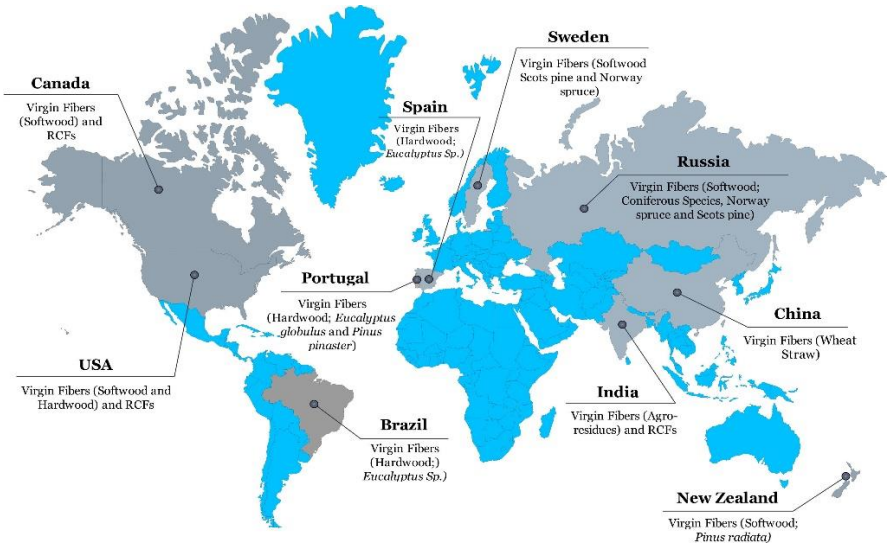

Figure 2 – P&P main raw materials in a few major P&P producers (8).

Concerning the paper production process, it comprises several stages, namely wood handling and debarking, pulping, bleaching and papermaking. The process starts with raw material preparation, namely wood handling and debarking. The wood logs are delivered to the factory where the bark is first removed by means of a drum or a hydraulic debarker. The bark is then used to produce steam through burning. After debarking, the logs are processed into chips and screening is performed to separate the acceptable size chips for pulping from the small and oversized ones. The fines are burned together with the bark to produce steam, while the larger ones are resized in a "rechipper". Then, the properly sized chips are transported through conveyor belts or in pipes using an airveying system to the digester.

In the pulping stage, the main goal is to extract the cellulose fibers from the rest of the raw material (e.g. wood or straw). This can be achieved by chemical, mechanical and semi-chemical techniques. In this review, more attention will be given to the kraft pulping process since it represented half of the pulp produced worldwide in 2016 (4,5).

Figure 3 shows a simplified scheme of this dominant pulp production process. It consists of a combination of the above-mentioned techniques in order to obtain a soft pulp, suitable for the production of a wide range of finished or semi-finished paper products (4,9).

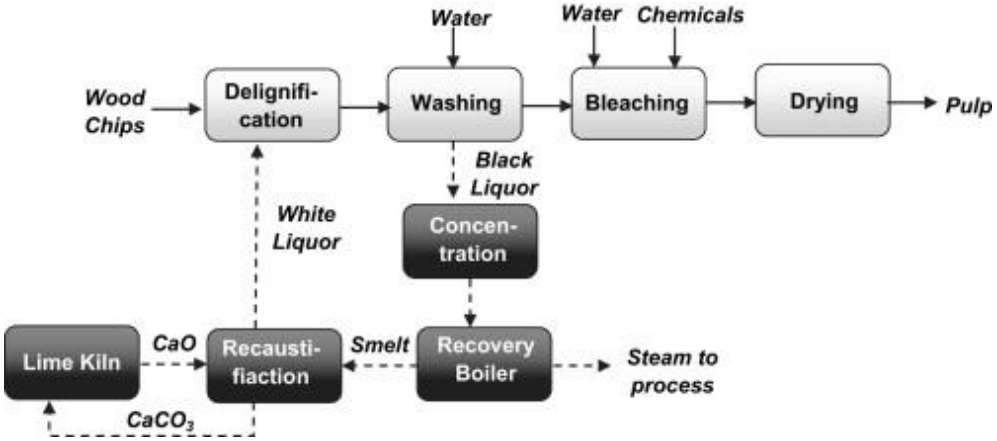

Figure 3 – Diagram of the kraft pulp production process (8).

The core process consists of a chemical delignification carried out in a digestor, allowing the segregation of the cellulose fibers from the raw material matrix, giving rise to the pulp (9). To accomplished this, sodium hydroxide (NaOH) and sodium sulphide (Na_2S) (white liquor) are mixed with the raw material and heated at temperatures from 150 °C to 165 °C. In this step, temperature maintenance is important otherwise, if it is too high, the chips are irregularly delignified (4).

After wood dissolution in the white liquor, cellulose fibers are washed, chemically bleached, drained, pressed and thermally dried. The liquid fraction originated after washing, the black liquor, is collected, concentrated and burnt in the recovery boilers to create steam. The spent inorganic fraction, composed of sodium carbonate (Na_2CO_3) and Na_2S, is collected and recaustified with quick lime, produced in a lime kiln, to recycle the white liquor (9). Thus, the kraft recovery system allows the reuse of the inorganic pulping chemicals and the use of the organic part for steam and power generation. Bearing in mind that about 200 Mt of the black liquor dry solids are burned every year, allowing the recovery of 50 Mt of cooking chemicals and the production of 700 Mt of high-pressure steam, this makes black liquor the fifth most important fuel and the first bio-fuel in the world (10).

With the aim of evaluating pulp quality, the kappa index is calculated. This is an essential parameter because it allows knowing the effectiveness of the digestion process of the wood, that is, the determination of the residual lignin content in the raw pulp (the achieved delignification degree) (4). Lignin is susceptible to suffer oxidation, in particular, its aromatic rings, by oxidizing agents, such as potassium permanganate ($KMnO_4$). Thus, the procedure for calculating the kappa index consists of the determination of the volume of a $KMnO_4$ solution required to oxidize a known amount of dry pulp, as is described in NP 3186/95. Additionally, the consumption of the $KMnO_4$ solution also depends on other variables such as the temperature and reaction time, as well as the amount and concentration of the added $KMnO_4$ solution (11).

As it was mentioned before, after pulping, the resulting brown pulp must be brightened and must undergo some changes before the papermaking stage. To achieve this, elemental chlorine-free (ECF)

or total chlorine-free (TCF) bleaching processes can be performed. The former comprises an acidic stage (pH 2–3), using ClO_2 and H_2SO_4, and an alkaline stage (pH > 10) with the use of NaOH, frequently blended with oxygen (O_2) and hydrogen peroxide (H_2O_2).

In the case of TCF, different combination patterns using H_2SO_4, O_3, O_2, chelating agents and/or H_2O_2 are performed (12). Due to its oxidizing capacity, H_2O_2 has been extensively employed in this process. This compound helps the delignification of chemical pulps during the bleaching stage, predominantly for decreasing the kappa number and increasing the brightness and stability of the produced pulps. In this case, chelating agents or acids are used to remove trace metal ions that interfere with the stability of the H_2O_2. Metal ions make the H_2O_2 consumption to increase and also promote the formation of radical species that can attack pulp carbohydrates, reducing pulp yield (13).

The bleaching process ends with pulp washing to remove the bleaching agents and hardly biodegradable compounds (7).

The ideal bleaching process would perform the oxidation by means of non-chlorinated substances, such as O_2, H_2O_2 and O_3. However, regardless of being less hostile to the environment than ECF, TCF represents only 5 % of the world's bleached chemical pulp production, mostly due to the increased costs, together with less strength and brightness of the produced pulp. Thus, nowadays ECF is considered the standard bleaching process (7).

Kraft pulping technique presents some characteristics that differentiate it from others. These are the capacity of handling almost all types of softwood and hardwood, the great strength of the produced pulp and the high magnitude of chemical recovery (about 97 %), which makes the process very profitable (10).

Papermaking is the final stage regarding paper production. The produced pulp is combined with materials like fillers, sizing agents, dyes and resins to give rise to the final paper sheet. Next, pressure is applied to the final mixture in order to dehydrate it, and air or heat are even used to dry the newly formed paper sheets (7).

1.4. Water usage

As was mentioned, the P&P industry is one of the most water-dependent industries. During the production process, water is used in nearly all the principal steps (**Figure 4**), since it is a carrier for the cellulose fibers after their extraction from the wood chips. It is also consumed for cleaning the equipment, as heat exchange fluid, as a lubricant, as steam and as a sealant in vacuum systems (14).

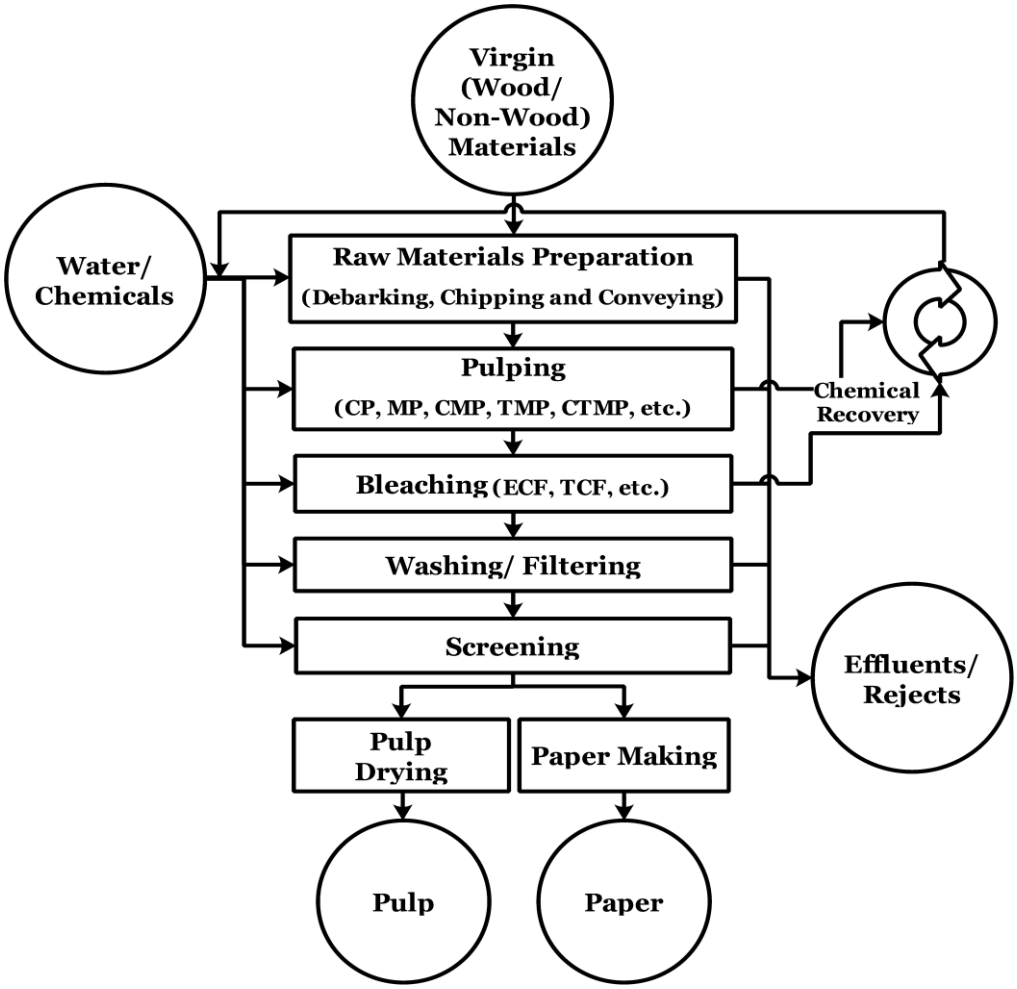

Figure 4 - Representation of the P&P production process with water usage (7).

P&P industry is responsible for controlling its water consumption due to the environmental restrictions imposed by the legislation and social responsibility. Factors that can affect water consumption by this industry are the production volume, the raw materials used and technologies applied in the pulp, papermaking and associated processes (13,14).

At the beginning of the last century, the total amount of water that was fed in the process varied between 200 m^3/ton and 1000 m^3/ton of paper. So, in order to increase efficiency and reduce the environmental impact, the P&P industry has been making important efforts to reduce the consumption of water resources through more rigorous management. These efforts have resulted in a decrease in the overall volume consumed by this industry to 5 m^3/ton up to >100 m^3/ton. For instance, in Germany, a reduction to 13 m^3/ton of produced paper has already been reported (7,8,14).

Although a great volume of freshwater is consumed, just a small part of the water fed into the production is lost. The remaining water is returned to the environment. As an example, in the United States, 11 % of the water fed into the system is lost by evaporation, while 88 % are returned to the aquatic systems and 1 % is incorporated into final products or solid waste (8), as shown in **Figure 5**.

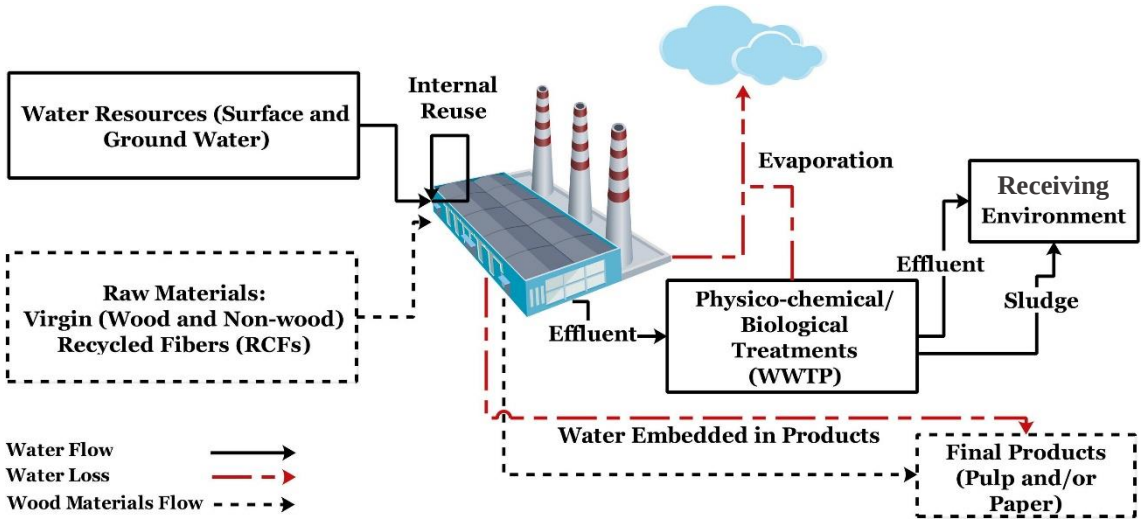

Figure 5 – Scheme of the water and raw materials flow, use and fate in a P&P mill (7).

Freshwater is usually obtained from ground and surface waters and before being used (e.g. as steam and water) it undergoes two pre-treatments. From the total of this freshwater that is being fed, 65 %, since it is in contact with the pulp, is screened and demineralized for steam production and for application in several process sections (**Table 1**). The remaining 35 % is screened for the purpose of cooling, scrubbing and housekeeping. Between Winter and Summer, the overall water consumption rises 18 % in order to counteract the greater heating of the equipment (9).

Table 1 – Water consumption by process section (8).

Treated water	**m^3/adt***	***Screened water***	**m^3/adt***
Delignification & Washing	10.1	Bleaching	24
Bleaching	30.7	Recaustification	4.6
Concentration	1.0	Non-process uses	11.1
Drying	10.7	Unaccounted water	6.4
Recaustification	2.0		
Deaeration	4.8		
Steam exports	0.0		
Boilers	0.2		
Non-process uses	4.6		
Total process consumption		110.1	

*adt – air dry tonne of cellulose pulp.

Besides environmental legislation, water scarcity and the need to reduce costs have been the main drivers for a significant reduction in the use of fresh water for the use of recycled water. However, this replacement can have some drawbacks. These can be associated with the possible risks of accumulating contaminants in process waters and others, which may arise due to the lack of knowledge of the chemistry involved. These risks can cause several problems in the process and in

the final quality of the product. Still, with technological advances, water recycling is a promising option (14).

1.5. Wastewater composition

Considering the volume of water used by the P&P industry, a large amount of effluent will also be generated. Actually, per ton of paper, the process generates from 10 m^3 to 50 m^3 of wastewater, corresponding to 90 % of the water used in the process (14,15).

As it is shown in **Figure 6**, this industry is the sector that produces the greatest amount of industrial effluent. In view of this, environmental legislation has imposed stricter limits to this industrial sector regarding the volume of wastewater produced and its toxicity (16).

The characteristics of the discharge effluents in the P&P industry are highly variable due to the dependence of parameters as the production scale, the raw materials (e.g. hardwood, softwood and recycled paper), the amount of circulating water and the chosen process technology and management practices that are used. Conventional wastewater treatment methods used have proven not to be totally efficient in pollutants removal. Consequently, efforts are being made by P&P companies in order to change to more effective techniques to meet the current environmental standards (7,16).

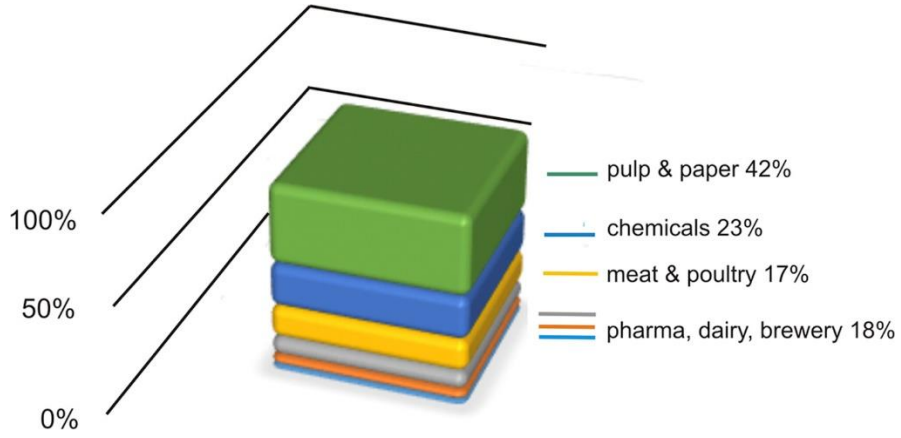

Figure 6 – Global production of wastewater in several industrial sectors (15).

As shown in **Figure 4** (page 7), almost all stages in paper manufacture are responsible for the increase in the overall amount of effluent produced. These effluents have characteristics of toxicity due to being rich in fibers, fillers and chemicals, which are derived from the raw materials or additives (e.g. surfactants, bleaching agents and biocides) used during the papermaking process (**Figure 7**).

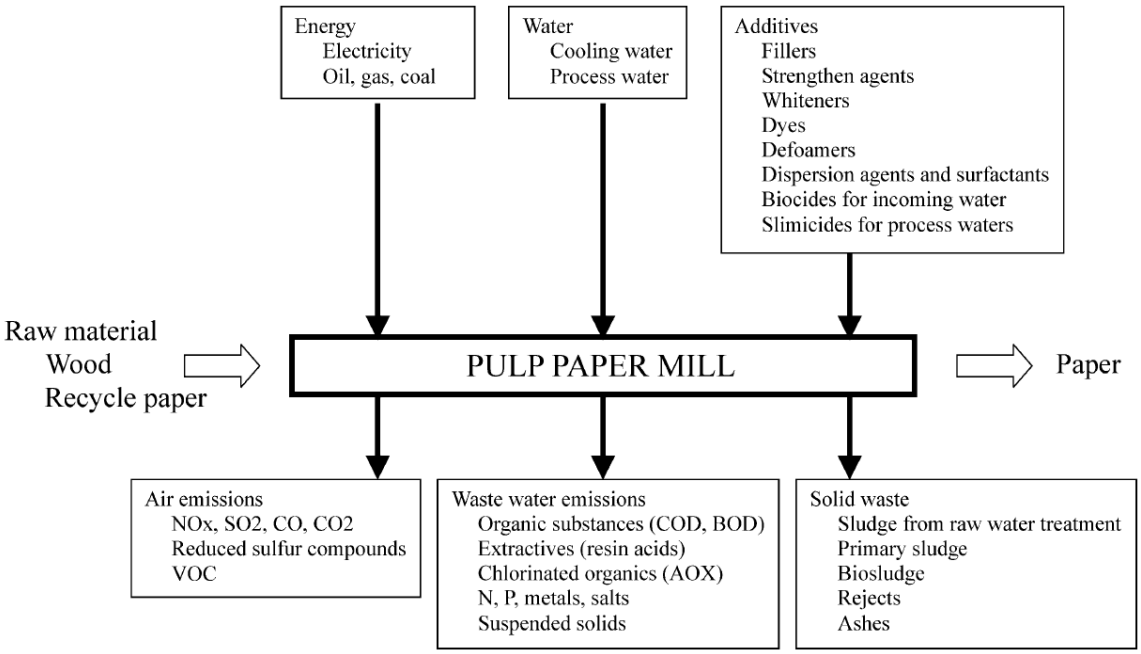

Figure 7 – General schematic representation of mass streams present in the paper industry (14).

The stages that have the greatest contribution to the increase of toxicity of the effluents are wood preparation, pulping and pulp washing, screening, washing, bleaching, paper production and coating operations. Compounds such as lignins, stilbenes, phenols, dioxins, chlorides, furans, phenols and sulphur compounds were found in these effluents (15–17).

To begin with, the debarking stage consists of the removal of soil and bark by wet process, allowing a huge elimination of organic matter from the wood into the circulating effluent. At this stage, the generated effluents are very colored due to the high tannin content, contributing to up to 50 % of the chemical oxygen demand (COD) of debarking wastewater. Resin acids, which naturally occur in the resin of tree wood, might also be present in the debarking waters (7).

The effluent from the pulping process is rich in lignin and lignin degradation products. Additionally, some of the cooking chemicals (e.g. NaOH and Na_2S in the case of the kraft process) can also be accumulated depending on the method chosen (16). This stage is also responsible for a high COD concentration (from 1000 mg/L to 6000 mg/L), that persists even after bleaching, and comprises carbohydrates, organic acids and easily degraded compounds (7).

The bleaching stage contributes to the hazardous waste with compounds such as adsorbable organic halogens (AOX), chlorinated organic compounds (e.g. dioxins or furans), chlorinated lignosulfonic acids, phenols and vestigial concentrations of toxic compounds such as DDT, polychlorinated biphenyls and polychlorinated dibenzodioxins (7).

Papermaking step also includes some delignification processes. As a consequence, some lignin derivatives and natural wood extractives (e.g. acids, tannins, alkaloids, waxes, fats and phenols) are present in the effluents generated at this stage and very often are recalcitrant to degradation (16).

Furthermore, in the step of paper production itself, more chemicals (e.g. dyes, resins and fillers like titanium dioxide, calcium carbonate and sizing agents like rosin and starch) are employed to finish the whole process, making them be present in the wastewaters (7).

Although organic compounds are naturally present in water due to natural decay (humic acid, fulvic acid, amines and urea) and other synthetic sources (e.g. detergents, fertilizers, etc.), these interfere with a great number of processes in aquatic systems and with water quality. An excess of organic compounds can affect the receiving aquatic systems due to the depletion effect exerted on the dissolved oxygen concentration. Organic materials use oxygen for degradation. High organic loads released into the environment can be critical for oxygen concentration, creating hostile conditions for living organisms. Additionally, organic matter favors the growth of microorganisms, which also contribute to the decline of the dissolved oxygen concentration (18,19).

As was described, the P&P industry contributes to the release of high amounts of organic compounds into the water. More than 200–300 different organic compounds might be present in its effluents, contributing to a high COD and a low biodegradability (defined as the ratio between biochemical oxygen demand (BOD) and COD).

Table 2 – Typical values of effluents from P&P production processes (6).

Process	COD (mg/L)	BOD (mg/L)
Pulpwood storage, debarking and chipping	1275	556
Kraft cooking section	1669	460
Bleaching	3680	352
Papermaking	1116	641

The chronic and toxic effects, which may arise from the release of these effluents into the environment, even after conventional treatment, continue to be the main problem regarding these industry effluents. Consequently, process and wastewaters must be monitored, with the purpose of minimizing the discharge of hazardous compounds into the environment and to find new and more efficient processes. Taking this into account, new analytical techniques are arising in order to control these compounds in the water in conformity with the European Standards for water quality (7,15).

1.6. Nitrogen in wastewater

Nitrogen is the most abundant element in the Earth's atmosphere and is the fourth most present in cellular biomass. Microorganisms are crucial for the nitrogen cycle, being totally responsible for some of the redox conversion reactions present in it. One of the most important functions of microorganisms is the conversion of non-reactive nitrogen, N_2, into useable forms of nitrogen (e.g. ammonia (NH_3) or nitrate (NO_3^-)), making N_2 available to be used by other microorganisms (20).

Besides the use of this element for protein, nucleic acids and cellular components synthesis, bacteria have in their protoplasm nitrogen in several oxidation states, namely organic nitrogen (R-NH_3), ammonia (NH_3), nitrogen gas (N_2), nitrous oxide (N_2O), nitrite (NO_2^-), and nitrate (NO_3^-). The interconversion reactions of the various nitrogen species are shown in **Figure 8**.

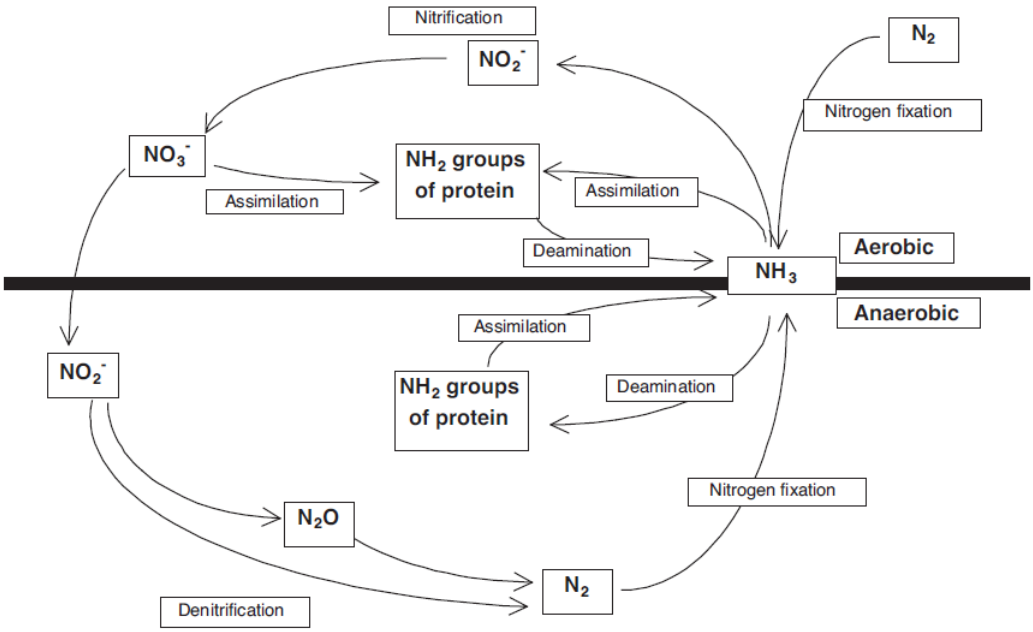

Figure 8 – Conversion reactions between the various nitrogen forms in nature (20).

Besides this important role, nitrogen release in excess into the water has a high fertilizer effect. Reactive forms of nitrogen are extremely soluble in water, which causes them to eventually reach watercourses and coastal areas. There, they lead to an excess of algae growth, a process called eutrophication, which prevents the sunlight to penetrate in the water and thus inhibiting the activity of living organisms. Additionally, algae deposit on the bottom of the aquatic systems at their end of life, where aerobic bacteria perform their degradation by consuming oxygen, leading to a decrease in its concentration and creating anoxic regions that inhibit the development of life (21).

Regarding the P&P industry, its effluents undergo treatment before being released into the receiving aquatic systems. The main goals of the conventional treatment technology have been the removal of suspended solids, organic carbon and toxic compounds. With the increase of environmental concerns, some measures have been applied, in particular, the reduction of water consumption, the introduction of processes like ECF/TCF bleaching and a more wide-ranging biological wastewater treatment, that have resulted in the decrease of the discharge of BOD, AOX and other toxic compounds. Additionally, nitrogen concentration is no exception, and this industry has been compelled to reduce its concentration in its wastewater.

However, the reduction of nitrogen in the discharge effluents from the P&P industry is not an easy task since its levels are often very low, reflecting the low amount present in wood species. This

small amount of nitrogen present in wood ends up in the wastewater due to dissolution during the pulping and bleaching processes. Additionally, numerous additives containing nitrogen can also contribute to the nitrogen effluent concentration. Regarding the kraft process, the major contributing process stream for the overall nitrogen content is the foul condensate stream, containing great amounts of ammonium nitrogen (NH_4^+) which, under alkaline conditions becomes a gas (NH_3), and this stream is a collecting point for volatile compounds.

Nevertheless, this concentration of nitrogen is still very low, leading to nutrient limitation in wastewater, which is required for the growth of bacteria during conventional treatment. In a nutrient-deficient situation, microorganisms cannot fulfil their needs for the enzymatic and cellular activities, compromising their ability to remove organic pollutants and leading to a high BOD value in the effluent. Therefore, nutrients, in particular, nitrogen species, have been added to the wastewater in order to secure the best treatment performance (22).

In a wastewater treatment system, nutrients usually accumulate within the biomass agglomerates, thus an effective reduction in the nitrogen concentration in the discharge effluent should comprise the suspended solids removal and the optimization of nutrient supplementation. Therefore, the sources of nutrient input in the wastewater should be considered in the assessment of the discharge concentrations, in order to increase the efficiency of the wastewater treatment and protect the receiving environments (22).

In the last decades, the effect of eutrophication has been detected in 60 % of lakes and aquatic systems. This is not the only drawback influencing the receiving ecosystems, as the effects of endocrine disruptors are known. Regarding this, not much attention has been given by the scientific community to the possible contribution of the P&P industry to the increase of nutrient supplementation, which only recently has been subject to more regulatory legalization.

Consequently, in order to minimize the negative effects of nutrient supplementation, it is important to have knowledge of the nutrient requirements of the effluents and to monitor the discharge of representative compounds that may be present (22).

1.7.Quantification of Organic Carbon in water

In water and wastewater, organic carbon is represented by numerous organic compounds present in several oxidation steps, being able to be further oxidized by biological or chemical processes. Techniques such as BOD, COD and TOC are different approaches that can be performed in order to assess the organic fraction in a water sample (23). However, the BOD test is a five-day test, so it presents the time disadvantage. Additionally, it corresponds to the biochemical oxidation of organic matter, carried out primarily by microorganisms, whereas the COD parameter has a wider range as regards the oxidation capacity of organic compounds and so, more similar to the TOC technique. So,

in the present work, only COD and TOC will be studied and, consequently, BOD will not be taken into account in this bibliographic review.

1.7.1. Chemical Oxygen Demand

Chemical oxygen demand (COD) is a measuring method for the organic matter present in water. COD assay is a quick and undemanding way to determine the amount of oxygen equivalent to the organic matter in water susceptible to oxidation, making COD a convenient indicator of the organic pollution in water (24).

Chemically, COD is defined as the oxygen mass concentration that is consumed during organic matter degradation by the equivalent amount of a strong oxidizing agent (23,25). Potassium dichromate ($K_2Cr_2O_7$) or potassium permanganate ($KMnO_4$) can be used as oxidizing agents. While the former is more employed for water quality assessment in heavily or moderately polluted water bodies (e.g. sewage or wastewater), the latter is more suitable for relatively clean water bodies (e.g. surface or river water) (24).

Two stages are included in COD determination by standard methods. These are oxidation and the quantification of used oxidants during the reaction. Standard methods are divided into closed digestion and spectrophotometric methods, and reflux digestion and $K_2Cr_2O_7$ titration. In the closed digestion method, the amount of $K_2Cr_2O_7$ used is determined by measuring the absorbance of Cr^{3+}; this approach produces less harmful wastes and is more cost-effective in reagents usage. In contrast, in the open digestion technique, the excess of $K_2Cr_2O_7$ is titrated against ferrous ammonium sulfate (Mohr's salt), using ferroin as an indicator, and a great variety of wastes can be analysed (23,24).

Three main chemicals are used in the standard analysis, the oxidant ($Cr_2O_7^{2-}$), the catalyst (Ag^+) and the sulfuric acid (H_2SO_4) and, usually, potassium hydrogen phthalate (KHP) is used as a model compound for organic matter (**Equation 1**). Silver sulfate (Ag_2SO_4) is used as a catalyst to increase the oxidation efficiency of organic compounds.

$$2KC_8H_5O_4(aq) + 10K_2Cr_2O_7(aq) + 41H_2SO_4(aq) \rightarrow 16CO_2\,(g) + 46H_2O\,(l) + 10Cr_2(SO_4)_3(aq) + 11K_2SO_4(aq) \quad \text{Eq. 1}$$

When dichromate is replaced by oxygen as an oxidizing agent, the reaction is translated by **Equation 2**.

$$2KC_8H_5O_4(aq) + 15O_2(g) + H_2SO_4(aq) \rightarrow 16CO_2(g) + 6H_2O(l) + K_2SO_4(aq) \quad \text{Eq.2}$$

However, halogens, more precisely chloride, work as blockers of the silver effect. Chloride reacts with silver ion to precipitate silver chloride, inhibiting silver catalytic activity. This interference can be overcome, although not fully, by sample treatment with mercuric sulfate ($HgSO_4$) before the oxidation process (**Equation 3**) that makes chloride unavailable and therefore eliminating interference (23,24).

$$Hg^{2+}(aq) + 2Cl^{-}(aq) \rightarrow HgCl_2(s) \quad \text{Eq. 3}$$

Although it is normally present in low concentrations in wastewater samples, nitrite (NO_2^-) is able to work as interference when significant amounts of nitrite are present. In this situation, sulfamic acid should be added, generating nitrogen, according to **Equation 4**. Moreover, reduced inorganic species (e.g. ferrous iron, sulfide) are also oxidized in assay conditions. Usually, corrections of the obtained COD value are performed when it is substantially present in the sample (23).

$$NaNO_2(aq) + H_3NSO_3(aq) \rightarrow N_2(g) + NaHSO_4(aq) + H_2O(l) \quad \text{Eq.4}$$

Although standard methods for COD determination are the most employed in wastewater treatment and environmental programs worldwide, they still present a great number of drawbacks. Low detection sensitivity, long digestion and titration times and the production of hazardous wastes (e.g. mercury, hexavalent chromium, sulfuric acid, silver and acids) increase the environmental impact related to these methods and reduce their application for COD measurements (24).

Due to not being totally satisfactory, improvements have been made in COD oxidation methods. For instance, radiation-based technologies, such as microwave and ultrasound energy, have been exploited as alternatives to the conventional heating systems, i.e., electric furnace and hot plate (24). Although these have demonstrated to be suitable for less refractory organic pollutants and less polluted water samples, these methods have associated the safety risks of radiation techniques, have proved to be inefficient in the oxidation of several pollutants and do not discard completely the use of toxic chemicals (24).

Additionally, the set-out methods in the legislation remain the standard, which makes it difficult for the new to become a reality as COD analysis methods (25).

1.7.2. Total Organic Carbon

As was referred, the COD technique is not the most suitable method for wastewater monitoring due to the use and generation of hazardous chemicals. Consequently, new alternative techniques are needed for the replacement of the COD methods (26).

The total organic carbon (TOC) method arises as a faster and possibly more precise substitute technique to the COD and BOD tests (26). Although the information provided is different, TOC ends up being the most direct representation of total organic carbon content in water. It is a non-specific indicator of the amount of carbon bound in an organic compound and it can work as an analytic parameter in order to assure water quality (18,19).

The values of TOC in water samples may vary between 0.1 mg/L and 25 mg/L, for drinking water samples and >100 mg/L, for wastewater. It must be remembered that the determination of TOC is independent of the organic matter oxidation state (23).

Usually, TOC instruments establish a TOC value by analysing several fractions of total carbon (TC). In **Table 3** the total carbon fractions are described

Table 3 – Fractions of total carbon (22).

Fraction	***Designation***	***Description***
Total	TOC	All carbon atoms covalently bonded in organic molecules
Inorganic	IC	Carbonate, bicarbonate, and dissolved CO_2
Dissolved	DOC	TOC fraction that passes through a filter with pores of 0.45 μm
Particulate	-	TOC fraction retained by a 0.45 μm filter
Purgeable/volatile	POC	TOC fraction removed from an aqueous solution by gas stripping under specified conditions
Nonpurgeable	NPOC	TOC fraction not removed by gas stripping

Measurements of TOC can be performed directly or indirectly. The direct method is more suitable when inorganic carbon (IC) fraction is present in greater quantity than TOC. To quantify the TOC value in this situation, IC interference has to be eliminated before analysis. One of the ways of performing this is by decreasing the sample pH (≤ 2), which transforms the IC content into CO_2, subsequently removed by purging the sample with a purified gas like O_2. Because sample purging also eliminates POC, the value that is actually assessed is nonpurgeable organic carbon (NPOC) (23).

On the other hand, when IC is less than TOC, the latter can be determined by separately measuring TC and IC fractions and then evaluate the difference between these values (TC – IC = TOC). When samples are richer in IC, the difference method (DM) is not the most appropriate for TOC determination due to the larger error associated (23,27).

1.7.2.1.Methods for the determination of Total Organic Carbon

As a potential alternative to the above-mentioned traditional methods, TOC has aroused great interest, causing the establishment of more precise detection techniques turning it even more reliable and accurate, particularly for more complex matrix such as industrial wastewaters (26).

Several methods can be used for TOC determination. As the sensitivity range of the methods overlaps, other factors might be responsible for the chosen method, like precision, ease of use, cost-effectiveness and the production of hazardous wastes.

Essentially, during the analysis, organic carbon fraction is converted to CO_2 which is then measured. In order to perform the reaction, two types of methods can be used. One method uses high temperature, typically 680 °C to 950 °C, together with catalysts and oxygen or air. The other methods

use lower temperatures (<100 °C) with ultraviolet irradiation and/or one or more chemical oxidants. The generated CO_2 is quantified by spectrophotometry, conductivity or coulometric titration (23).

1.7.2.2. Persulfate-Ultraviolet or Heated-Persulfate Oxidation method

This technique is based on the use of persulfate to carry out the oxidation of organic compounds into CO_2, together with UV radiation or heat in order to activate the reagents used. Represents a very accurate and quick way of determining low carbon levels (<1 mg/L C) in water. Actually, this method can measure TOC levels as low as 0.010 mg/L C.

The procedure consists of the oxidation, by persulfate, of organic carbon to CO_2 in the presence of heat or UV light. The CO_2 released is purged from the sample, dried and measured by a nondispersive infrared (NDIR) detector, by conductivity change or coulometrically titrated. The measurement by conductivity consists of the filtration of the liquid stream by a membrane which only allows the CO_2 transition into high-purity water, where a conductivity change is verified and related to the CO_2 presence.

The persulfate oxidation method has its interferences. The efficiency of the conversion can be affected by several factors, generating a great variation in the oxidation time within the organic compounds. In order to verify the oxidation efficiency of the organic compounds, a test must be performed previously with compounds representative of the sample matrix to predict possible results. Additionally, in order to evaluate the oxidation efficiency of the instrument, some compounds that are resistant to oxidation and measurable in trace levels, can be added to the matrix together with other compounds, allowing to evaluate the oxidation capacity of the analyser. Some of these compounds are urea, n-butanol, acetic acid, tartaric acid, nicotinic acid and pyridine.

Another source of interference might be the presence of chloride in the samples. Chloride is preferably oxidized (i.e. $2Cl^- \rightarrow Cl_2(g) + 2e^-$) and may inhibit the oxidation of organic compounds when present above 0.05 %. To reduce this inhibition is usually added mercuric nitrate to the persulfate solution in the UV-persulfate systems. This procedure needs an appropriate discharging method of the effluents to avoid possible contaminations of the receiving aquatic system. In the case of heated-persulfate devices, the oxidation time and/or the amount of persulfate solution is normally increased (23).

1.7.2.3. Wet-Oxidation method

Wet-oxidation is a measuring technique of organic matter by persulfate oxidation. First, the sample is acidified and purged for the IC removal; then it is oxidized with persulfate at a temperature between 116 ºC and 130 ºC in an autoclave. After this reaction, the released CO_2 is quantified by a nondispersive infrared (NDIR) spectrometer.

This method is not appropriate for the quantification of volatile organic compounds but is normally used for water and seawater samples, mixtures of water-suspended sediments and wastewater comprising at least 0.1 mg/L of NPOC (23).

1.7.2.4. High-Temperature Combustion method

High-Temperature Combustion (HTC) method is carried out by a specific instrument named TOC analyser. Samples with a high level of organic compounds in suspension or containing high amounts of halogens (500 mg/L) can be analysed by this method. Standards documents EN 1484:1997 (27) and ISO 20236:2018 (28) specify a method and guidance for the determination of TOC in several water types (from freshwater to wastewater) after high-temperature combustion.

HTC general procedure can be separated into three parts, sampling, oxidation and detection (29). Sampling consists of the sample suction from its container, followed by its injection into the combustion tube of the analyser, packed with an oxidative catalyst (e.g. cobalt oxide, barium chromate or platinum group metals), where the oxidation of organic compounds takes place. Water is evaporated and the oxidation reaction of the organic carbon to CO_2 is accomplished. The generated CO_2 released from organic and inorganic carbon oxidation is detected by an NDIR detector. This procedure allows the determination of TC. In order to obtain TOC by difference, IC must be measured separately or removed first by acidification and sparging (23).

For IC determination, the sample can be injected in a chamber for acidification. In acidic conditions, IC forms, excluding organic, are converted into CO_2, which is quantified in the NDIR detector. Instead, IC can be also removed by acidification before sample injection, being CO_2 removed by purging. However, this method also removes POC so, for a correct TOC determination, POC has to be determined (29).

The combustion temperature is a decisive parameter for the success of the method. Because some carbonates are only decomposed above 950 ºC, low-temperature systems must use acidification at the same time to decompose these carbonates. However, low-temperature systems have the advantage of reducing the diffusion of dissolved salts, which results in obtaining lower blank values in the results. This is precisely the greatest limitation of the HTC method, which gives higher and more variable blank values. With the aim of having lower blanks, the analysers suppliers have developed catalysts and procedures in order to refine the method (23).

Some stages of this method can contribute to organic content losses, which may influence the overall result. For instance, the removal by purging of the CO_2 generated from the IC content leads to the loss of volatile organic substances. In this situation, it is advisable to determine TOC separately. Additionally, during sample mixing these substances can also partly escape.

Another concern is related to particulate samples. Large diameter particles might fail to enter the injection needle and the analysis does not give the correct value. When only DOC is to be determined, the sample must be filtrated. However, depending on the physical properties of the carbon compounds and the desorption or adsorption of the carbonaceous material on the filter, some DOC content can still be lost or added (23).

1.7.3. Relationship between the TOC and COD parameters

As it was mentioned before, TOC presents several advantages when compared with COD, which makes this parameter a possible substitute for COD, either in routine laboratory analysis or in the characterization of effluents. Although TOC does not return the same information as COD, correlations of the two parameters can be established, allowing the use of TOC in water analysis and avoiding the need for safe disposal of the spent COD waste (23,24,26).

The limit values set for the organic matter concentration in wastewater are COD values. So, efforts have been made in the last years to arrange a suitable correlation between COD and TOC, in order to replace the COD test by TOC analysis. Additionally, since the characteristics of each effluent are highly variable, specific correlations between these two parameters should be established for each effluent and properly validated every season. (26,30).

In some cases, it is possible to calculate a correlation factor that allows the prediction of the COD values from the TOC ones, without performing the COD test and consequently avoiding all the disadvantages inherent to the latter. The theoretical correlation factor can be assessed based on **Equation 5**, which describes the carbon oxidation with oxygen, where COD is represented by the oxygen amount and TOC by the carbon amount.

$$C + O_2 \rightarrow CO_2 \qquad \text{Eq. 5}$$

The values of COD and TOC are usually expressed in mass concentrations so the molar masses are used to calculate the ratio C/O, which is the conversion factor TOC/COD. For instance, based on **Equation 5** and assuming 1000 mg/L for both parameters, the correlation factor can be calculated by **Equations 6** to **8**:

$$C\text{: } 1000\ \left(\frac{mg}{L}\right) \times \frac{1\times10^3}{12\times10^3}\left(\frac{mmol}{mg}\right) = 83.33\ mmol/L \qquad \text{Eq. 6}$$

$$O_2\text{: } 1000\ \left(\frac{mg}{L}\right) \times \frac{1\times10^3}{32\times10^3}\left(\frac{mmol}{mg}\right) = 31.25\ mmol/L \qquad \text{Eq. 7}$$

$$CF = \frac{TOC}{COD} = \frac{83.33}{31.25} = 2.667 \qquad \text{Eq. 8}$$

This method is of easy application in liquid matrices whose composition is well defined. However, for the specific case of the current work, in which the effluents of pulp and paper industry

are the object of study, it is difficult to predict a theoretical factor that relates these two parameters, since the matrix of these effluents is composed by a different number of organic compounds that alter the ratio between carbon and oxygen atoms, having a direct influence on the correlation factor (30). In this condition, the correlation between the two parameters can be established by interpolation of the TOC and COD values. For that, several values of the two parameters from the effluents to be analysed should be obtained over time. Then, the two data sets should be analysed statistically and the correlation between TOC and COD for each effluent should be established, if verified, as well as **Equation 9:**

$$TOC = a + b \cdot COD \qquad \text{Eq. 9}$$

where b is the slope and a the intercept of the linear regression curve which reproduces the relationship between the two parameters.

1.8. Nitrogen determination

As it was mentioned, several nitrogen species comprise the nitrogen cycle, being converted into each other. These forms are present in water and wastewater (23).

Total bound nitrogen (TNb) defines the pollution in water by nitrogen compounds, comprising ammonia, nitrites, nitrates, organic and aromatic nitrogen compounds. As an analytical parameter for water, techniques that allow its assessment in several compounds and samples are very important tools in environmental research and monitoring programs (31).

The assessment of this parameter in a sample can be performed through the oxidation of all digestible nitrogen species present to nitrate, followed by the latter quantification (23). Based on this principle, two approaches for the quantification of TNb will be discussed in more detail. Moreover, the HTC method is also an alternative for the quantification of TNb and will also be reviewed here.

1.8.1. Persulfate Oxidation Method

This method consists of the oxidation of all nitrogen compounds to nitrate through the use of persulfate. To achieve this, alkaline oxidation is conducted at a temperature between 100 ºC and 110 ºC to convert all the organic and inorganic nitrogen species in nitrate. Nitrate is then reduced to nitrite through the use of a cadmium column, which is then quantified (23).

Although it is a simple technique with low cost of instrumentation, the high potential for contaminations due to manual operation (32) and the long-time analysis, which invalidate monitoring purposes (33), are important drawbacks of this technique.

1.8.2. UV photo-oxidation Method

UV oxidation is another alternative method for the assessment of TNb. It comprises the use of UV light in the presence of an oxidizing agent (e.g. H_2O_2 or persulfate ion) to convert the nitrogen compounds in nitrate. The latter is then determined by direct detection in the UV region, by reduction with a cadmium column or sponge, or with a Devarda alloy (33).

It has also been reported the integration of on-line devices using UV oxidation in this type of method. Additionally, flow analysis has been used in conjunction with this technique, giving it useful characteristics such as automation, ruggedness and portability, enabling its use in monitoring environmental programs. Thus, the determination of nitrogen using flow analysis combined with on-line UV digestion has been widely used (32).

Nevertheless, the UV oxidation method also presents some drawbacks considering the determination of TN, like efficiency decrease, when digesting some nitrogen organic compounds, and incomplete oxidation or loss of nitrogen, when the organic nitrogen compounds are highly concentrated in samples (34).

1.8.3. High-Temperature Combustion Method

This method is similar to the last mentioned for the organic carbon assessment. HTC methods for nitrogen determination have an increased efficiency and are much less time-consuming when compared with wet chemical digestion/oxidation methods (e.g. persulfate digestion, etc). In this technique, all the present nitrogen compounds are oxidized to NO_x compounds using a catalyst (e.g. Pt). The released NO_x is quantified by a chemiluminescent or electrochemical detector.

Although the HTC method highly depends upon the activity, selectivity and stability of the catalyst, it represents an opportunity to perform a fast and reproducible analysis of the nitrogen compounds in a large number of samples (31).

1.9.Vario TOC Select analyser

Vario TOC Select is a TOC analyser supplied by the *Elementar* company. One of the main advantages of this equipment is the possibility of measuring all parameters, such as TOC, NPOC, TC, TIC, DOC, POC and Total bound Nitrogen (TNb), in the same unit. Samples like drinking water, industrial effluents or solids can be also analysed with this equipment shown in **Figure 9**.

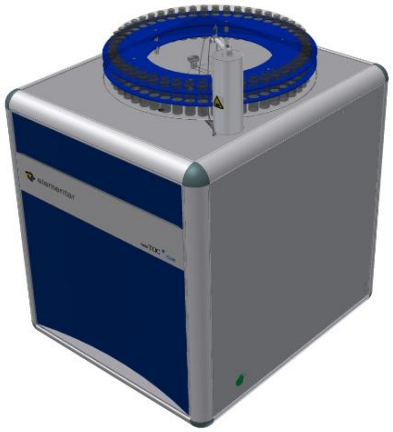

Figure 9 – Image of Vario TOC analyser from Elementar company.

The technique of analysis is the high-temperature combustion allowing to fully oxidize the sample and convert carbon in CO_2, which is then detected quantitatively by means of an NDIR detector. In the case of nitrogen, after oxidation, the generated NO is detected by means of an electrochemical cell (EC) down to ppb level.

The oxidation in this equipment is performed at 850 ºC using platinum (Pt) to allow efficient analysis of organic compounds. The high combustion temperature is crucial for quantitative oxidation of bound or dissolved carbon to CO_2. Moreover, when compared to others, this method guarantees that even stable compounds, particles or salt-containing solutions will be completely detected.

The TOC analyser from *Elementar* comprises the following five functional units:

- Sample insertion mechanics – comprised by the sample vials that carry the sample during the analysis, and the multiway valve that holds the sample being analysed and transports it into the combustion tube or the sparger;
- Furnace and reaction zone – comprising the furnace, that holds the combustion tube at a constant temperature depending on the operating mode, and the combustion tube where the sample combustion takes place;
- Separator – comprised by the sparger, that separates the IC from TC by the acidification with a 1% phosphoric acid solution (H_3PO_4), and the halogen absorber for the absorption of halogen;
- Cooling and drying unit – comprising a condenser and magnesium perchlorate ($Mg(ClO_4)_2$) for the separation of water from the measuring gas;
- Detector – NDIR detector that converts an optical signal to an electrical signal.

Figure 10 shows a representation of the sample path during analysis. The process of IC analysis in the direct mode begins with the injection of the acid solution of 1 % H_3PO_4 into the sparger. Then,

the NDIR detector performs autozero alignment of the measuring signal and the system is ready for sample measurement. The multiway valve goes to two different positions, the suction of the sample and the waste. Afterwards, the syringe is filled with the corresponding injection volume, the multiway valve goes to the 4th position and the sample is injected into the sparger, allowing the IC conversion in CO_2.

In the case of TC direct measurement, the process is slightly different because, after the syringe is filled with the sample injection volume, the multiway valve goes to the 3rd position and the sample is injected into the combustion tube. The combustion products are transferred by the gas flow into the separation unit, where the volatile halogen compounds are removed by bounding to the absorption reagent (e.g. brass wool). The water that might be present is successively separated by a condenser, a measuring gas drying and a transition through a magnesium perchlorate absorption tube. Regarding TNb measurement, the sample is determined at the same time by injection into the combustion tube and the NO produced is quantified.

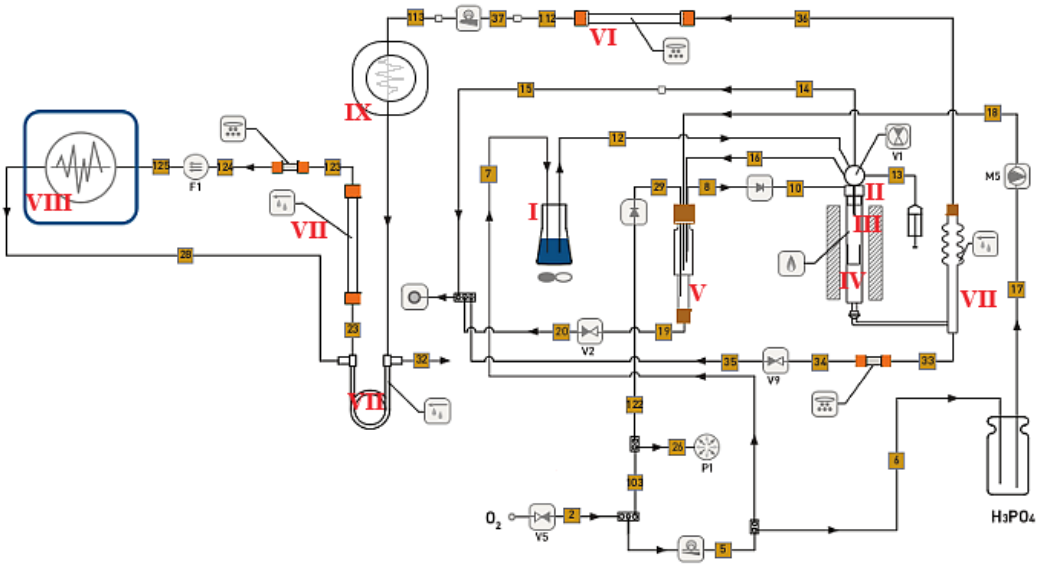

Figure 10 – Schematic representation of the Vario TOC analyser operation. I - sample vial; II - multiway valve; III - furnace; IV - combustion tube; V - sparger; VI - halogen absorber; VII - condenser, permaPure, magnesium perchlorate; VIII – measuring cell; IX – electrochemical cell (34).

In order to determine the absolute content of the sample being analysed, the NDIR detector generates an electrical signal in accordance with the concentration of CO_2 entering the detector. This signal is digitalized and integrated and the absolute element content is calculated from this and the calibration coefficients of the elements. In the case of TNb measurement, this is carried out in an electrochemical cell (EC) by detecting the generated NO (35,36).

1.9.1. Features of TNb analysis by analyser

As mentioned above, the determination of the TNb parameter by the Vario TOC Select analyser is performed by high-temperature combustion (850 °C) of the sample. The nitrogen present in the

sample is oxidized to nitric oxide (NO), which is then read in an electrochemical cell (EC). The signal is digitized and integrated, resulting in an area value that, according to the calibration set for the parameter, is transformed into the weight of total nitrogen.

The main purpose of the equipment is the TOC analysis. Thus, there is no method that allows the analysis of nitrogen individually, being this parameter always analysed together with carbon. In samples with a high TOC content, this might be a problem as it may lead to the determination of lower nitrogen values. Depending on the analysis conditions, organic carbon present in the sample is oxidized not only to CO_2 but also to carbon monoxide (CO). CO is a reducing agent and therefore reacts with NO, forming CO_2 and N_2. The latter is not detected by the EC but CO_2 is detected in the infrared detector. Thus, high concentrations of TOC in the samples lead to the default detection of nitrogen present.

To overcome this interference, it is necessary to mimic, as far as possible, the composition of the nitrogen species present in the sample matrices when developing the standard calibration solution. For this it must be taken into consideration that:

- nitrogen in nitrate (NO_3^-) form has a recovery in the order of 100 %;
- ammonium (NH_4^+) nitrogen has a recovery of about 90 %

Elementar company also advises the use of NH_4Cl to account for the ammonium fraction and $NaNO_3$ to represent the nitrate part. The standard calibration solution shall be composed of these two reagents, the proportions of which shall be similar to their proportions in the matrix of the samples to be analysed. If the composition of the samples is unknown, a solution taking into account 50 % N-ammonium and 50 % N-nitrate is recommended.

Given the above assumptions, TNb calibration will vary depending on the matrix of the samples under analysis.

1.10. Validation of analytical methods

Analytical practices involve several manipulations likely to accumulate errors (gross, systematic and/or random), which in some situations may significantly alter the final result. In addition, it is necessary to determine whether the method used is the most appropriate for a particular purpose. Thus, detection and quantification procedures shall only be performed by analytical methods that have been properly validated (37,38).

Validation practices are a crucial laboratory instrument when it comes to best practices implementation. This type of practices allows to have confidence in the results returned by a certain technique in the quantification of a given compound, and that it meets all requirements for the expected analytical application (38).

Analytical method validation is performed when some situations are verified. For instance, when a new method is employed in a certain procedure and its viability needs to be assessed, when it is intended to use an already existing method for the first time in a laboratory or, when an already existing method is going to be applied in a different way (e.g. different instrumentation or concentration ranges) or to a new sample (39).

The proper validation procedure depends on the type of method and the assessment of several parameters, namely the working range, linearity, analytical limits (e.g. limit of detection and limit of quantification), sensitivity, precision and intermediate precision (IP) and trueness. However, the determination of all these parameters is not always performed. For instance, in quantitative analysis, and specifically in vestigial concentration ranges, the above-mentioned parameters need to be well established but, when higher concentration ranges are used, analytical limits have no important meaning. Thus, it is the choice of the analysis methodology that will determine which parameters to be evaluated during the design of the validation strategy (37).

1.10.1. Work range

The definition of the working range is the starting point of all calibration procedures. Its assessment can be performed differently for first and second-order models. However, in this review, only first-order models will be covered (37).

According to ISO 8466–1 for the calibration of analytical methods in water quality assessment, the working range should be defined in accordance with the sample concentration range, and the most frequent concentration should be in the midpoint of the calibration curve (40).

Once the working range has been established it can be evaluated by the homogeneity of variances (HV) test. For that purpose, ten measurements of the highest and lowest concentrations shall be performed, and the mean and variance values associated with each of the concentration sets determined by **Equation 10** and **Equation 11**, respectively:

$$\bar{y}_i = \frac{\sum_{j=1}^{10} y_{i,j}}{n_i} \qquad \text{Eq. 10}$$

$$s_i^2 = \frac{\sum_{j=1}^{10} (y_{i,j} - \bar{y}_i)^2}{n_i - 1} \qquad \text{Eq. 11}$$

for i=1 or i=10,

being,

i – the correspondent standard solution (i=1,…,10),

j – the replicate number and

n – the total number of replicates.

Then, the variances of both concentration limits of the work range are tested through the *F-test* in order to assess the existence or not of statistically significant differences between the limits of the range of work. First de PG value is calculated through the **Equation 12** or **13.**

$$PG = \frac{s_{10}^2}{s_1^2}, \quad s_{10}^2 > s_1^2 \qquad \text{Eq. 12}$$

or

$$PG = \frac{s_1^2}{s_{10}^2}, \quad s_1^2 > s_{10}^2 \qquad \text{Eq. 13}$$

Afterwards, the calculated PG value is compared with the tabled value from *Fisher* distribution (F_{critical}) for n-1 degrees of freedom (DF), and two conclusions can be achieved:

- If PG ≤ F_{critical}, it can be concluded that the difference between the variances is not significant;
- If PG > F_{critical}, the difference between the variances is significative, usually the applied solution is to reduce the work range until the PG ≤ *F* condition is verified (37).

However, the reduction of the working range is not the perfect solution when the variance is not similar in all the points of the calibration line, because it means that one will not get the most out of the analytical capability of an equipment or method. Thus, another solution for surpass the lack of homoscedasticity is the application to the data the weighting of the linear regression (41).

1.10.2. Linearity Assessment

This parameter is part of the evaluation of the calibration curve that is used to establish the relationship between the equipment signal and the analyte concentration in a sample. The linearity of the calibration curve used in a particular analytical method can be assessed using ISO 8466-1 as a reference to perform first-order linear regressions, using the least squares method, and ISO 8466-2, in case second-order polynomial is applied to the calibration function. In the case of the statistical linearity test, namely the Mandel's test, the calibration data is used to calculate the corresponding first- and second-degree functions, as well as the residual standard deviations, $S_{y/x}$ and S_{y2}, respectively. Then, the difference of the variances is calculated from **Equation 14:**

$$DS^2 = (N-2)S_{y/x}^2 - (N-3)S_{y2}^2 \qquad \text{Eq. 14}$$

where *N* corresponds to the number of calibration standards. The residual standard deviations, $S_{y/x}$ and S_{y2} are given by **Equations 15** and **16**, respectively:

$$S_{y/x} = \sqrt{\frac{\sum_{i=1}^{N}[y_i-(a+b\cdot x_i)]^2}{N-2}} \qquad \text{Eq. 15}$$

$$S_{y2} = \sqrt{\frac{\sum_{i=1}^{N}[y_i-(a+b\cdot x_i+c\cdot x_i^2)]^2}{N-3}} \quad \text{Eq. 16}$$

being,

a, *b* and *c* – coefficients of the calibration function;

i – subscript of the concentration levels, where *i* = 1, 2, ..., *N*.

Then, the PG test value is calculated in order to perform the *F*-test, according to the **Equation 17**.

$$\text{PG} = \frac{DS^2}{S_{y2}^2} \quad \text{Eq. 17}$$

Afterwards, the calculated PG value is compared with the tabulated *F* value of the Snedecor/Fisher distribution, to a confidence level of 95 %, and if:

- PG ≤ *F*, the second-degree calibration function does not lead to a better adjustment, concluding that the one that fits the best is the first-degree calibration function;
- PG > *F*, the working range should be reduced as much as possible to use the 1st degree calibration function or, alternatively, the second-degree calibration function.

Using the values of the standards concentrations as independent variables (*x*) and the measured instrumental signal as dependent variables (*y*), the calculation of the calibration coefficients (*a* and *b*) of the first-order linear regression is carried out by **Equation 18**.

$$y = a + bx \quad \text{Eq. 18}$$

The coefficients of the linear regression line, slope (*b*) and intercept (*a*), are given by **Equations 19** and **20:**

$$b = \frac{\sum_{i=1}^{N}[(x_i-\bar{x})\cdot(y_i-\bar{y})]}{\sum_{i=1}^{N}(x_i-\bar{x})^2} \quad \text{Eq. 19}$$

$$a = \bar{y} - b \cdot \bar{x} \quad \text{Eq. 20}$$

where,

x_i – individual values of the standards concentration (*i*=1, …, *N*),

$\bar{x}$ – mean of x values,

y_i – individual values of the instrumental signal of each concentration standard x_i (*i*=1, …, *N*) and

$\bar{y}$ - mean of y values.

The coefficients values correspond to an estimation of the true values because they are subjected to the predictable method dispersion. The accuracy quantification of the calculated linear regression

line is carried out by calculating the residual standard deviation ($S_{y/x}$) of the regression line through the **Equation 21**:

$$S_{y/x} = \sqrt{\frac{\sum_{i=1}^{N}(y_i - \hat{y}_i)^2}{N-2}} = \sqrt{\frac{\sum_{i=1}^{N}[y_i - (a+bx_i)]^2}{N-2}} \quad \text{Eq. 21}$$

where,

$\hat{y}_i$ – estimated value from y_i results of the corresponding concentration standards x_i.

Another parameter used to evaluate linearity is the correlation coefficient (r). This statistical parameter measures how well the experimental points, namely the set of ordered and independent pairs (x_i, y_i), fit a straight line (39). It can be calculated through **Equation 22:**

$$r = \frac{\sum_{i=1}^{N}\{(x_i - \bar{x})\cdot(y_i - \bar{y})\}}{\sqrt{[\sum_{i=1}^{N}(x_i - \bar{x})^2 \cdot \{\sum_{i=1}^{N}(y_i - \bar{y})^2\}]}} \quad \text{Eq. 22}$$

and can only take values in the range $-1 \leq r \leq 1$, where $r = -1$ represents a perfect negative correlation and $r = 1$ represents a perfect positive correlation. Conversely, when there is no linear correlation between x and y, r value is close to zero. In chemical analysis, in order to a correlation be accepted, the absolute value of the correlation factor should be greater than 0.995, however this condition is variable and dependent on the laboratory in question.

Nevertheless, attention should be given to the lack of homoscedasticity in the analytical curve since the application of linear regression in these situations can lead to the propagation of significant errors and loss of accuracy (37,38,40).

1.10.2.1. Weighted Linear Regression

During the establishment of the working range, when large differences between the lowest and the highest concentration pattern are verified, the homoscedasticity condition is not fulfilled. This means that the variance of the extreme points of the calibration line is significantly different (41).

In this situation, weighted least squares linear regression model (WLSLR) can be performed once it allows an unbiased prediction for calibration when the data random errors are not constant through all the determinations of the calibration curve. WLSLR works by incorporating "weights" to each data point inversely proportional to the corresponding error, causing the calibration curve to be closer to the points associated with a smaller error than those corresponding to higher concentrations, which are associated to larger errors. The appropriate weighting factor, w_i, can be calculated through the inverse of variances (s_i^{-2}) through **Equation 23** (39)**:**

$$w_i = \frac{s_i^{-2}}{\sum_i \frac{s_i^{-2}}{n}} \quad \text{Eq. 23}$$

These weights can be applied to the calculation of the several equation parameters of the linear regression in order to convert it into a WLSLR. Thus, the calculation of the *a* and *b* parameters according to WLSLR is given by **Equations 24** and **25**, respectively (39):

$$b_w = \frac{\sum_i w_i x_i y_i - n\bar{X}_W \bar{y}_W}{\sum_i w_i x_i^2 - n\bar{X}_w^2} \quad \text{Eq. 24}$$

$$a_w = \bar{Y}_w - b_w \bar{X}_w \quad \text{Eq. 25}$$

The correlation coefficient (r_w) and the estimate of the residual standard deviation of the WLSLR can be calculated, respectively, by **Equation 26** and **27** (38):

$$r_w = \frac{\sum w_i \sum_i w_i x_i y_i - \sum w_i x_i \sum w_i y_i}{\sqrt{\sum w_i \sum w_i x_i^2 - (\sum w_i x_i)^2}\sqrt{\sum w_i \sum w_i y_i^2 - (\sum w_i y_i)^2}} \quad \text{Eq. 26}$$

$$S_{(y/x)w} = \sqrt{\frac{\sum_i^N w_i (y_i - \hat{y}_i)^2}{N-2}} \quad \text{Eq. 27}$$

1.10.3. Analytical limits

Nowadays, the measurement of trace and ultra-trace quantities of a certain analyte are possible due to instrumental techniques that allow its detection and determination. The assessment of very low concentrations of compounds has shown the importance, in environmental or biological terms for instance, of those small concentrations that until now would have been neglected. Therefore, statistical techniques that allow its calculation and evaluation are of great importance (39).

1.10.3.1. Limit of Detection

Qualitatively, the limit of detection (LD) of an analyte corresponds to the minimal concentration which generates an instrument signal that is capable of being distinguished from the blank with reasonable statistical certainty. A reading below the LD cannot be interpreted as the absence of the analyte being measured. The only true assumption that can be made is that, with a defined probability, the analyte concentration will be under a certain value (37,39). Usually, the value of LD is given by **Equation 28**:

$$\text{LD} = \bar{X}_0 + 3.3\sigma_0 \quad \text{Eq. 28}$$

where,

$\bar{X}_0$ – mean of the measured content of a series of independently prepared blanks or trace standards (between 10 and 20 trials);

σ_0 – standard deviation associated to $\bar{X}_0$.

However, when an analysis method of an analyte follows a linear calibration, LD can be calculated using the slope of the calibration curve (*b*) and the residual standard deviation of the calibration curve ($S_{y/x}$) as it is shown in **Equation 29** (37):

$$\text{LD} = \frac{K \cdot S_{y/x}}{b} \qquad \text{Eq. 29}$$

where K takes the value of 3.3 for a normal distribution and a confidence level of 99.7 %.

1.10.3.2. Limit of Quantification

Unlike qualitative detection by LD, limit of quantification (LQ) corresponds to the minimum amount of analyte that can accurately be quantified (39). Ideally, it should match the lowest concentration calibration standard. LQ value can be determined by **Equation 30**:

$$\text{LQ} = \bar{X}_0 + 10\sigma_0 \qquad \text{Eq. 30}$$

being,

$\bar{X}_0$ – mean of the measured content of a series of independently prepared blanks (between 10 and 20 trials), read over several working days;

σ_0 – standard deviation associated to $\bar{X}_0$.

Additionally, as for the LD, when the quantification method involves the use of a linear calibration, LQ can be calculated with the slope of the calibration curve (*b*) and the residual standard deviation of the calibration curve ($S_{y/x}$) through **Equation 31**:

$$\text{LQ} = \frac{K \cdot S_{y/x}}{b} \qquad \text{Eq. 31}$$

where K usually takes the value of 10. After its determination, LQ should be tested. This can be performed through the analysis of several standard solutions whose concentration is similar or close to the determined. In order to be accepted, according to the International Union of Pure and Applied Chemistry (IUPAC), the coefficient of variation (standard deviation divided by the mean values) for these standards should not exceed 10 % (37).

1.10.4. Sensitivity

Sensitivity evaluates an analytical method for its ability to distinguish small changes in the concentration of the analyte being measured. It is usually defined as the quotient between the increase

of the read value (ΔL) and the change in the concentration (ΔC) responsible for the increase (**Equation 32**):

$$Sensitivity = \frac{\Delta L}{\Delta C} = b \qquad \text{Eq. 32}$$

Nevertheless, if a linear model defines the calibration curve it means that the sensitivity will be similar over the entire working range and, thus, its value corresponds to the slope of the calibration line. This parameter is very useful to evaluate the method sensitivity for several analytes or to compare the analysis capability of several methods towards one compound (37,39).

1.10.5. Precision

Precision is a parameter that assesses the agreement between replicate measurements over several independent analyses of the same sample, or in similar or standard samples. Its evaluation is performed in two ways, within one assay by repeatability, and between several assays through reproducibility. Moreover, there is a third measurement regarding precision assessment, an intermediate condition named as intermediate precision (IP) or intra-laboratory variability (37).

When performing validation of methods, it is advised not to use reference materials, that may be atypically homogeneous. It is preferable to use "real" test materials and precision should be assessed in several points over the method working range (39).

1.10.5.1. Repeatability

Repeatability is part of precision evaluation of a method. It consists in the precision evaluation under similar conditions (e.g. the same laboratory, the same analyst, the same equipment and measurement procedure and the same type of reagents) during a short period of time.

The repeatability limit (Δr) is defined as the value under which the difference of two replicates (x_i, x_{i-1}) should be located, usually with 95 % of probability. Therefore, all results in accordance with the condition $|x_i - x_{i-1}| \leq \Delta r$ will be accepted as precise.

The repeatability of a method can be assessed within the laboratory itself or assessed by interlaboratory assays. In the first approach ten measurements ($n \geq 10$) on the same sample or standard are required, while in the second approach, the number of measurements may be lower ($n \geq 2$). Then, the repeatability of a method can be calculated through the variance by the **Equation 33**:

$$S_{ri}^2 = \frac{\sum_{w=1}^{p}[(n_{wi}-1)\cdot S_{wi}^2]}{\sum_{w=1}^{p}(n_{wi}-1)} \qquad \text{Eq. 33}$$

being,

S^2_{ri} – repeatability variance associated with the results, for each Laboratory,

S^2_{wi} – variance associated with the results, for each Laboratory,

$(n_{wi}-1)$ – number of DF in the series of analyses,

p – number of participating laboratories and

i – the concentration level being analysed.

Then, the repeatability limit (Δr), for a 95 % confidence level, can be evaluated according to **Equation 34**:

$$\Delta r = t_{critical} \cdot \sqrt{2} \cdot S_{ri} \qquad \text{Eq. 34}$$

being,

S_{ri} – standard deviation of repeatability associated with the results and

$t_{critical}$ – t student value for $n_{wi}-1$ DF and the desirable confidant level.

The Variation Coefficient of repeatability (CVr), for each level of concentrations, expressed as a percentage, is given by **Equation 35**:

$$\mathrm{CV}_r = \frac{S_{ri}}{\bar{X}} \times 100 \qquad \text{Eq. 35}$$

being $\bar{X}$, the mean of the considered values.

1.10.5.2. Reproducibility

Opposing to repeatability, reproducibility evaluates the precision of a method between laboratories, i.e., under different test conditions, namely different equipment and analysts, on the same sample and over longer periods of time, and usually it is associated with larger random errors. This parameter is obtained through interlaboratory tests to the same sample.

The reproducibility variance (37,39) is calculated through the sum of the interlaboratory variance (S^2_{Li}; variance of the systematic errors) and the repeatability variance (S^2_{ri}; random error variances) by **Equation 36**:

$$S^2_{Ri} = S^2_{Li} + S^2_{ri} \qquad \text{Eq. 36}$$

Moreover, the reproducibility limit (R), for a confidence level of 95 %, can be assessed by **Equation 34** but using the standard deviation of reproducibility (S_{Ri}) associated to the results considered, for each Laboratory, as well as the coefficient of variation of reproducibility (CV_R) by **Equation 35** (37).

1.10.5.3. Intermediate Precision

The intermediate precision (IP) is almost a compromise between the precision measurements mentioned above and is the most accepted way to calculate the results variability of a given method in the laboratory.

It consists of measuring precision within a single laboratory over a longer period of time, by keeping several parameters constant, like the samples or standard solutions and the method, and varying only one or a few specific measuring conditions, such as the equipment, the analysts, with new or the old calibration. Thus, IP measures de the dispersion of results according to the variation of the chosen interest parameters.

Essentially, under the predetermined conditions, several measurements (*n*) are carried out on several samples, covering different concentration ranges. Then, from the obtained results and after the removal of the outliers, the IP calculation can be performed through Range (*R*) Control Charts or by the **Equation 37:**

$$Si_{(\,)} = \sqrt{\frac{1}{t(n-1)} \sum_{j=1}^{t} \sum_{k=1}^{n} (y_{jk} - \bar{y}_j)^2} \qquad \text{Eq. 37}$$

being,

$Si_{(\,)}$ – standard deviation of the IP (intermediate conditions are identified between parentheses),

t – the total number of samples included in the assay,

n – number of tests per sample,

j – sample number (from 1 to *t*),

k – number of the obtained result for the sample *j* (from 1 to *n*),

y_{jk} – the individual result (*k*) for the sample *j* (from 1 to *t*) and

$\bar{y}_j$ – the mean of the results of sample *j* (form 1 to *t*).

Although control charts may be a way of assessing IP, they have other functions. For a better understanding and, due to their relevance, they will be covered in a separate chapter.

1.10.6. Trueness

This validation parameter translates the agreement between the mean of a set of analytical results and a reference accepted value for the analyte being measured (42). A high trueness level is achieved when the method is not affected by systematic errors or bias. One way to test a method for the extent to which it is affected by bias is through the use of certified reference material (CRM). In this situation the method is used to analyse the analyte content of a reference material and the obtained value is evaluated by the relative error calculation, hypothesis test (t-test), by the "Z-score" test or by the standard error calculation (39).

1.10.6.1. Relative error

The relative error (*Er*) is one of the ways of assessing the trueness of a certain method. The acceptance degree of the relative error is dependent of each laboratory, but usually an *Er* value of less than or equal to 5% is acceptable. It can be calculated through **Equation 38:**

$$Er = \frac{(X_{lab} - X_v)}{X_v} \cdot 100 \qquad \text{Eq. 38}$$

being,

X_{lab} – the experimental obtained value and

X_v – the certified value from the CRM.

1.10.6.2. Student's *t*-test

Another way to assess the presence of systematic errors in a certain method is through the hypothesis test approach, where the *t* value can be calculated by **Equation 39:**

$$t = \frac{|X_{lab} - X_v|}{S_{Xlab}/\sqrt{N}} \qquad \text{Eq. 39}$$

being,

X_{lab} – the experimental value obtained in the CRM analysis,

N – the number of tested samples and

S_{xlab} – the standard deviation associated to the experimental values (X_{lab}).

The value of t_{exp} that was calculated is compared, in absolute value, with the critical *t* value (for N-1 FD) and one of two decisions can be made:

- $| t_{exp} | > t_{critical}$, test is not acceptable as the existence of systematic errors was statistically shown;
- $| t_{exp} | \leq t_{critical}$, test is accepted as the existence of systematic errors was not statistically shown.

1.10.6.3. Z-score

The Z value corresponds to a way of evaluating the result of a laboratory CRM analysis. The value of Z is calculated from **Equation 40**:

$$Z = \frac{(X_{lab} - X_v)}{S} \qquad \text{Eq. 40}$$

being,

S – the uncertainty of the CRM or another unit of internal deviation.

Then, according to the calculated Z value, the test result might be considered satisfactory ($Z \geq -2$ or $Z \leq 2$), questionable ($-3 \leq Z \leq 3$) or incorrect ($Z < -3$ or $Z > 3$).

1.10.6.4. Standard Error

Standard error (*En*) consists in verifying if the certified value (*Xv*), obtained from CRM analysis or from the participation in interlaboratory tests (43), is comprised in the uncertainty determined for the experimental result (U_{lab}) obtained by the laboratory (X_{lab}) and if $|En| \leq 1$, U_{lab} was well assessed. When the latter condition is not verified, the uncertainty range might be underestimated. The determination of the standard error can be performed through the **Equation 41**:

$$En = \frac{(X_{lab} - X_v)}{\sqrt{U_{lab}^2 + U_{ref}^2}} \qquad \text{Eq. 41}$$

being,

U_{ref} – expanded uncertainty associated with the conventionally true or reference value (X_v).

The rate of recurrence to the analysis with CRM is variable. It depends on the regularity with which analyses are performed and whether these are routine or more punctual, the degree of confidence required for the result, the techniques used and the knowledge of the samples analysed (37,39).

1.11. Comparative Tests

Comparative testing is an important tool during the method validation process as it allows comparing results obtained by two different methods. They are often used to assess the closeness of results obtained with a reference method to an internal test method.

There are several types of comparative tests, namely, average *t*-test, paired *t*-test and the linear regression test between two test methods. Though, in the present literature review only the first two will be covered in more detail (37).

Averages *t*-test

This test studies the proximity of the results obtained by two methods, but the comparison is performed around the average values, i.e., it is not necessary that the number of samples be equal for both the methodologies. It consists in comparing two t values, one experimentally calculated, t_{exp}, and another tabulated, $t_{critical}$. If $|t_{exp}| \leq t_{critical}$ it is concluded that the results from both methods do not present statistically significant deviations, therefore both methods can be used for analysis.

The test consists of collecting values from both methods, n_1 and n_2, on the same sample. Then, the mean results of the replicates (X_{m1} and X_{m2}) and the associated standard deviations (S_1 and S_2) for each method are calculated and the t_{exp} is assessed by one of two ways described below. But first, an analysis of variances is performed to verify whether or not significant differences exist between the variances of the two methods and for that **Equations 42** and **43** are used.

$$F_{exp} = \frac{S_1^2}{S_2^2} \quad \text{Eq. 42}$$

$$F_{exp} = \frac{S_2^2}{S_1^2} \quad \text{Eq. 43}$$

The choice between **Equation 42** or **43** is performed consonant S_1^2 is larger or smaller than S_2^2, respectively. The calculated F_{exp} values are compared with the Fisher-Senedecor ($F_{critical}$) distribution for n-1 DF. When $F_{exp} < F_{critical}$, t_{exp} is calculated by **Equation 44**:

$$t_{exp} = \frac{\{X_{m1} - X_{m2}\}}{S \cdot \sqrt{\frac{1}{n1} + \frac{1}{n2}}} \quad \text{Eq. 44}$$

where S is calculated by the square root of S^2, which is calculated by **Equation 45**.

$$S^2 = \frac{(n1-1)\cdot S_1^2 + (n2-1)\cdot S_2^2}{\{n1+n2-2\}} \quad \text{Eq. 45}$$

Then, the t_{exp} value is compared with $t_{critical}$ for a DF number equal to $n_1 + n_2 - 2$. On the other hand, when $F_{exp} > F_{critical}$, t_{exp} is calculated by **Equation 46**:

$$t_{exp} = \frac{\{X_{m1} - X_{m2}\}}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}} \quad \text{Eq. 46}$$

and then, once again, the t_{exp} is related with $t_{critical}$ for a number of DF given by **Equation 47** (37).

$$DF = \left\{ \frac{\left[\left(S_1^2/n_1\right) + \left(S_2^2/n_2\right)\right]^2}{\left[\frac{\left(S_1^2/n_1\right)^2}{n_1+1} + \frac{\left(S_2^2/n_2\right)^2}{n_2+1}\right]} \right\} - 2 \qquad \text{Eq. 47}$$

Paired *t*-test

This statistical test is used to compare two analysis methods on the same or similar samples over the same concentration range (37). It differs from the previous average *t*-test because the previous does not separate the variation between the two methods, from that between samples. In the paired *t*-test this problem is overcome by looking at the average of the differences, $\bar{d}$, between each pair of results given by the two methods (39). This form of calculation only requires the number of tests to be equal for both methods (37).

As in the averages *t*-test, this test also presupposes the comparison between the two *t* values (t_{exp} and $t_{critical}$) being taken as acceptance criteria, $| t_{exp} | \leq t_{critical}$, i.e., it is concluded that the results obtained by both methods do not present significant differences if $| t_{exp} |$ is less than $t_{critical}$ value, for a certain confidence level and *N*-1 DF (37). The absolute value of the experimental *t* is obtained by **Equation 48** (37):

$$\left|t_{exp}\right| = \frac{D_m}{S_m} \cdot \sqrt{N} \qquad \text{Eq. 48}$$

where,

D_m – mean of differences (D_i);

D_i – difference between the values obtained, by both methods, for the same sample;

S_m – standard deviation associated with mean differences (D_i).

N – number of tested samples.

1.12. Control Charts

In many production industries, the traditional approach is to put quality control at the end of production, i.e., to look for possible defects in the final product, rather than focusing on a preventive strategy to avoid unnecessary production. This can be carried out by early stage data analysis, so that preventive measures can be taken in the process.

Control charts (CC) are a tool for statistically controlling a process graphically. They allow one to check whether a particular process remains stable and acceptable, in order to ensure that the final product follows the specific requirements.

There are several types of CCs, among which are Shewhart CC of averages, ranges, and cumulative sum charts. Shewhart CCs are a statistical measure, represented graphically and constructed from the data obtained from a given process. Data are collected in subgroups, and in each subgroup one or more variables are evaluated, namely mean and range, standard deviation or other accounting characteristics. The values of each subgroup are plotted against the subgroup number as shown in **Figure 11**. Additionally, in the CCs, three more levels are represented, namely the central (*CL*) and lower (L_{CL}) and upper control limits (U_{CL}) (**Figure 11**).

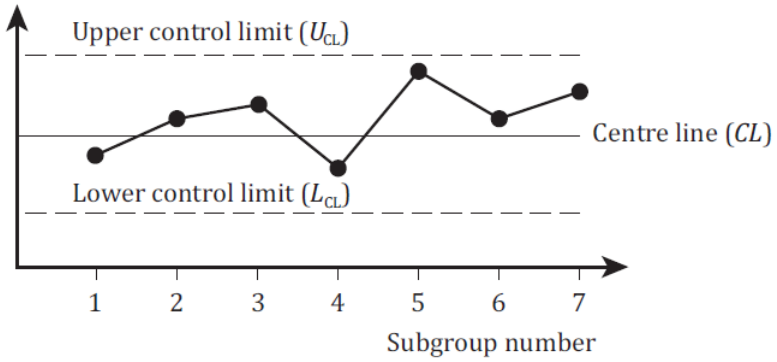

Figure 11 – Representation of a control chart.

These limits are statistically determined and placed on either side of the centreline (*CL*), at a distance of 3σ. Assuming that the values represented follow a normal distribution, the 3σ limits indicate that 99.7 % of the values are likely to be within the control limits, assuming that the process is under statistical control. When a value or a series of values has an unusual pattern, it is no longer possible to assume the statistical control of the process and in these situations, measures should be taken in order to define the cause. However, in rare occasions, when no assignable cause can be found for a point outside the limits, it is concluded that it is due to random effects, being the process still in control.

Essentially, Shewhart CCs can be resumed in two types, attribute and variable CCs, and for each of them two distinct situations can be verified:

- when pre-specified process parameters values are given.
- when no pre-specified process parameter values are given;

Pre-specified process values may result from process-specific prerequisites, target values, or estimated values that have been determined from data collected over time when the process is in control.

In the second case, and the one that will be given more relevance in the present work, the CCs graphic is plotted from data collected from process samples. Here, the main goal is to find if the variations, between subgroups, of the analysed features (*X*, *R*, etc) are due to not occasional causes.

The purpose is to detect those random variations in order to bring the process to a stage of statistical control.

As mentioned above, in addition to whether values can be pre-specified or not, CCs can be constructed from attributes or variables. Regarding the first, these are based in the observation within the considerate subgroups of the existence or absence of some characteristic (or attribute) and to assess how many units in the subgroup have or have not the attribute.

Concerning to variable CCs, namely *X*, *R* and *s* charts, they correspond to the most common application of CCs. Variable CC are particularly advantageous for most processes, because the information they offer is process specific, allowing to predict possible process problems. Additionally, most processes have measurable features which generate data for a possible application of CCs. Moreover, when compared to attribute CCs, these require a smaller subgroup size and so less data is needed, which entails less cost and decreases the time between determining the problem cause and taking the corrective action. Thus, variable CCs correspond to a visual way of assessing a process performance, by describing it in terms of mean and variability, which is why they are usually analysed in pairs.

Several variable CCs can be named, in particular:

1. average ($\bar{X}$) chart and range (R) or standard deviation (s) chart;
2. individuals (X) and moving range (R_m);
3. median ($\tilde{X}$) chart and range (R) chart.

In the case of the first group, the one that will be given more relevance in this work, it comprises two groups of CCs namely, average ($\bar{X}$) chart and range (R), and average ($\bar{X}$) and standard deviation (s) chart. The first set is usually applied when the subgroup size is small ($n < 10$). In the case of larger subgroups, the $\bar{X}$ and s CCs are more suitable, since the larger the sample size, the range control charts become less efficient in estimating the standard deviation of the process.

As mentioned above, in addition to the classification between variables and attributes, may be classified according to whether their control limits are pre-specified or estimated. **Table 4** summarizes the calculation formulas for the control limits of the various variable CCs. The values of the several factors (A, B, c, D) used in the calculation of the limits depend on the size of subgroup (n) and are available in ISO 7870-2:2013 for the construction of Shewhart CCs (44).

Table 4 – Formulae for the control limit estimation for Shewhart variables control charts (44).

Control Chart Type	Pre-specified control limits		Estimated control limits	
	Centre line	U_{CL} and L_{CL}	Centre line	U_{CL} and L_{CL}
$\bar{X}$	μ_0	$\mu_0 \pm A\sigma_0$	$\bar{\bar{X}}$	$\bar{\bar{X}} \pm A_2\bar{R}$ or $\bar{\bar{X}} \pm A_3\bar{s}$
R	$d_2\sigma_0$	$D_2\sigma_0$, $D_1\sigma_0$	$\bar{R}$	$D_4\bar{R}$, $D_3\bar{R}$
s	$c_4\sigma_0$	$B_6\sigma_0$, $B_5\sigma_0$	$\bar{s}$	$B_4\bar{s}$, $B_3\bar{s}$

Note: μ_0 and σ_0 are pre-specified values.

1.13. Uncertainty of analytical measurements

The word *uncertainty* could be associated with doubt. However, the confidence in a certain measurement increases with the information of the uncertainty. This is a non-negative parameter related to a measurement result which characterizes the dispersion of the quantity values that could satisfactorily represent that measurement. That is, the range of values that the analyst believes to be similar to the measurand (45).

An important distinction is the difference between error and uncertainty. These two parameters are often erroneously applied as representing the same, however, error is defined as the difference between a result and the measurand true value, that is, the error represents a single value, whereas uncertainty comprises a range of values where the true value of the measurand can be found. Once the uncertainty is estimated for an analytical procedure and defined sample type, may be applied to all determinations so described (45).

Regarding the error, three types can occur in a laboratory. These are gross, random and systematic errors. The former type concerns to those errors that make an analysis unfeasible when they occur. The breakdown of an instrument, the use of a contaminated reagent, the discard or dropping of a critical sample are some examples of this type of errors.

Random errors are related to the repeatability or reproducibility, affecting the precision of an experiment. These errors prevent the replicates of a measurement from having an acceptable degree of satisfaction with each other and, as they are related to the analyst technique and the equipment, can be minimized but not eliminated. On the contrary, systematic errors are a component of the error independent from the number of measurements and therefore cannot be minimized by its increasing. Instead, they can be rectified by the use of standard materials and methods and generally affect the trueness of a method. During a series of analysis of the same measurand, the systematic error remains invariable or varies in an expected way. The sum of all systematic errors inherent to a method is called bias, an overall deviation of a value from its true value, even when random errors are low (39,45).

The uncertainty of a measurement is usually influenced by these two error components, random and systematic (e.g. taking into account that a measurement in which procedure a gross error happened does not go forward) and several forms can be applied to calculate the uncertainty of a measurement, namely:

- The bottom-up approach or
- The empirical approaches where the calculation of uncertainty is based:
    - On in-lab validation and/or quality control data from the test method or
    - On validation and/or quality control data which takes into account interlaboratory comparison test data.

The calculation of measurement uncertainty by one of these approaches is at the discretion of each laboratory. However, this choice is dependent on the information available at the time and whether the technique chosen for the uncertainty assessment is duly validated and is adequate to evaluate the test method under study (43).

1.13.1. Bottom-up approach

The estimation of uncertainty through this approach involves several steps as shown in **Figure 12**. The first stage consists in the specification of the measurand and all sources that may add uncertainty to the measurement. The value of a measurand (y) depends on the various input quantities (*N*) that contribute to its calculation and therefore add uncertainty to the measured value.

For this, all sources of uncertainty must be identified in order to proper perform the calculation. The Ishikawa diagrams (also called cause-and-effect diagrams) can be an important tool in identifying the various sources of uncertainty. For instance, in **Figure 13** is shown the cause and effect diagram of the preparation of a standard cadmium solution.

After the identification of all uncertainty components, the next step involves the size determination of each component associated with all uncertainty sources identified, either by measurement or by consultation of tabulated values (43,45).

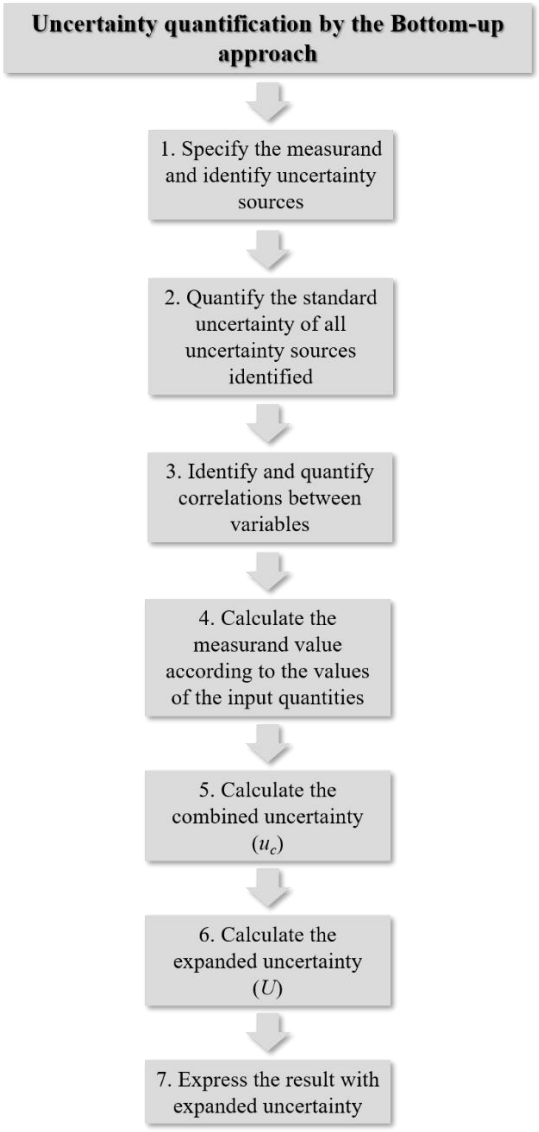

Figure 12 – Steps of the bottom-up approach for the calculation of measurement uncertainties.

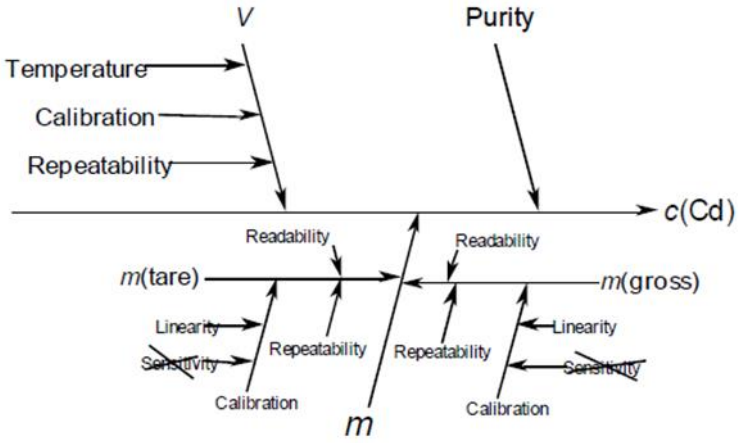

Figure 13 – Cause and effect diagram of the uncertainties in cadmium standard preparation (45).

Before combining all the contribution sources for uncertainty, all should be expressed as standard deviations. This can be calculated through the use of statistic tools to derive from experimental test results (type A evaluation), or using other means (type B evaluation) such as manufacturer's specifications, calibration certificates, test reports, values defining acceptance criteria or maximum acceptable errors.

Then, the contribution of all uncertainty components associated with the value of the measurand is combined by calculating the combined uncertainty (e.g. the standard uncertainty associated with y, u_c (y)). For this calculation, all components whose contribution to the uncertainty is less than one-fifth of the value of the highest uncertainty component can be neglected (43). The combined uncertainty can be performed by two ways. When the result of a test is given only by the addition and/or subtraction of the various input quantities, the calculation of the combined uncertainty (assuming as input quantities the variables *p*, *q* and *r*) is given by **Equation 49**:

$$u_c(y) = \sqrt{u(p)^2 + u(q)^2 + u(r)^2} \qquad \text{Eq. 49}$$

On the other hand, if the multiplication and/or division of the input quantities is used to obtain the final measurement result, the **Equation 50** can be used to estimate the combined uncertainty of the final result.

$$\frac{u_c(y)}{y} = \sqrt{(\frac{u(p)}{p})^2 + (\frac{u(q)}{q})^2 + (\frac{u(r)}{r})^2} \qquad \text{Eq. 50}$$

In the case of a situation where the measurement result is obtained by an expression combining both multiplication and/or division and addition and/or subtraction, a mixture of the above rules is used for the calculation of the combined uncertainty.

The previous **Equations 49** and **50** are applied to perform the combination of the associated uncertainties to each one of the independent input quantities, whose ways to assess will be reviewed next, namely for evaluating the most common inputs.

Uncertainty associated with weighing

In most cases, the uncertainty associated with a weighing, *u*(m) is given by **Equation 51**:

$$u(m) = \sqrt{u(tare)^2 + u'(m)^2} \qquad \text{Eq. 51}$$

being *u*(tare) the standard uncertainty associated to the tare of the used container, and *u*'(m) the standard uncertainty of the total mass (e.g. the tare and the reagent mass). If the tare and the gross mass are in the same scale zone, *u*(m) is given by **Equation 52**:

$$u(m) = \sqrt{2 \times u'(m)^2} \qquad \text{Eq. 52}$$

being *u*'(m) obtained directly from the scale calibration certificate, by dividing the expanded uncertainty at the work points, by the expansion factor, k (45,46).

Standard uncertainty associated with atomic and molecular weight

The standard uncertainty associated to the molecular weight is assessed by combining the standard atomic uncertainty for each chemical element, presented as that comprises the molecule. These are calculated, assuming a rectangular distribution, by **Equation 53**:

$$u(atomic) = \frac{uncertainty}{\sqrt{3}} \quad \text{Eq. 53}$$

being the uncertainty value available in a table provided by IUPAC (47), which contains the uncertainties for a large number of elements, as a confidence interval (e.g. ± *a*), together with their atomic weight. Afterwards, the all the atomic uncertainties are combined according to **Equation 49** to assess molecular uncertainty (45,46).

Standard uncertainty associated with purity

The standard uncertainty associated with the reagent purity is usually mentioned in its supplier's certificate, usually as a confidence interval (e.g. ± *a*). And, in order to obtain the standard uncertainty associated with purity, *u*(pur), if no further information is available, it is assumed that this quantity takes a rectangular distribution and is obtained by **Equation 54** (45,46).

$$u(pur) = \frac{a}{\sqrt{3}} \quad \text{Eq. 54}$$

Then, the final stage concerns to the calculation of the expanded uncertainty (*U*), i.e., the presentation of the uncertainty of a measurement as an interval with a certain level of confidence. This interval is expected to contain the set of values that may reasonably correspond to the value of the measurand (43). This topic will be discussed in more detail later in the empirical approaches to the calculation of uncertainties, as well as the presentation of the results.

1.13.2. Empirical approaches

Besides the step by step approach, there are other ways to calculate the uncertainty of a measurement. The measurement uncertainty is inherent in each of the individual measurement results. However, if the end result of a measurement is derived from several measurements that result from a controlled measurement process, the estimation of the measurement uncertainty for each individual measurement is generally not necessary. According to ISO 11352, it is assumed that for a set of non-floating measurement results the measurement uncertainty estimation can be applied to

the whole set. This is the principle of the empirical techniques, that are based in validation data or/and in the quality control of an analytical method. The data is obtained in the laboratory and interlaboratory environment and the uncertainty is calculated through the use of global performance parameters obtained in the concerned laboratory.

In this type of approach, the uncertainty calculation is not given by an expression that combines the several components contribution to the measurement uncertainty. Here, the uncertainty calculation is based on the two major error contributions, random and systematic corresponding to the standard uncertainty associated with precision and trueness respectively (**Figure 14**). These two uncertainty components are present throughout the assay method, in the sample matrix and in the concentration level of the analyte.

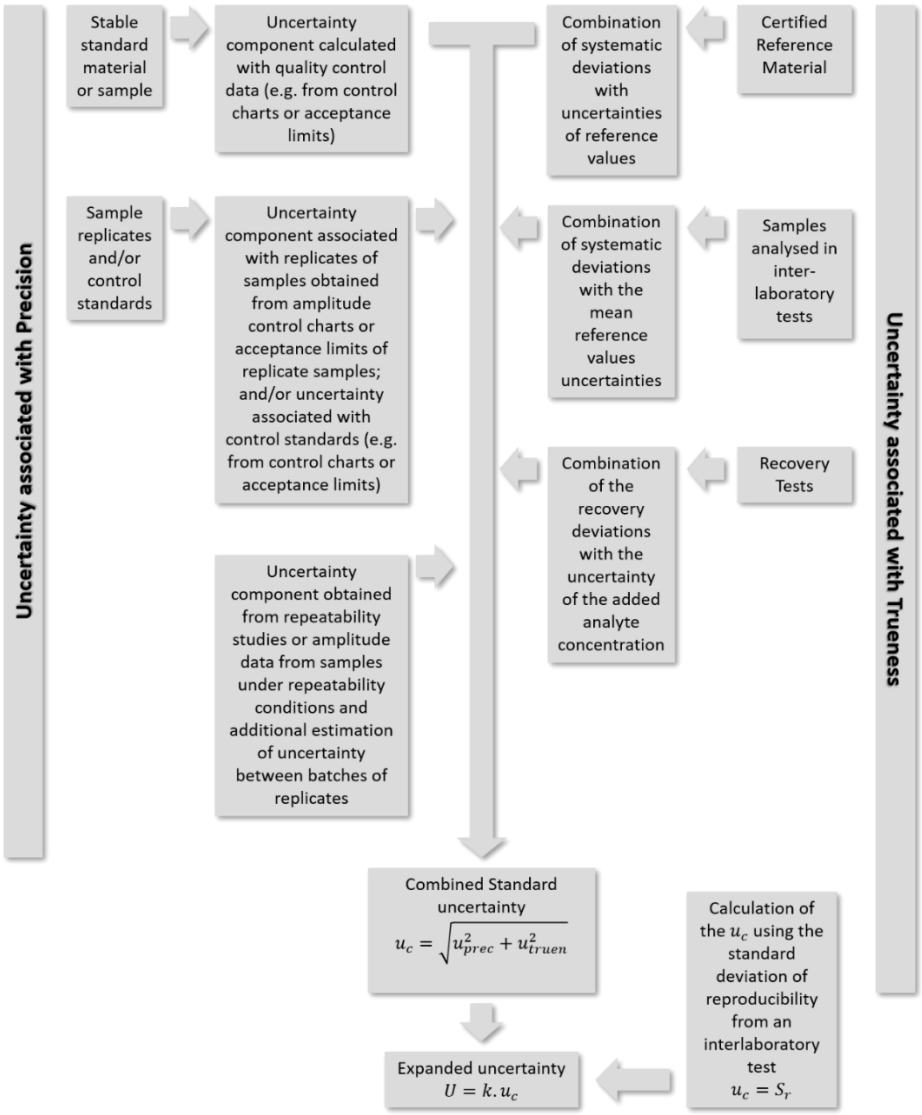

Figure 14 - Approaches for uncertainty estimation based on validation and quality control data (43).

Uncertainty calculation can be performed in relative or absolute terms. However, when opting for one of the forms, all components must be in the same dimension.

1.13.2.1. Standard uncertainty associated with precision

This uncertainty component corresponds to a significant part of the overall uncertainty of a method and therefore should be assessed throughout the method. The uncertainty associated to the precision of the results is calculated through the IP.

The standard uncertainty associated to the precision can be deduced directly by the absolute standard deviation of precision ($s_{precision}$), quantified accordingly to the **Equation 55**:

$$u_{precision} = s_{precision} \quad \text{Eq. 55}$$

The relative standard uncertainty associated with precision is calculated using **Equation 56**:

$$u'_{precision} = \frac{u_{precision}}{\bar{y}} = \frac{s_{precision}}{\bar{y}} \quad \text{Eq. 56}$$

where $\frac{s_{precision}}{\bar{y}}$ corresponds to the relative standard deviation regarding $\bar{y}$ mean value.

Several options can be used for the quantification of this uncertainty component, namely for different analysts or concentration levels, that allow to estimate a standard deviation, since no significant statistical differences can be verified between the dispersions of those analysts or concentrations.

Several data sources can be used to perform the calculation of the uncertainty of precision, namely:

- a stable sample or material;
- unstable samples;
- several samples and/or control standards (CSs).

The laboratory can even use information from its own quality control, such as the standard deviation used to define the limits of a control chart, values from acceptance criteria limits and ranges of replicates, in order to account for precision uncertainty components.

Of the many forms previously described, one can highlight the calculation of the precision uncertainty of a method through the data gathered in the analysis, either from CSs or from sample replicates. However, the combination of these two elements of analysis, although it may lead to an overestimated uncertainty, is complementary and allows to broadly characterize the dispersion involved in a method. On the one hand, sample analysis may not be as representative of the entire process as the CSs. On the other hand, the sole contribution of the latter may exclude possible matrix effects, which may be caused by interfering agents present exclusively in the sample matrix. Thus,

whenever possible, the uncertainty of the precision of a method should be obtained by combining CSs and sample replicates.,

In order to calculate the standard uncertainty associated with the combination of sample replicate data and CSs/materials, **Equation 57** is used:

$$u_{precision} = \sqrt{u^2_{preci,samp} + u^2_{preci,CS}} \quad \text{Eq. 57}$$

where,

$u_{preci,samp}$ – standard precision uncertainty associated with sample replicates;

$u_{preci,CS}$ – standard uncertainty of precision associated with standards.

Regarding the uncertainty component that is evaluated by sample replicates, there are several ways to measure it depending on how the replicate data are obtained. If duplicates of different samples are used for the assessment uncertainty, this is given by **Equation 58**:

$$u_{preci,samp} = s_{precision} = \sqrt{\frac{\sum_{j=1}^{t}(y_{j1} - y_{j2})^2}{2t}} \quad \text{Eq. 58}$$

being,

t – number of samples analysed in duplicate;

$(y_{j1} - y_{j2})$ – the difference between the duplicates of the sample j.

About the uncertainty component evaluated by the CSs, as with samples, there are several ways to measure their uncertainty, depending on the data currently available. One way to assess it can be by calculating the absolute standard deviation directly from a set of replicates as presented in **Equation 59**:

$$u_{preci,CS} = s_{precision} \quad \text{Eq. 59}$$

Both results of **Equations 58** and **59** can be used as presented, i.e., in its absolute form, or one can choose to present it as relative standard uncertainty by dividing the final result by the mean or central value of the measurand, as shown in **Equation 56** (43).

1.13.2.2. Standard uncertainty associated with trueness

As it was already mentioned, trueness evaluates the influence of systematic errors of a method. The uncertainty generated by this error component can be measured through the difference between the mean of multiple replicate assay results and one reference value, for which interlaboratory assays, CRMs or spiked samples and may be used, being these presented in descending order of relevance.

Although bias is ideally measured against a reference material, in practice is more common its assessment from spike recovery. In such assays a known amount of standard solution is added (*spike*) to the previously analysed sample and the method ability to recover the added analyte is assessed (43,45).

The standard uncertainty associated to trueness of the test method is assessed from two components:

- the difference between the observed values and the reference values verified in the spiked samples;
- the standard uncertainty of the concentration of the added solution.

The uncertainty associated to these two components can be combined as in **Equation 60**:

$$u'_{trueness} = \sqrt{b_{rms}^2 + u'^2_{add}} \quad \text{Eq. 60}$$

where,

b_{rms} – square root of the average of the recovery tests deviations (**Equation 61)**;

u'_{add} – uncertainty associated with the concentration of the added analyte (**Equation 64**).

Regarding the b_{rms} component, this can be calculated through **Equation 61**:

$$b_{rms} = \sqrt{\frac{\sum_{i=1}^{n_N} (b'_i)^2}{n}} \quad \text{Eq. 61}$$

being,

n – number of recovery trials.

b'_i – recovery deviation, calculated by **Equation 62**, when the deviation is calculated considering full recovery, or by **Equation 63**, if instead the average recovery is considered for the calculation.

$$b'_i = \frac{REC_i - 100\ \%}{100\ \%} \quad \text{Eq. 62}$$

$$b'_i = \frac{REC_i - N_m}{N_m} \quad \text{Eq. 63}$$

Where,

REC_i – individual recovery value (%);

N_m – recovery mean value of the test method (%).

Moreover, recovery trials shall be performed on at least six different samples from the matrix that is being assessed, wherein the confidence in the calculated uncertainty value increases with the number of tests performed.

About the uncertainty associated with the added analyte concentration, u'_{add}, it also depends on two components, namely the uncertainty associated to the added volume and the one associated to the added solution concentration, traduced by the following **Equation 64**:

$$u'_{add} = \sqrt{u'^2_v + u'^2_{conc}} \quad \text{Eq. 64}$$

being,

u'_v – relative standard uncertainty associated to the added volume;

u'_{conc} – relative standard uncertainty associated to the concentration of the spiking solution.

Regarding u'_v, just as the uncertainty associated with an analysis method has a random and a systematic component, so does the uncertainty associated with the added volume, and both must be taken into account in the calculation. The component of systematic errors may be calculated by the maximum permissible error (MPE) or the tolerance associated with the volume value. As this value is usually given without any indication of the distribution associated, it is considered that the volume systematic component, $u_{v,sys}$, is translated by a rectangular distribution and is therefore calculated by **Equation 65**:

$$u_{v,sys} = \frac{MPE/\text{tolerance}}{\sqrt{3}} \quad \text{Eq. 65}$$

The volume component associated with random errors ($u_{v,rep}$) is usually quantified by each laboratory, under repeatability conditions, and corresponds to a standard deviation. Thus, the uncertainty associated to the added volume is given by the following **Equation 66**:

$$u_v = \sqrt{u^2_{v,sys} + u^2_{v,rep}} \quad \text{Eq. 66}$$

being,

$u_{v,sys}$ – systematic uncertainty component of the added volume;

$u_{v,rep}$ – random uncertainty component of the added volume (repeatability conditions).

Regarding u'_{conc}, it can be obtained directly from the certificate of analysis, if a CRM is used, or calculated using the bottom-up methodology, in case the spiking solution is prepared in the laboratory. Taking the latter approach into account, it comprises the uncertainty quantification of each the associated and most frequent unit steps in chemical analysis (43), which were reviewed in **section 1.13.1.**.

1.13.3. Combined standard uncertainty

The standard uncertainties associated with each component (precision and trueness) and estimated by validation and/or quality control data, are merged in the combined standard uncertainty calculation, u_c, which resumes the overall uncertainty of a method. When the test method is applicable to several concentrations, the uncertainty components should be accounted for as relative standard uncertainties. In this case, the combined standard uncertainty, $u_c(y)$, of a given measure, y, is given by **Equation 67**:

$$\frac{u_c(y)}{y} = \sqrt{u'_{precision}{}^2 + u'_{trueness}{}^2} \qquad \text{Eq. 67}$$

On the other hand, when the test method is applicable to a limited number of concentrations, the combined standard uncertainty calculation is performed by the combination of the two components as standard uncertainties (43).

1.13.4. Expanded uncertainty

As it was mentioned, the culmination of the calculation of the uncertainty associated with a measurand corresponds to the expanded uncertainty (U). It is represented by an interval comprising all the values most likely to correspond to a measurand, with a certain level of confidence. In order to calculate this interval, combined standard uncertainty is multiplied by a coverage factor (k) by the **Equation 68:**

$$U = k \cdot u_c(y) \qquad \text{Eq. 68}$$

Taking into account that the best estimate of the measurement result is y, the limits of the range of values that can be attributed to the measurand are given by $y - U$ and $y + U$. The coverage factor determines the level of confidence in the interval of values and usually, varies between 2 and 3, depending if the intended confidence level is, respectively, 95.44 % or 99.72 %. However, the latter is less frequent.

The desired level of confidence is not the only determining aspect about choosing the coverage factor value. When the expanded uncertainty to be calculated is originated from a high number of tests, the coverage factor used for the calculation should correspond to 2. However, this may not be the best choice for the calculation of the expanded uncertainty when the calculations performed for the combined uncertainty took into account a reduced number of trials (less than six). Here, k value must be deduced from the two-tailed Student's *t* table with a level of confidence of 95 %, for *n*-1 DF.

Regarding the presentation of the final result, there is some information that is required. It is recommended a list of all the uncertainty components included in the final calculation and the way they were handled, a description of all the methods and values (including constants) used to calculate

the measurement result and its uncertainty. Moreover, the important steps comprised in the calculation process should be easily noticed and be described in a way that is easy to repeat the measurement if necessary. And, unless otherwise is required, the value of a measurand should be presented together with the expanded uncertainty for a coverage factor of two (k=2) which, in the case of a normal distribution, corresponds to a confidence level of 95.44 %:

"**(Result** ± ***U*)** [units]"

In case the report includes both analytical and sampling data and the calculation of the uncertainty value took only the analytical data it is important to refer that the uncertainty calculation does not include the sampling stage (43).

2. Materials and Methods

During the present work, the validation of the NPOC method, with the analyser Vario TOC Select, was performed. The study started with the validation of the difference method (DM) for the assessment of TOC. However, the process could not be completed due to technical problems that will be properly addressed during the development of this work.

Additionally, in order to evaluate the TOC/COD relationship in wastewater from the P&P industry, the TOC value was assessed by the DM and also by the direct method (NPOC). With the purpose of establishing this relationship, COD value was also evaluated by the use of a determination kit.

A new TNb calibration according with the superficial water matrix was also carried out to increase the analyser ability of evaluating this type of samples, so it will also be reviewed.

This chapter will then describe the materials and methods used to carry out the TC, TIC, NPOC, TNb and COD determination methods.

2.1. General principle of TOC analysis

TOC parameter can be determined by two methods, difference (TC – TIC = TOC) and direct method (NPOC).

Since water samples, in addition to organic carbon, also contain CO_2 and carbonates (TIC fraction), TOC determination by the direct method (NPOC) must be performed after TIC elimination. In this method, the sample is acidified and purged with a carrier gas free from CO_2 and organic compounds (e.g. O_2, as foreseen on the RAIZ TOC analyser). Thus, what is accounted for, in the end, is only NPOC.

TOC determination can also be performed indirectly. For this, the individual determination of TC and TIC is required, being TOC obtained by their difference (TC – TIC = TOC). The DM is usually used when samples are rich in volatile organic compounds (e.g. benzene, toluene, etc.), avoiding losing these compounds by *stripping*, which consists in the loss of more volatile components of a liquid mixture upon contact with a gas). This method is also suitable for samples where the inorganic fraction is smaller or at least similar to the organic one.

2.1.1. Vario TOC Select analyser operation fundaments

As mentioned in **1.9.**, the analyser acquired by RAIZ detects organic and inorganic carbon by means of CO_2. In the case of total carbon (TC), this is generated by total sample combustion at 850 °C, whereas for TIC, CO_2 is generated by sample acidification and subsequent purging by O_2. In the

case of TOC direct determination (e.g. NPOC), the oxidation of the carbon present is made by combustion, as for TC.

For both parameters (TC and TIC), the amount of CO_2 generated is detected in a non-dispersive infrared detector (NDIR) and is calculated from the peak area that is produced by CO_2 upon reaching the detector. Then, by means of the established calibration, the area is converted to carbon weight content and, therefore, to concentration.

After the entrance of the resulting CO_2 from sample's oxidation in the NDIR detector, a measuring electrical signal is generated, depending on the sample content in C and/or N. The signal is digitalized and integrated, resulting in an area value. If so, the blank value is discounted and, depending on the parameter being measured and the set of coefficients selected, the absolute content of the sample is calculated.

In order to have confidence in the results, a relationship must be established between the equipment detector signal and the sample content in C and/or N – calibration. The Vario TOC Select analyser software had an already established calibration for each one of the parameters, which allows the mass content, *y*, of each parameter to be calculated from the area, *x*, generated by the CO_2 upon its entrance in the detector. Thus, by taking into account the peak area, the several calibration curves are defined according to **Equation 69**:

$$y = a + b \cdot x \qquad \text{Eq. 69}$$

where,

y – mass content of the parameter (mg);

a and *b* – calibration coefficients of the calibration curve defined for each one of the parameters (available in the software of Vario TOC Select analyser);

x – peak area.

Then, from the calculated mass content value and the respective injection volume, the parameter concentration in the sample is given by the **Equation 70**:

$$C = \frac{y \cdot 1000000}{v} \qquad \text{Eq. 70}$$

being,

C – parameter concentration (mg/L);

y – mass content of the parameter (mg);

v – injection volume (µL).

Table 5 defines the calibration ranges in which the relationship between weight content and area, for each one of the parameters on the left, is linear and established according to **Equation 69**.

Table 5 - Calibration ranges defined for each one of the analysed parameters.

Analysed parameter	Calibration range (µg)
TC	2.00 – 12.00
TIC	1.00 – 6.00
NPOC	0.30 – 3.00
TNb	0.30 – 3.00

The fact that the calibration relationship is established between detector signal (area) and weight, rather than concentration, as commonly found in other equipments, makes it possible to perform calibration from one single standard solution. Due to the possibility of the analyser autosampler, calibration can be carried out in this way by using different injection volumes. This is possible because the sample volume has no influence on the signal size due to the use of a mass flow controller. It only depends on the content of C and/or N. The default TC and TIC calibrations were performed from a single standard solution by programming the equipment for the injection of different volumes, which correspond to different mass contents of each one of the parameters.

Although, calibration can also be performed from different standard solutions using the same injection volume. NPOC and TNb calibrations were performed this way. However, this is a more time-consuming way.

2.1.2. Software analysis methods

For the equipment to analyse a sample, whether blank, standard solution or real sample, it is necessary to define which is the method to be used. The analyser software has several predefined methods for the analysis of the different parameters involved in the TOC analysis.

Each method is associated with a set of coefficients that define the calibration curve (**Equation 69**) used in the interpolation calculation of the peak area into mass content of the parameter. **Table 6** describes the most commonly used methods, as well as the type of coefficients used for interpolation. The method *TC_RAIZ* was created when the analyser was already in RAIZ laboratory, to allow the individual analysis of TC, because no method was available in the software.

Table 6 - Analysis methods, description and associated coefficients for carbon determination.

Method	Description	Associated coefficients*
TC_RAIZ	Sample is directly injected in the furnace; no distinction is made between TOC and TIC.	TC
NPOC	Sample is directly injected in the furnace. At the beginning of the analysis, with the autosampler is possible to acidify the samples automatically. As suggested by the manufacturer of the analyser, the acidification acid should be a 10 % (w/v) HCl solution. Then, the needle moves to the position set for the acid vial (i.e. nº 32), removes acid which is then injected into each one of the vials whose analysis method is NPOC. After the acidification of the last vial, one minute elapses until the start of the analysis. If acidification cannot be performed automatically, samples have to be externally acidified to pH < 2 with a suitable acid (e.g. HCl), prior to measurement, in due time.	NPOC
TIC	Sample is injected in the *sparger* and TIC is purged by acidification with 10 % (w/v) H_3PO_4. Used when the analysis takes place according to the DM (TC-TIC=TOC).	TIC
TIC/TC	Sample is injected in the *sparger* and TIC is purged and measured; then, TC is determined by sample injection into the furnace. From their difference results TOC (TC – TIC=TOC).	TIC e TC

*The coefficient values of the analyser calibration curves for each parameter (i.e. TC, TIC, etc.) are available in the equipment software.

2.1.2.1. Methods extensions and specifications

Besides the analysis methods described above, Vario TOC Select software provides the possibility of adding specifications and extensions to the already existing methods, that change the analysis conditions. In **Table 7** these are resumed.

Table 7 - Analysis methods specifications and extensions.

Specification	Extension	
Precise vs *Fast*	*Particle*	*TNb analysis*
In both cases, the sample is injected into the furnace, but in the *precise* the first measurement runs in background (*dummy peak*) and it is not taken into account in the software calculations. Thus, it is almost completely avoided that remnants of previous measurements may affect the next sample measurements, but it takes longer than *fast* (15 vs 12 minutes). **NOTE**: methods with the *fast* specification are useful for performing cleaning runs between samples and standards solutions.	Activates a magnet rotation below the position of the sample under analysis; by introducing a magnet into the sample vial, creates agitation and prevents sedimentation of suspended particles. **NOTE**: automatically controlled function.	Simultaneous determination of total nitrogen by sample injection into the furnace.

2.2. TOC determination with the analyser

The following will describe the solutions and procedures used in determining the TOC parameter with the Vario TOC Select analyser.

2.2.1. Reagents

2.2.1.1.Water used in the trials

The water used for the preparation of all equipment solutions, samples and blanks corresponded to ultra-pure type 1 water, obtained through a Merck-Millipore purification system.

2.2.1.2. Stock solution for TOC/NPOC determination – [organic carbon] = 1000 mg/L

According with EN 1484:1997 for TOC determination in water, 2.125 g of $C_8H_5O_4K$ (100.00 % ± 0.05 %), obtained from Merck Millipore, were weighted in a Mettler-Toledo analytical scale and dissolved in a 1 L volumetric flask, after being dried at a temperature between 105 °C and 120 °C, for 1 hour. The solution volume was made up with water (**2.2.1.1.**) and was stored in a tightly closed bottle in refrigerated conditions. With these precautions, the solution can be stable for two months (27).

2.2.1.3. Stock solution for TIC determination – [inorganic carbon] = 1000 mg/L

This solution was made like the one described before, but by measuring 4.415 g of Na_2CO_3 (> 99.8 %), purchased from Chem-lab, in a Mettler-Toledo analytical scale, after previously dried, for 1 hour, at the temperature of 285 °C. Then, 3.500 g of $NaHCO_3$ (> 99.7 %), acquired from Panreac AppliChem, were also weighted in a Mettler-Toledo analytical scale, and added, after being for 2 hours in a desiccator. Water (**2.2.1.1.**) was used to make up the solution volume. This solution is stable for two weeks, at room temperature, and was also prepared according to the EN 1484:1997 for TOC determination in water (27).

2.2.1.4. Standard solution and difference method calibration (TC – TIC = TOC)

To perform calibration according with the DM, a mixed 20/10 mg/L TC/TIC standard solution, respectively, was prepared. From each of the solutions prepared in **2.2.1.2.** and **2.2.1.3.**, 2 mL were pipetted into a 200 mL volumetric flask and its volume was completed with water (**2.2.1.1.**). For this determination, a new fresh solution was prepared whenever calibration by this method was to be performed. By this way storage is not relevant.

The calibration of these two parameters, TC and TIC, was performed together and by different injection volumes of the TC/TIC standard, namely, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mL. Regarding TC,

these volumes correspond to the weights of 2, 4, 6, 8, 10 and 12 µg, respectively. For TIC, the weights that make up the calibration curve are 1, 2, 3, 4, 5 and 6 µg. **Table 8** summarizes the calibration procedure for carbon assessment by the DM.

2.2.1.5. Standard solutions and direct method calibration (NPOC)

To perform calibration according to the NPOC method, 10 mg/L NPOC standard solution was prepared. For this purpose, 2 mL of the solution **2.2.1.2.** were pipetted into a 200 mL flask and its volume was fulfilled with water (**2.2.1.1.**).

As mentioned in **2.1.1.**, NPOC calibration was performed by injecting the same volume (0.3 mL) of different standard solutions. So, six NPOC standard solutions were prepared by diluting 2, 5, 5, 10, 15 and 20 mL of the 10 mg/L standard solution, in 25 mL volumetric flasks, except the second standard solution, which was prepared in a 50 mL volumetric flask. The 10 mg/L solution also corresponded to the last standard of the NPOC curve. Thus, the final NPOC calibration standards are 0.8, 1, 2, 4, 6, 8 and 10 mg/L, corresponding to the mass contents of 0.24, 0.3, 0.6, 1.2, 1.8, 2.4 and 3 µg. **Table 8** summarizes the calibration procedure for carbon assessment by the direct method.

As for the DM, these solutions were prepared to carry out NPOC method validation with the TOC analyser. Therefore, the NPOC standards were prepared whenever calibration by this method was to be performed. Like before, the way of storage is not relevant.

Table 8 - Summary of calibration procedure for carbon assessment methods.

Method	Parameter	Standard (µg)	Standard solution (mg/L)	Injection volume (mL)
Difference	TC/TIC	2.00/1.00	20.0 TC/10.0 TIC	0.100
		4.00/2.00		0.200
		6.00/3.00		0.300
		8.00/4.00		0.400
		10.00/5.00		0.500
		12.00/6.00		0.600
Direct	NPOC	0.24	0.80	0.300
		0.30	1.00	
		0.60	2.00	
		1.20	4.00	
		1.80	6.00	
		2.40	8.00	
		3.00	10.00	

2.2.1.6.Phosphoric acid (H_3PO_4) 10 % (w/v) solution for TIC determination

In order to prepare this solution, to a 500 mL graduated glass flask, 35 mL (≈34.6 mL) of 85 % (w/w) commercial H_3PO_4, purchased from Panreac AppliChem, were added. Its final volume was made up with water (**2.2.1.1.**) and the final solution was stirred. This acidic solution is not of rigorous concentration and was prepared to provide the analyser the acidic solution to carry out TIC reaction.

2.2.2. Sample preparation and analysis

For TOC determination in water and wastewater, the samples were analysed on arrival day at RAIZ. If not possible, the samples were stored in the refrigerator at a temperature between 2 °C and 5 °C.

Although samples arrived from the factory were already acidified, when its analysis was to be performed by the direct method, pH was always verified by pH-indicator strips from Merck Millipore in order to assure that pH < 2.

To assure that the collected sample portion was as representative as possible of a given sample, homogenization was carried out throughout the sample preparation process for analysis. If prior dilution was required, the sample volume to be diluted was pipetted under agitation, in order to perform adequate homogenization. This agitation was carried out using a magnetic stirrer combined with a stir plate. Then, after sample dilution, transport of the volume portion to be introduced in the vial for analysis was also performed under agitation. Finally, the aliquot injection of the sample to be analysed was also done with agitation, since the analyser provides it automatically.

2.2.3. Calculations and expression of TOC results

A. Determination through the difference method

The weight content of both TC and TIC of each sample was obtained by entering the area value in the respective calibration curves and then applying the injection volume and the dilution factor of the sample to the calculation, as shown in **Equation 71**:

$$TC/TIC\ (mg/L) = \frac{\left[{(Area - a)}/{b}\right]}{V_{injection}} \times f \qquad \text{Eq. 71}$$

where,

a – intercept of the TC/TIC calibration curve;

b – slope of the TC/TIC calibration curve;

$V_{injection}$ – injection volume used for the sample analysis (mL);

f – dilution factor used in the sample.

Then, the sample TOC value was determined by **Equation 72**:

$$TOC(mg/L) = TC - TIC \quad \text{Eq. 72}$$

According to the Portuguese Accreditation Institute (IPAC), when an A result is assessed from the sum of several parcels (A = X + Y), as is the case, and any of them is lower than the LQ (X < LQ), the final result is equivalent to that of the quantifiable portion (A = Y), not considering the lower portion in the calculation, but indicating it (i.e. X < 5 mg/L (LQ)). In the present study, it was chosen to present the final result according to this rule, if any of the plots are in the referred conditions (48).

B. Determination through the direct method

From the day calibration curve, NPOC content of the samples was calculated from the following **Equation 73**:

$$NPOC\ (mg/L) = \frac{\left[{}^{(Area-a)}/_{b}\right]}{V_{injection}} \times f \quad \text{Eq. 73}$$

where,

a – intercept of the NPOC calibration curve;

b – slope of the NPOC calibration curve;

$V_{injection}$ – injection volume used for the sample analysis (mL);

f – dilution factor used in the sample.

2.3. TNb determination with the analyser

The analyser purchased by RAIZ has the ability to analyse TNb. However, the process is not independent from the carbon analysis. TNb analysis is an extension that can be coupled with other methods for carbon analysis, that is, when nitrogen is analysed carbon is also analysed.

Additionally, as mentioned in section **1.9.1.**, it is necessary to mimic the nitrogen species composition of the sample in the preparation of the standard calibration solutions. So, the equipment must also be calibrated for the various types of matrices compositions.

In the view of this, in the present work, the exemplified calibration procedure concerns to the calibration according to the TNb composition of surface water so, all next solutions were prepared in consideration of this. However, if there is a need to calibrate the analyser to another matrix, the actions to be performed in the software, that are described here, are equivalent. Only parameters, such as the injection volume, as well as concentration and composition of stock and standard solutions may vary.

2.3.1. Reagents

2.3.1.1. Standard stock solution for TNb determination – [TNb] = 500 mg/L

Given the greater complexity and susceptibility of the method to interferences, the standard stock solution was prepared in accordance with the TOC analyser manual, which advises for the use of ammonium chloride (NH_4Cl) and sodium nitrate ($NaNO_3$) as reagents to prepare these solutions.

Regarding the surface water matrix, the proportion found of nitrogen species was approximately 65 % of NO_3^- and 35 % of NH_4^+. Therefore, to prepare a 500 mg/L TNb stock solution in a 1000 mL volumetric flask, 1.973 g of $NaNO_3$ (99.4 %) and 0.667 g of NH_4Cl (99.7 %), both purchased from Panreac AppliChem, were weighed in a Mettler-Toledo analytical scale. These reagents were previously dried for 1 hour at the temperature of 105 °C. The flask volume was made up with water (**2.2.1.1.**).

2.3.1.2. Standard solutions and TNb calibration

A standard solution with a concentration of 10 mg/L of NPOC and 10 mg/L of TNb was prepared. For that purpose, 2 mL of the solution **2.2.1.2.** and 4 mL of the solution **2.3.1.1.** were pipetted to a 200 mL volumetric flask. The volume was made up with water (**2.2.1.1.**) and the solution was mixed thoroughly. From this standard, the TNb calibration standards were also prepared with the concentrations 1, 2, 4, 6 and 8 mg/L. Solutions 2, 4, 6 and 8 mg/L were prepared by pipetting 5, 10, 15 and 20 mL into 25 mL volumetric flasks. The 1 mg/L solution was prepared by pipetting 5 mL to a 50 mL volumetric flask. Their volumes were made up with water (**2.2.1.1.**) and homogenized. The 10 mg/L NPOC/TNb standard solution also corresponds to the last standard solution of the TNb calibration curve.

The new TNb calibration was performed by injecting 0.100 mL of each of the above-mentioned standard solutions, corresponding to the TNb weight contents presented in **Table 9**, which also summarizes the calibration conditions carried out for TNb.

Table 9 - Summary of calibration conditions for TNb assessment method.

Parameter	Standards (µg)	Standard solution (mg/L)	Injection volume (mL)
TNb	0.10	1.0	0.100
	0.20	2.0	
	0.40	4.0	
	0.60	6.0	
	0.80	8.0	
	1.00	10.0	

2.4. COD determination by digestion and spectrophotometric detection

The following section describes the procedures and solutions used for the assessment of COD by digestion and spectrophotometric detection.

2.4.1. Reagents

2.4.1.1. COD determination kit – sealed COD tubes

As suggested by ISO 15705:2002 for COD determination, wherever possible, ready-to-use sealed tubes should be used. The COD method involves the use of hazardous reagents and its handling can be minimized by the use of sealed tubes (49).

There are several types of commercial kits, with spectrophotometric detection, covering different analytical ranges. In the present work, HACH LCK 314 tubes for the concentration range of 15 mg/L – 150 mg/L, were used. These have mercury (II) sulphate ($HgSO_4$) in the composition, in order to suppress chloride concentrations up to 1000 mg/L.

2.4.1.2. Stock standard solution – [COD] = 10000 mg/L

In order to prepare the stock standard solution, 4.251 g of $C_8H_5O_4K$, previously dried at a temperature of 105 °C, for 2 hours, were weighed in a Mettler-Toledo analytical scale and dissolved in 500 mL of water (**2.2.1.1.**). This solution should be stored refrigerated, at a temperature between 2 °C and 8 °C, and has a shelf life of one month**.** Alternatively to refrigeration, and to prevent degradation by microbiological contamination, 2 mL of dilute sulfuric acid (H_2SO_4) may be added before making up the solution volume (49).

2.4.1.3. Standard solutions for calibration in the COD range of 15 mg/L – 100 mg/L

The volumes 1.5, 3, 9, 12 and 15 mL of the 10000 mg/L stock solution (**2.4.1.2.**), were separately diluted in 1000 mL of water (**2.2.1.1.**). The final solutions have a COD concentration of 15, 30, 60, 90, 120 and 150 mg/L, respectively, and a shelf life of one month. These solutions were stored at a temperature between 2 °C and 8 °C (49).

2.4.1.4. Silver nitrate solution – [$AgNO_3$] = 0.1 mol/L

To prepare this solution, 17.0 g of silver nitrate ($AgNO_3$**)** were weighed in a Mettler-Toledo analytical scale and dissolved in 1000 mL of water (**2.2.1.1.**). This solution was stored in a black glass bottle and is stable for six months at room temperature (49).

2.4.1.5. Potassium chromate solution – 5 % (w/v)

For the preparation of this solution, 5.0 g of potassium chromate (K_2CrO_4) were weighed in a Mettler-Toledo analytical scale and dissolved in 100 mL of water (**2.2.1.1.**). Then, the 0.1 mol/L $AgNO_3$ (**2.4.1.4.**) solution was added dropwise, until a red silver chromate precipitate was produced. The solution was finally filtrated and is stable at room temperature for 12 months (49).

2.4.2. Equipment

2.4.2.1. Digestor for sample digestion

According with ISO 15705:2002, the device must be capable of maintaining uniform temperature of (150 ± 5) °C along the entire tube length, without causing local overheating. Additionally, the digester must have the test capacity for 10 tubes, simultaneously. The tubes insertion holes must be of such diameter and height that ensure close contact of its walls with the heating block and proper heating of the tube, respectively (49).

In the present work, to carry out the samples and/or standards digestion, a heating block HT 200S from HACH was used, which meets the required necessities. It has 12 digestion compartments for test tubes and allows the test tubes heating from 40 °C to 150 °C or 170 °C. It is also programmable for digestion times from 5 min to 240 min.

2.4.2.2. Spectrophotometer for COD determination

The recommendation of ISO 15705:2002 is that the detection equipment, used after sample digestion, must be able to measure up to (600 ± 20) nm of wavelength. Also, the spectrophotometer must allow the reaction tube to be directly inserted in it. Thus, the solution does not need to be transferred to a separate cuvette for sample reading and the risk of environmental contamination by the toxic chemicals used in this method is minimized (49).

In the present work, a HACH spectrophotometer, model DR 2800, was used. It allowed reading in the visible region over a range of 340 nm to 900 nm.

2.4.2.3. Automatic pipette

In the COD method, a (2.00 ± 0.02) mL automatic pipette, purchased from Sartorius, was used to perform sample dilutions and to add them to the digestion tubes.

2.4.3. Calibration and spectrophotometric verification

The COD method was already implemented in the RAIZ laboratory and so equipment calibration in each analysis was not necessary. However, it is common practice to evaluate the

calibration that is in effect at the time. This assessment is made by measuring, in each analysis, the two CSs corresponding to the extremes of the calibration curve.

In the present work, only the low concentration COD range (15 mg/L to 150 mg/L) was used and, therefore, the CSs evaluated were 15 mg/L (method LQ) and 150 mg/L. These solutions were prepared by diluting the $C_8H_5O_4K$ stock standard (**2.4.1.2.**) from a different lot from that used to prepare the calibration standards. If these CSs do not meet the requirements set out in RAIZ's Internal Quality Control (± 10 % of the theoretical value of the CS), a new calibration curve must be performed.

2.4.4. Analytical procedure for COD measurement in samples

The present method is suitable for samples containing up to 1000 mg/L of chloride, as this is an interferent of the method. Thus, prior to sample digestion, the chloride concentration was always checked as follows, to detect concentrations above 1000 mg/L.

2.4.4.1. Chloride screening test

In a stoppered vial, 2.0 mL of test sample were added, together with two drops of K_2CrO_4 solution (**2.4.1.5.**). The mixture was slightly stirred and 0.5 mL of the $AgNO_3$ solution (**2.4.1.4.**) were added. The vial was stoppered and its content was mixed completely. If:

- the solution turned red, chloride concentration was below 1000 mg/L. So, the sample did not require further dilution and COD test could be performed;
- the solution stayed yellow, the chloride concentration was above 1000 mg/L. Consequently, sample required further dilution before carrying out the COD test.

In the present work, when the second situation occurred, in order to perform the COD test, the sample was diluted and the chloride concentration test was performed again, successively, until the sample colour was red, meaning that the chloride concentration was less than 1000 mg/L. This was the dilution factor applied to the sample to perform the COD test. In **Figure 15** are presented the tubes with the effluent samples after the performance of chloride screening test.

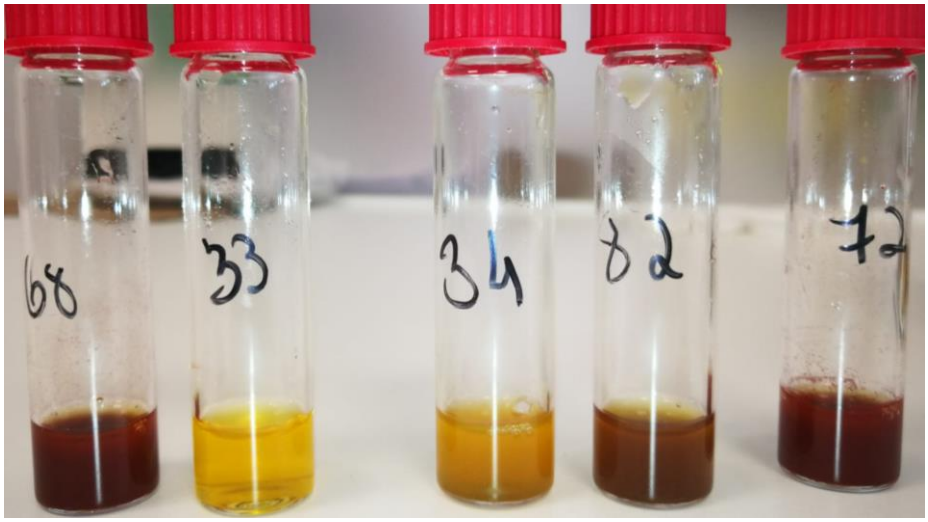

Figure 15 – Appearance of real samples after the chloride test. Yellow samples need further dilution; red samples have the appropriate dilution for COD measurement.

2.4.4.2. Sample digestion and spectrophotometric detection

After checking the chloride concentration, the samples were vigorously stirred and 2.0 mL of each sample were pipetted into a HACH commercial kit digestion tube. If diluted samples (by chloride concentration or COD concentration itself) were tested, 2.0 mL were also pipetted. Before and after pipetting the aliquot, the digestion tubes were shaken according to the manufacturer's instructions, as shown in **Figure 16**.

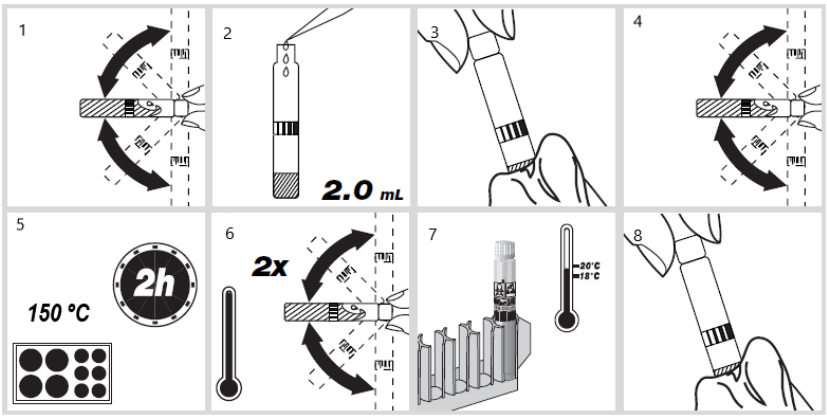

Figure 16 – Schematic representation of COD test procedure with HACH commercial kit (1 to 8 operations) (50).

Additionally, in each analysis series, besides the analysed samples, three more digestion tubes were added. A blank assay, consisting of sample dilution water (i.e. milli-Q water), and the two CSs, 15 mg/L and 150 mg/L, were analysed to validate the applicable calibration curve.

The tubes were all placed in the heating block and their contents refluxed for 2 hours at the temperature of 150 °C. After digestion, tubes were agitated and allowed to cool to room temperature, before measuring their absorbance. After cooling, if the digested samples were found to be clear in

appearance, their absorbance on the spectrophotometer (**2.4.2.2.**) was measured at the wavelength of 448 nm. Then, the results were obtained by comparison against the calibration graph that was in effect.

2.4.5. Calculations and expression of the results

From the calibration curve, the COD content of the samples was calculated from the following formula (**Equation 74**):

$$[COD] = \frac{(Abs - a)}{b} \times f \qquad \text{Eq. 74}$$

where,

[*COD*] – chemical oxygen demand (mg/L O_2);
Abs – sample absorbance after digestion;
b – slope of the calibration curve in effect;
a – intercept of the calibration curve in effect;
f – dilution factor applied to the sample.

2.5. Validation of NPOC method

2.5.1. Work range

The work range of NPOC method was already defined by the manufacturer and was assessed by the homogeneity of variances test. For that, 12 individual measurements of the lowest standard (0.3 µg) and the highest standard (3 µg) were carried out.

To begin with, due to the volumetric material available at the time, 200 mL of a 20 mg/L intermediate standard solution were prepared. Thereafter, from this solution were prepared 200 mL of each of the standards to be tested, with the final concentrations of 10 mg/L and 1 mg/L.

Finally, the variances associated with the measured signal of each standard were tested by performing the PG test, in order to verify the possible existence of significant differences between them.

2.5.2. Linearity and Sensitivity

Regarding linearity, it was assessed using a statistical model according to ISO 8466 – 1, for linear calibration functions, and to ISO 8466 – 2, for non-linear calibration functions. For that, several calibration curves were carried out, during the validation stage of the NPOC method, and the Mandel's test performed (40). Additionally, their graphical representation, accompanied by the correlation coefficient, was performed, having been established as an acceptance criterion $r \geq 0.999$.

Although the latter is a good indicator of correlation, it is not necessarily of linearity. At the same time, sensitivity was evaluated by the slope of the same calibration curves.

For the purposes mentioned above, standard NPOC calibration solutions were prepared fresh on each day of analysis.

2.5.3. Analytical Limits

Since the method involves a linear calibration, the analytical limits were estimated according to **Equations 28** and **29**, through the slope of the calibration curve and its residual standard deviation. To achieve this purpose, from two calibration curves, namely the factory and one carried out at RAIZ laboratory, the respective theoretical limits of quantification and detection were calculated and their arithmetic mean was performed. The calibration curve performed at RAIZ laboratory was carried out according to the method described in section **2.2.1.5.**, as it was the factory calibration curve.

The assessed theoretical LQ value was tested by HV test, as well as one weight level higher, but lower than the already established first standard of the NPOC calibration curve.

Then, it was tested over time by the analysis of CSs. As a criterion for the acceptance of CSs and, therefore, the LQ, a relative error of 10 % or less was established.

2.5.4. Repeatability

The repeatability assessment was performed individually for both types of treated and untreated effluent. Ten independent measurements of the same sample were performed at the lower end of the NPOC calibration curve and free of outliers. To assess the presence of extreme values, the Grubbs' test was used, carried out according to ISO 5725-2 (42). Then, the repeatability limit and the corresponding coefficient of variation were calculated.

2.5.5. Intermediate precision

To evaluate this parameter, range CCs were used in this study. For this, several measurements in duplicate were performed on samples of two matrices, treated and untreated effluent. The range control charts were then constructed based on the data collected from the samples and, in accordance with ISO 7870 – 2:2013, for Shewhart control charts construction (44).

2.5.6. Reproducibility

The assessment of this precision component was carried out by participating in an interlaboratory trial. The sample delivered by the organizing laboratory consisted in water from a urban waste water treatment plant, already acidified with sulfuric acid (H_2SO_4) (pH < 2) and, therefore, no further acidification was required. The sample was first diluted (1:10) and the analysis

was carried out with agitation and in triplicate by the NPOC method, since the trial organizer requested the submission of three measurements.

The reproducibility of the method was estimated by calculating the coefficient of variation of reproducibility (CV_R). Besides the estimation of the reproducibility, it was possible to evaluate the laboratory performance against other laboratories through the obtained value of Z – score.

2.5.7. Assessment of uncertainty associated with trueness

Trueness of NPOC method was assessed for two types of sample composition by spiked samples, namely treated (effluent) and untreated (influent) effluent. For that, six samples of each matrix were analysed in duplicate, both the spiked and the real sample. The dilution factors applied to the samples were 1:100 and 1:125 for treated and untreated effluent, respectively. The 100 mg/L NPOC spiking solution was prepared by dilution from the stock solution prepared in **2.2.1.2.**. Samples were fortified with 4 mL and 5 mL of this solution, depending on whether it corresponded to treated or untreated effluent, respectively.

As noted above, the analyser calibration is performed in weight instead of concentration. In this way, the percent recovery calculations were done in weight. Each duplicate was analysed in triplicate, whose injection volume was 0.200 mL. Thus, three area values resulted from the analysis of each duplicate and where converted in weight through interpolation on the daily calibration curve.

The average NPOC weight per duplicate and, therefore, the average NPOC weight per sample were calculated using **Equations 75** and **76**:

$$m_1/m_2 = \frac{\sum_{i=1}^{n} m_i}{n} \qquad \text{Eq. 75}$$

$$m_{sample} = \frac{m_1 + m_2}{2} \qquad \text{Eq. 76}$$

Being,

$\sum_{i=1}^{n} m_i$ – sum of the weight values corresponding to the volume injections in each duplicate;

n – number of injections per duplicate;

m_1/m_2 – the NPOC weight of the first and second duplicates;

m_{sample} – final NPOC weight of sample/spiked sample.

The same procedure was carried out for the fortified samples. The volume of the spike corresponded to 4 mL and 5 mL, depending on whether the sample corresponded to effluent or

influent, respectively. **Table 10** shows the carbon weight increments corresponding to the added spiking solution volumes, either in the final volume of the spiked sample or in the injection volume.

Table 10 – Used volumes of spiking solution and the corresponding weight increase.

V_{spike} (mL)	V_{final} (mL)	$m_{incremented}$ in V_{final} (µg)	$m_{incremented}$ in $V_{injection}$ (µg)
4	200	400	0.4
5	250	500	0.4

Thus, in the recovery percentage calculation of each sample used was considered the mass increase in the injection volume and **Equation 77** was applied:

$$\%\ REC = \frac{m_{spiked\ sample} - m_{sample}}{m_{increased}} \times 100\ \% \qquad \text{Eq. 77}$$

where,

$m_{spiked\ sample}$ – carbon mass of the spiked sample present in the injection volume (µg);

m_{sample} – carbon mass of the regular sample present in the injection volume (µg);

$m_{increased}$ – carbon mass increase in the injection volume corresponding to the added volume of spiking solution (µg);

% REC – recovery percentage of the sample.

2.5.8. Assessment of uncertainty associated with precision of NPOC method

Uncertainty associated with precision was evaluated by the analysis of several samples and CSs, representative of the whole process. The samples were analysed in duplicate, by matrix. Under these conditions, **Equation 58** was used to quantify the uncertainty associated with precision through the samples.

In order to make the accuracy uncertainty as representative as possible, the data from the three CSs (0.24, 0.30 and 3.0 µg), performed at each working session to control the calibration, was also used. From the several measurements, the absolute standard deviation was estimated, being this directly equivalent to the precision uncertainty, as indicated by **Equation 59**.

In order that the concentration would not affect the final precision uncertainty, both final values were used as relative standard uncertainty (**Equation 56**) and the global uncertainty was calculated according to **Equation 57**, by the combination of data from sample duplicates and CSs.

2.6. Correlation between water quality assessment parameters for wastewater from the P&P industry

Untreated influent and treated effluent samples were received weekly and analysed in the arrival day for their TOC or NPOC content, depending on the method used, in the Vario TOC Select analyser (**2.2.2.**). These were collected and received in vials filled to the top and previously acidified to pH 2. Still, if the analysis was performed by NPOC method, their pH was confirmed by a rapid test using pH-indicator strips, obtained from Merck Millipore. The samples were stored overnight at a temperature between 2 °C and 5 °C and COD analysis was carried out in the next day, in duplicate, according with section **2.4.4.**. To avoid errors arising from an unequal distribution of suspended solids, samples were homogenized before and during the conduction of the analytical analyses.

3. Results and discussion

3.1. Difference method validation

As it was mentioned in the previous section, the validation procedure involves the evaluation of certain parameters. Having the present work been developed in an accredited laboratory according to the NP EN ISO/IEC 17025:2018 normative reference, it is of paramount importance that the various parameters to be evaluated fulfil the acceptance criteria.

The first attempt, in the present work, was to validate the DM for TOC analysis. However, due to technical problems that will be addressed later in this paper, it was not possible to conclude the validation of the DM. So, the validation of the direct TOC analysis method (NPOC) was carried out. First the validated parameters of DM will be discussed and then the validation of the direct method will be described.

3.1.1. Work range

As mentioned in section **1.10.1.**, HV test consists in verifying the existence of significant differences between the variances of the extremes of each of the work ranges. For this, ten replicates of the extreme standards of each of the working ranges (TC:[2-12]µg and TIC:[1-6]µg) were performed, being the obtained results shown in **Table A1** (**Appendix A**). Then, the variances quotient of the lowest and highest standards (S_1^2 and S_{10}^2, respectively) was compared with the critical value of the Fisher-Senedecor distribution ($F_{critical}$).

Regarding the data in **Table 11**, it can be concluded that both PG values are greater than $F_{critical}$, for a confidence level of 99 % and nine DF ($f_1 = f_2 = 9$). So, the differences between the variances are considered significant and, according to ISO 8466-1, it can be established that there is no homogeneity of variances between the extremes of each of the work ranges (40).

Table 11 – Variables used to perform the HV test for TC and TIC parameters.

Work range (µg)	X_i (µg)	S_i^2	PG	$F_{critical}$
TC [2-12]	2	557.1	11.36	5.35
	12	6327.5		
TIC [1-6]	1	5632.2	12.33	5.35
	6	69459.8		

Furthermore, the dispersion and accuracy of the test results were evaluated by calculating the CV and *E*r, respectively (**Table A1 - Appendix A**). Regarding the latter, *Er* value of the highest and the lowest TC (-16.4 %) and TIC (26.9) standards, respectively, show a significant deviation from the reference value for the respective standards. Although this value is purely indicative, it shows

poor accuracy with regard to these standards. Still, it was expected that over the course of the method validation, accuracy could improve.

Regarding dispersion, none of the calculated CVs (**Table A1** – **Appendix A)** surpasses de 10 % value, as advised by IUPAC, and, as a consequence, the intra-day repeatability is considered satisfactory.

Nevertheless, the experimental values measured for each standard were plotted in order to observe the dispersion of the ten replicates around its theoretical value for both TC and TIC parameters. **Figures 17** and **18** show the graphical representation of the ten replicates measured used to carry out the HV statistical test. As can be seen from both **Figures 17** and **18**, the replicates of the higher standards (12 µg – TC; 6 µg – TIC) for both TC and TIC, present greater dispersion than the lower standards (2 µg – TC; 1 µg – TIC), which results in the greater variance associated with them. This supports the results of the statistical test performed, i.e., the heterogeneity of the data between the extremes of the ranges.

Moreover, several attempts were performed in order to reach an experimental value not significantly different from the theoretical value of the highest TC standard. However, in the several readings performed, the experimental value differed systematically and significantly from the theoretical value of the standard.

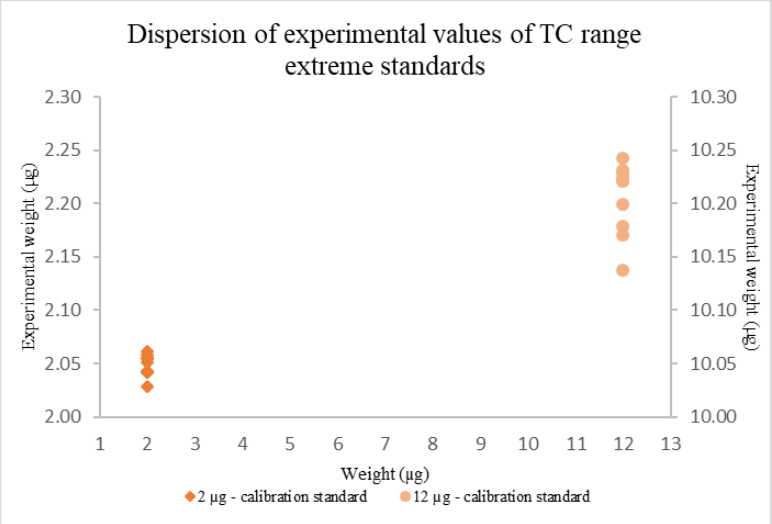

Figure 17 – Weight values of the TC extremes standards obtained in the HV test.

The same was performed for the TIC work range (**Figure 18**) and, although not as significant as for TC, the dispersion of the replicates around the theoretical value can be observed.

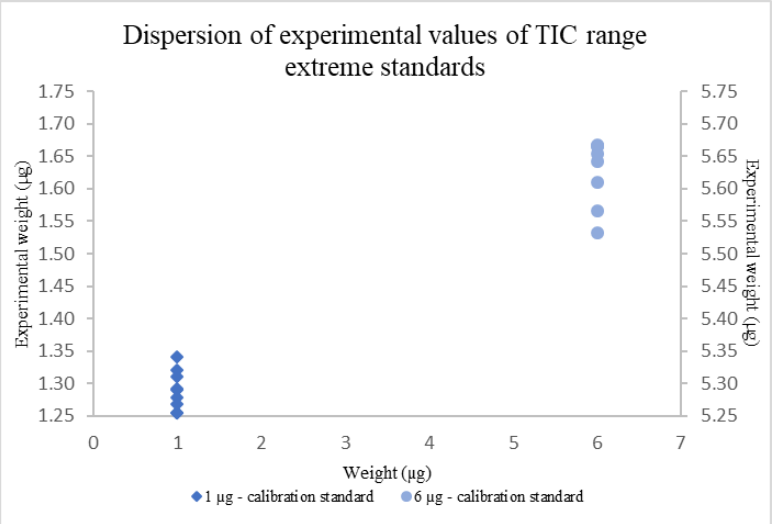

Figure 18 – Weight values of the TIC extremes standards obtained in the HV test.

Taking into account the heteroscedasticity in both work ranges, each of the TC and TIC work ranges was divided in two, i.e., an intermediate standard was tested in each of the working ranges and the HV test was performed again in an attempt to obtain a positive result. The area and weight results obtained for each standard are expressed in **Table A2** in **Appendix A**.

From these results, the *Fisher-Snedecor* test was performed to verify the homoscedasticity. The values required to perform the test are shown in **Table 12**. Regarding the TC work ranges, significant differences between the variances of the extremes of the lowest TC half-range (2 – 7.5 µg) were verified. However, the same did not occur for the 2nd TC range, since PG < $F_{critical}$. For TIC, HV was only confirmed in the 2nd half-range, because PG (1.37) < $F_{critical}$.

Table 12 – Variables used to perform the HV test for TC and TIC half working ranges.

Work range (µg)	X_i (µg)	S_i^2	PG	$F_{critical}$
TC [2 – 7.5]	2.0	557.0	6.72	5.35
	7.5	3746.0		
TC [7.5 – 12]	7.5	3746.0	1.69	5.35
	12.0	6327.5		
TIC [1 – 3.5]	1.0	5632.2	9.02	5.35
	3.5	50811.5		
TIC [3.5 – 6]	3.5	50811.5	1.37	5.35
	6.0	69459.8		

Regarding dispersion, CV values were not significant, namely 0.4 % and 2.5 % (**Table A2** – **Appendix A**) for the 7.5 µg and 3.5 µg standards, respectively. However, the *Er* value for the intermediate TC standard (7.5 µg) indicates a significant difference from the real value (-13.2 %).

To conclude, although HV has been proven in two of the four ranges, in the other two was not. One possible approach would have been to reduce each of the TC and TIC working ranges until the differences between the variances of the extremes were not significant. However, this is not an advantageous approach because it does not allow full use of the analytical capacity of the equipment. The other approach, applying a weighted linear regression (WLR) to the equipment data, was approved.

3.1.2. Linearity

Linearity is assessed by the Mandel's test according to ISO 8466-1 and ISO 8466-2 and by the graphical representation of the measured signal together with its correspondent correlation coefficient (*r*) (40,51).

After HV test, it was concluded that the best approach would be to apply the weighted linear regression to the TC and TIC calibration curves. Nevertheless, linearity was initially tested by performing Mandel's statistical test for two calibration curves, TC and TIC, in order to prove the linearity statistically. In addition to the statistical test, linearity was confirmed by the graphical representation and by *r* value. The two working ranges continued to be studied over time. However due to the need to apply to the results the weighting, Mandel's test could not be performed on all curves.

For this purpose, TC and TIC calibration curves were constructed according to section **2.2.1.4.**. In **Table 13** are presented the required values for the statistical test, performed according to ISO 8466, parts 1 and 2. According to Mandel's test, one method is of linear calibration if PG < $F_{critical}$, condition that was statistically proven for both TC and TIC. Additionally, **Figure 19** also show the calibration curves of both parameters, which together with their correlation coefficients (**Table 13**) prove linearity. In addition, both correlation coefficients are in accordance with the established acceptance criteria ($r \geq 0.999$).

Table 13 – Coefficients of the calibration curves and variables used in the Mandel's statistical test.

Parameter	*b*	*a*	*r*	$S_{y/x}$	S_y^2	DS^2	PG	$F_{critical}$
TC	2008.1	-9.8	0.99985	144.7	163.6	3382.0	0.13	10.13
TIC	2274.8	-339.4	0.99991	61.7	71.0	79.3	0.02	

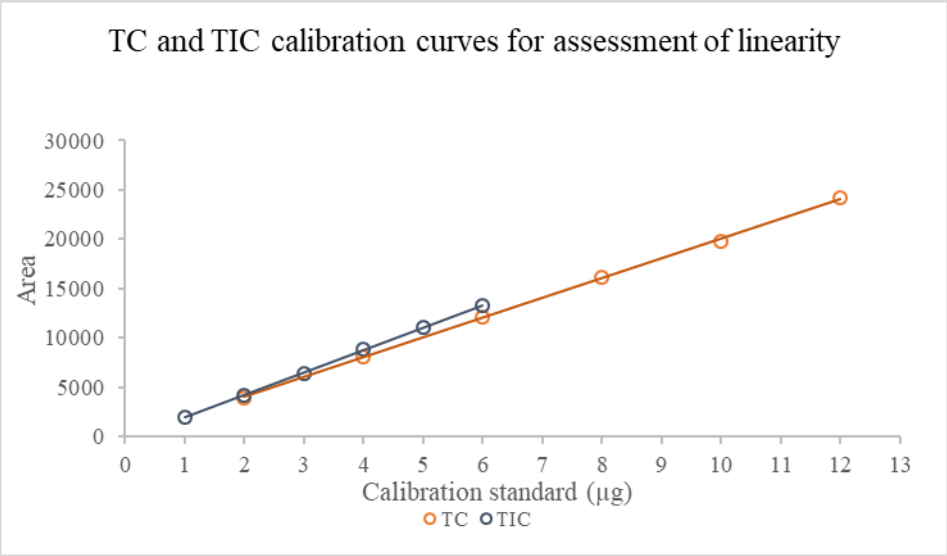

Figure 19 – TC and TIC calibration curves used for linearity assessment.

Although linearity has been demonstrated, from the calibration data used to perform the statistical test, shown in **Table 14**, it was concluded that for both TC and TIC, as the calibration standard value increases, the difference between the experimental value and its theoretical value increases. However, for TIC, this difference is acceptable, as the maximum *Er* value was found to be - 6.2 % (6 µg-standard), that is, in modulus is less than the 10 % value assumed as acceptance criteria for the standards. For TC this difference was very evident since from the 4 µg-standard the *Er* value is always greater than 10 % acceptance criteria.

Table 14 – Data from the TC and TIC calibration standards used to carry out Mandel's statistical test.

Parameter	Standard (µg)	$\overline{\text{Area}}$	$\overline{\text{Weight}}$ (µg)	*Er* (%)
TC	2	3945.2	1.9	-3.8
	4	8031.4	3.6	-8.9
	6	12125.0	5.4	-10.5
	8	16174.0	7.1	-11.6
	10	19841.6	8.6	-13.8
	12	24162.0	10.4	-13.5
TIC	1	1950.3	1.0	2.1
	2	4211.2	1.9	-3.0
	3	6399.2	2.8	-5.7
	4	8834.9	3.8	-4.5
	5	11063.4	4.7	-5.5
	6	13275.5	5.6	-6.2

Note: the area value is an average value, and so the weight value as well, because it results from three volume injections of the same vial, not from analysis replicates.

3.1.3. Application of weighted linear regression to calibration data

As mentioned, the heteroscedasticity of the data was confirmed and the choice of a new, more appropriate, calibration model to define the relationship between carbon mass and area was necessary. Reducing the working range is usually the most adopted solution for addressing extremes heteroscedasticity. However, the aim was not to work with a narrower calibration range, as it would imply a reduction in the method's analysis capability. In addition, in the RAIZ laboratory, the analysed samples correspond to different concentration ranges, which implies that the analyser must be used in all the interval values. So, in the present work a weighted least squares linear regression model was used.

To apply the weighted model to the calibration data, **Equations 23** to **27** were used (section **1.10.2.1.**). With that purpose, in each working session, the TC and TIC calibration standards were analysed, and the variance associated with the signal value (area) of each of the standards was calculated. Then, to establish the coefficients of the weighted calibration curve (*b*, *a* and *r*) of the day, a weighting factor was applied to each calibration point, that was inversely proportional to the corresponding y-direction (area) variance. For that, three determinations of each calibration standard were made. These did not result from replicates, but from different injections from the same vial. This procedure was applied to all calibration curves performed in the present work.

The weighting factor, *w*, used (**Equation 23**) was not very practical, as it required several determinations to be made for each calibration point and, as mentioned, a new calibration curve had to be performed at each work session. Consequently, in order to speed up the process, a spreadsheet was created in the excel program that allowed to apply the weighting to the data more efficiently, and has been duly validated. This sheet is represented in **Figure B1** (**Appendix B**), a view of this Excel spreadsheet.

Figures 20 and **21** show the TC and TIC calibrations, respectively, used to perform the Mandel's statistical test (**section 3.1.2.**), but the weighted linear regression applied to the calibration curve is represented. Thus, it was concluded that the adjustment of the weighted lines to the calibration of each of the parameters is good, which is also evidenced by the correlation coefficient values, both higher than 0.999 (**Table 15**). Therefore, despite the application of the weighting to the calibration data, good linearity was verified.

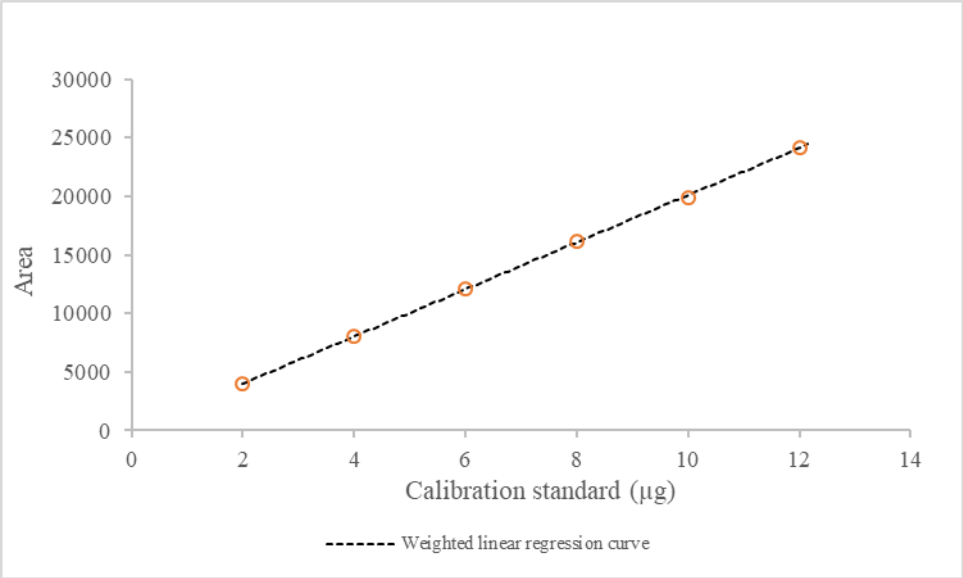

Figure 20 – Fitting of weighted linear regression to TC calibration data – area as a function of TC weight.

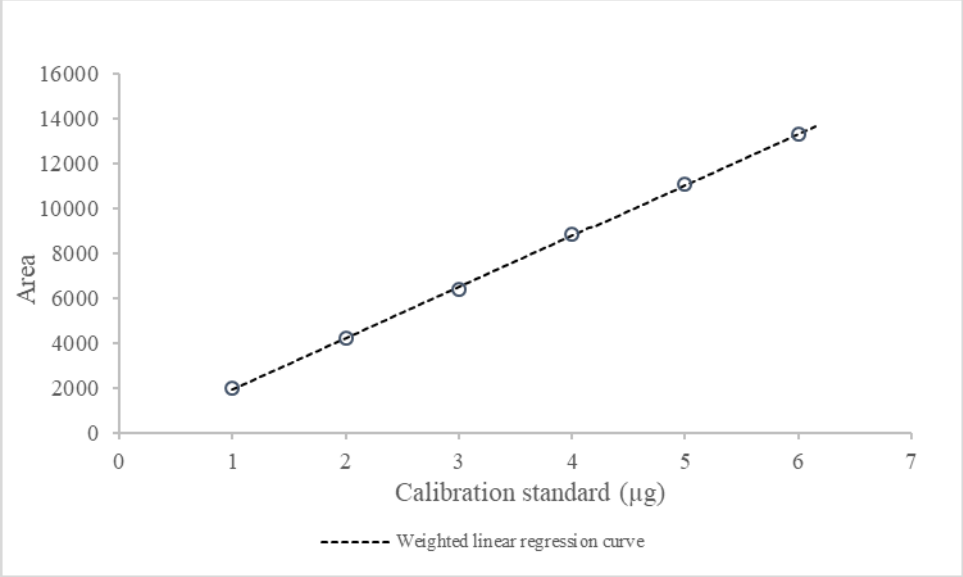

Figure 21 – Fitting of weighted linear regression to TIC calibration data – area as a function of TIC weight.

Additionally, in order to verify whether there were significant differences between the weighted and unweighted linear regression calibration curves, an averages *t*-test was performed to the respective slopes. The variance of each calibration curve was calculated from the respective residual standard deviation ($S_{y/x}$) for the performance of the *F*-test. The data proved to be heteroscedastic. Therefore, t_{exp} was calculated through **Equation 46** and compared to $t_{critical}$, for five DF (**Equation 47**) and a confidence level of 95 %. The values calculated for the statistical tests, as well as the parameters of each of the curves, are presented in **Table 15**.

Table 15 – Weighted and unweighted linear regression coefficients of TC and TIC calibration curves and calculated values for the slope comparison *t* test.

Parameter	Regression type	*b*	*a*	*r*	$S_{y/x}$	t_{exp}	$t_{critical}$
TC	LR	2008.1	-9.8	0.99985	144.7	0.001	2.57
	WLR	2011.4	-0.42	0.99988	11304.5		
TIC	LR	2274.8	-339.4	0.99991	61.7	0.005	2.57
	WLR	2270.5	-331.0	0.99990	2307.4		

Note: in the case of WLR, $S_{y/x}$ value is replaced by $S_{w(y/x)}$.

For both TC and TIC, the result of the statistical test was $t_{exp} < t_{critical}$, that is, there are no statistically significant differences between each of the linear regressions. Taking this in consideration, can be assumed that WLR calculations do not present any advantage regarding the unweighted methods. However, despite the similar coefficients, the WLR allows the measured calibration curve to pass closer the points with a reduced associated deviation, contributing to the accuracy of the method.

3.1.4. Averages *t*-test

Regarding the DM for TOC analysis, the international standard EN 1484:1997 indicates the use of sodium hydrogen carbonate ($NaHCO_3$) as TIC standard together with sodium carbonate (Na_2CO_3). However, the Vario TOC Select analyser manual does not recommend the use of sodium hydrogen carbonate ($NaHCO_3$) as TIC standard, if it is used in a mixture with potassium hydrogen phthalate ($C_8H_5O_4K$), due to the slightly acid character of the latter (27,36). Therefore, before evaluating the rest of the validation parameters, a comparison between the calibration curves, for both TC and TIC, carry out with the standard solution with and without $NaHCO_3$ was performed.

In view of this, and due to the fact that the calibration standard solution for both TC and TIC is a mixed TIC/TOC standard, several calibration curves for each of the TC and TIC work ranges were performed independently. Then, to assess possible differences in the calibration curves due to the use of $NaHCO_3$, a hypothesis test was carried out, namely a *t*-test. Through this it was possible to conclude whether the results from the two methods (calibration with and without $NaHCO_3$) presented significant statistical deviations or not.

Table 16 presents the results of the *t*-test performed for the TIC work range, with and without the contribution of $NaHCO_3$ in the calibration standard. Initially, for three TIC determinations of the calibration curve (extremes and middle of the range) representative areas were chosen (**Table A3** of **Appendix A**) and the corresponding weight values were calculated from the coefficients of the calibration curves used. These values are compiled in **Tables A4** and **A5** of **Appendix A** for TIC and TC, respectively.

Then, a HV test was performed to verify if the variances were significantly different for each weight determination, by the two methods. For each weight determination of the calibration curve, F_{exp} was found to be lower than $F_{critical}$, for a 95 % confidence level. It was concluded the existence of HV between the results obtained by each of the two methods. The result of the HV test is essential because it determines the t_{exp} way of calculation.

Thus, for every determination, a t_{exp} was calculated according to **Equation 44** and compared with the $t_{critical}$ for eight DF and a confidence level of 95 % (**Table 16**). It was found that $t_{exp} < t_{critical}$ for the three TIC determinations, which allowed to conclude that the use, or not, of $NaHCO_3$ in the preparation of TIC calibration curves is equivalent and, therefore, both approaches are possible and do not affect the performance of the calibration curves.

Table 16 – Results of the *t*-test performed to TIC work range to evaluate possible differences in the use of $NaHCO_3$.

Weight (µg)	F_{exp}	DF_{num}	DF_{denom}	$F_{critical}$	S^2	S	t_{exp}	$t_{critical}$
1	1.22	3	5	7.76	0.003	0.05	0.71	
3.5	1.32	5	3	14.88	0.03	0.17	1.20	2.31
6	1.45	5	3	14.88	0.08	0.29	1.28	

The same procedure was followed for TC. The *t*-test results for TC are displayed in **Table 17**. As for TIC, $t_{exp} < t_{critical}$ (six DF and a confidence level of 95 %) for TC. Thus, it was concluded that there were no significant differences between the two methods and, once again, both approaches are conceivable.

Table 17 – Results of the *t*-test performed to TC work range to evaluate possible differences in the $NaHCO_3$ use.

Weight (µg)	F_{exp}	DF_{num}	DF_{denom}	$F_{critical}$	S^2	S	t_{exp}	$t_{critical}$
2	3.78			39.25	0.009	0.09	-1.20	
7	3.38	4	2	39.25	0.03	0.16	0.20	2.45
12	2.90			39.25	0.06	0.24	0.71	

In the view of the *t*-test results for TIC and TC parameters and taking into account that the assay methods performed in RAIZ laboratory follow the international standards, it was decided that the methodology followed would be in accordance with EN 1484:1997, that is to use $NaHCO_3$ as reagent in the calibration solution together with Na_2CO_3 and $C_8H_5O_4K$. Thus, from this moment, all TC and TIC calibrations were performed from the calibration standard solution with $NaHCO_3$ as reagent too.

3.1.5. Sensitivity and analytical limits - dependence on the blank value

The equipment does the blank value discount automatically but only if the dilution factor is entered into the software (the form of blank discount is described in more detail in **Appendix C – Vario TOC Select Analyzer Operation/Maintenance Procedure**). However, at the beginning of the present work, was not given much importance by the analyser supplier's technicians to this issue. The information that was provided to RAIZ laboratory was that the discount would be automatically made, provided that the blank samples were analysed with the name identified by the analyser software (*Blank*).

The real form of the blank value discount was only understood after obtaining the results using the DM. Consequently, parameters such as LQ were incorrectly determined, because the calculation of the several calibration curves used to assess it were performed without the blank value discount. Thus, a comparison was made between the parameters without and with the blank value discount.

Tables 18 and **19** show the coefficient values (*a* and *b*) of various calibration curves used for the definition of the LQ of TIC and TC, respectively. In both **Tables 18** and **19**, in each parameter, the values in the left column refer to the calibration curves made without the blank rate value discount, unlike those on the right, where the blank rate was taken into account.

Table 18 – Values of the parameters used for TIC sensitivity and LQ assessment.

	b		***a***		$S_{(y/x)w}$		**LQ**	
	- B	+ B	- B	+ B	- B	+ B	- B	+ B
+ $NaHCO_3$	2275.8	2270.5	-331.0	-331.0	48.04	48.04	0.21	0.21
	2447.3	2443.8	-326.0	-326.0	41.33	41.33	0.17	0.17
	2447.3	2444.1	-302.9	-302.9	74.17	74.17	0.30	0.30
	2477.7	2471.9	-216.1	-216.1	81.70	81.70	0.33	0.33
- $NaHCO_3$	2399.7	2395.9	-387.6	-387.6	56.39	56.39	0.23	0.24
	2347.9	2364.9	-285.3	-356.1	48.03	48.03	0.20	0.20
	2582.4	2588.3	-321.2	-370.3	63.66	63.66	0.25	0.25
	2628.1	2624.9	-412.5	-412.5	79.80	79.80	0.30	0.30
	2483.1	2478.7	-317.9	-317.9	19.92	19.92	0.08	0.08

Note: all calibration curves presented have a $r \geq 0.999$. – B: value without the blank discount; + B: value with the blank discount.

In the present work, the LQ value calculation was performed from *b* and $S_{(y/x)w}$ (**Equation 29**). As can be observed, the $S_{(y/x)w}$ values do not practically vary for the totality of the curves of both TIC and TC. Thus, the slight variations that occur between the LQ values, before and after the blank value discount, might be due to the variations in the slope of the day's calibration curve. In order to verify whether there are significant differences between the slope values calculated with and without the

blank discount, a paired *t*-test was performed for both TC and TIC. Thus, the mean of the differences ($|\bar{d}|$) between the values, with and without the blank discount, were calculated to estimate the t_{exp} value, which was then compared to $t_{critical}$, for a confidence level of 95 % and a DF number of n-1. The test values of both parameters are shown in **Table 20**.

Table 19 – Values of the parameters used for TC sensitivity and LQ assessment.

	b		***a***		$S_{(y/x)w}$		**LQ**	
	- B	+ B	- B	+ B	- B	+ B	- B	+ B
+ $NaHCO_3$	2023.9	2011.4	-0.4	-0.4	118.52	118.53	0.59	0.59
	2022.2	2000.3	425.6	425.6	46.07	46.07	0.23	0.23
	2051.6	2030.3	279.8	279.8	259.36	259.36	1.26	1.28
	1952.3	1933.2	71.4	71.4	136.93	136.93	0.70	0.71
	1994.2	1979.5	123.0	123.0	143.47	143.47	0.72	0.72
- $NaHCO_3$	2058.6	2043.7	44.7	44.7	176.41	176.41	0.86	0.86
	2020.2	1997.4	-87.1	-87.1	374.83	374.83	0.47	0.48
	2073.3	2051.3	-162.5	-162.5	333.49	333.49	1.61	1.63

Note: all calibration curves presented have a $r \geq 0.999$. – B: value without the blank discount; + B: value with the blank discount.

As can be seen, in the case of TIC, no significant differences between the slopes of the two groups are verified ($t_{exp} < t_{critical}$). However, for TC, the *t*-test result, $t_{exp} > t_{critical}$, indicated that there are significant statistical differences between the values with and without the blank value discount.

Regarding the differences verified for TC, a new paired *t*-test was performed to the LQ values (**Tables 18** and **19**), to assess if the differences in slope affected the LQ. Although no differences were found for TIC regarding the slope, the new test was carried out for both parameters. The test values are also shown in **Table 20**.

Again, the TIC *t*-test result showed that there are no significant differences between the values with and without the blank value discount ($t_{exp} < t_{critical}$), in this case for the LQ. However, for TC, significant differences were found between the two groups of values, since $t_{exp} > t_{critical}$.

Table 20 – Values used in the paired *t*-tests to evaluate the slope and LQ differences due to blank value discount.

	TC		**TIC**	
	slope	**LQ**	**slope**	**LQ**
$\boldsymbol{|\bar{d}|}$	18.7	0.01	0.7	0.0003
S	4.0	0.01	7.5	0.001
n	8	8	9	9
$\boldsymbol{t_{exp}}$	13.09	2.39	0.28	2.00
$\boldsymbol{t_{critical}}$	2.36	2.36	2.31	2.31

It should be pointed out once again, that the blank value discount form was only known after the work with the DM had finished. Therefore, taking into account the result of the statistical test, and that the information regarding the blank value was obtained in the final stage of the present work, a study should be performed again to assess this validation parameter properly, for both TC and TIC.

Before evaluating the LQ values for both TC and TIC and taking into account that it was chosen to perform the calibration with the contribution of $NaHCO_3$ in the standard solution, the slopes of the calibration curves performed at RAIZ were compared with the default slope by a significance *t*-test performed between the slopes mean value of each parameter and the factory calibration slope (reference value). In **Table 21** are shown the *t*-test results and the slope values used to perform the test for TC and TIC. In **Figures 22** and **23** are shown the factory calibration curves together with the several calibration curves used to carry out the *t*-test, that were performed at RAIZ with $NaHCO_3$, whose coefficients are shown in **Tables 18** and **19**.

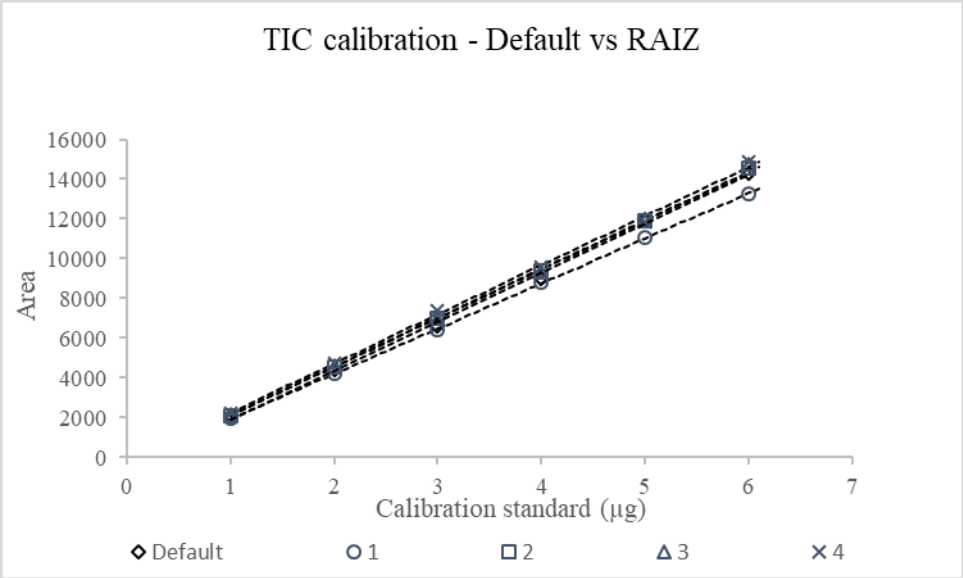

Figure 22 – Calibration curves performed on RAIZ compared to the default calibration curve for the TIC.

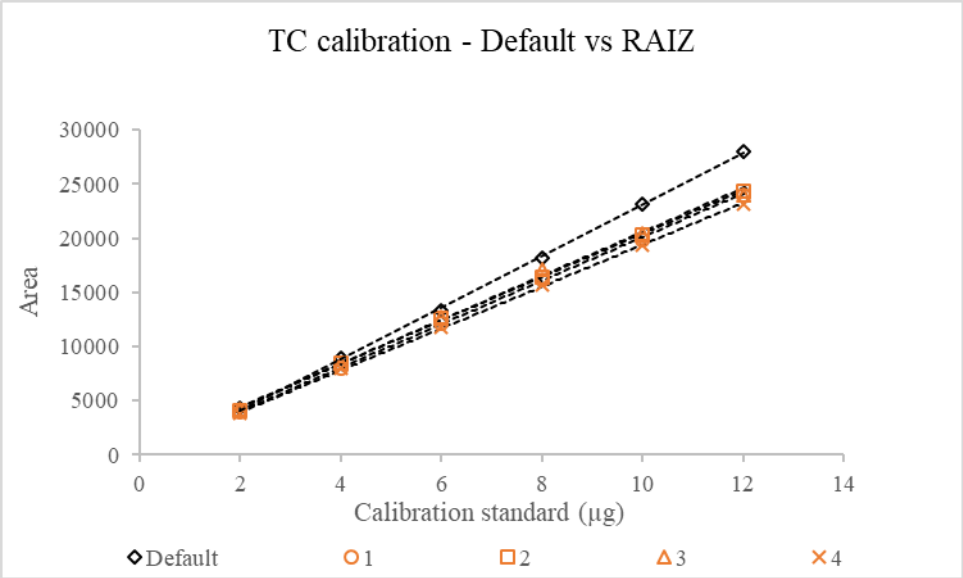

Figure 23 – Calibration curves performed on RAIZ compared to the default calibration curve for the TC.

As can be seen from the results obtained for TC, t_{exp} is higher than the $t_{critical}$, which means that the slopes of the calibration curves produced in RAIZ present significant differences from the default slope, the reference value. The result is in accordance with **Figure 23**, as a clear deviation of the several calibration curves from the factory one can be observed.

Regarding TIC, the result of the statistical test, $t_{exp} < t_{critical}$, (**Table 21**) reinforces what is observed in the TIC calibration curves (**Figure 22**), since, despite some variation, RAIZ curves are superimposed on the default calibration curve.

Table 21 - Slope values of the TC and TIC calibration curves and their deviation from the factory calibration (default).

Parameter	No.	Date	*b*	$b_{default}$	t_{exp}	$t_{critical}$
TC	1	06/nov	2011.4	2374.3	18.01	3.18
	2	21/nov	2000.3			
	3	23/nov	2030.3			
	4	03/dez	1933.2			
	Mean	-	1993.8			
	s	-	42.3			
TIC	1	05/nov	2270.5	2459.5	1.12	3.18
	2	20/nov	2443.8			
	3	21/nov	2444.1			
	4	26/nov	2471.9			
	Mean	-	2407.6			
	s	-	92.3			

Although TC deviation was significant and systematic, the method evaluation was continued, assuming that the area values for the same amount of carbon would be improved in the TC calibration curves performed in RAIZ laboratory.

3.1.6. Assessment of the Limit of Quantification

The weight determinations to add to TC and TIC calibration curves, corresponding to the LQ, were chosen, taking into account the above calibration curves and, before understanding the real operation mode of the analyser for the blank value discount. However, as can be seen from the values in **Tables 18** and **19**, the blank value hardly changes the calculated LQ values.

Taking into account the above calculated LQ values for TIC (**Table 18**), it was decided to evaluate the determinations 0.3 µg and 0.6 µg. The values chosen are not only related to the obtained results, but also with the available material to prepare the solutions and the injection volumes to be defined in the analyser software. In addition, the choice of two determinations relates to the fact that if one proved to be poorly accurate, the other could yield good results, avoiding starting the process of the LQ definition from the beginning again, and all the associated costs. Regarding the TC parameter, the same assumptions were made in choosing the determinations 0.6 µg and 1.05 µg.

For this purpose, ten individual measurements of each determination of TIC and TC were performed to assess their accuracy and precision. Results are presented in **Table 22**.

Table 22 – Weight values measured for evaluation of LQ determinations for TC and TIC parameters.

Replicate	**TC**		**TIC**	
no.	**0.60 (µg)**	**1.05 (µg)**	**0.30 (µg)**	**0.60 (µg)**
1	0.64	1.13	0.47	0.91
2	0.67	1.16	0.47	0.88
3	0.69	1.14	0.47	0.91
4	0.64	1.12	0.47	0.92
5	0.64	1.14	0.46	0.91
6	0.67	1.15	0.47	0.91
7	0.64	1.14	0.47	0.90
8	0.63	1.15	0.47	0.91
9	0.66	1.14	0.46	0.89
10	0.61	-*	-*	-*
Mean	0.65	1.14	0.47	0.91
s	0.02	0.01	0.01	0.01
***Er* (%)**	8.3	8.6	55.6	50.8
CV (%)	3.5	1.2	1.2	1.2

Note: The values with *, namely the tenth measurement, which are not presented for the 1.5, 0.3 and 0.6 µg standards refer to outliers that were removed after the performance of Grubbs' test.

Precision was satisfactory for the four chosen standards, since the respective variation coefficients, CV, are less than 10 % for all standards. The accuracy assessed by the *Er* value is acceptable for both TC standards (< 10 %). However, TIC standards showed very high values regarding *Er*. Even so, since precision values were acceptable, it was decided to keep the determinations of both parameters on the respective calibration curves in order to test their accuracy over time.

The calibration of both parameters was performed through a single standard solution, and the different standards were established through different injection volumes. Therefore, the establishment of the new determinations required a new standard solution to be prepared by diluting the TIC/TC standard of 10 mg/L and 20 mg/L, respectively. This new standard solution had the concentration 1.5 mg/L and 3 mg/L for TIC and TC, respectively. **Table 23** summarizes the injection volumes and solutions used to perform the calibration process, for TIC and TC, after using the new determinations on each one of the calibration curves.

Table 23 – TC and TIC calibration procedure after adding the LQ weight determinations to the calibration curves.

Standard solution (mg/L)	**TC**		**TIC**	
	$V_{injection}$ (mL)	**Standard (µg)**	**$V_{injection}$ (mL)**	**Standard (µg)**
3.0 TC/1.5 TIC	0.200	0.60	0.200	0.30
	0.350	1.05	0.400	0.60
20.0 TC/10.0 TIC	0.100	2.00	0.100	1.00
	0.200	4.00	0.200	2.00
	0.300	6.00	0.300	3.00
	0.400	8.00	0.400	4.00
	0.500	10.00	0.500	5.00
	0.600	12.00	0.600	6.00

Although the LQ was tested everyday, over time, by the analysis of CSs, a calibration curve was initially performed for each of the parameters (TC and TIC) in order to test linearity of the new calibration curves. The coefficients of the two obtained curves are presented in **Table 24** and **Figures 24** and **25** refer to the TC and TIC calibration curves obtained, respectively.

Table 24 - Coefficients of TC and TIC calibration curves after inclusion of LQ values.

Parameter	***b***	***a***	***r***
TC	1900.6	185.1	0.99967
TIC	2236.4	104.0	0.99954

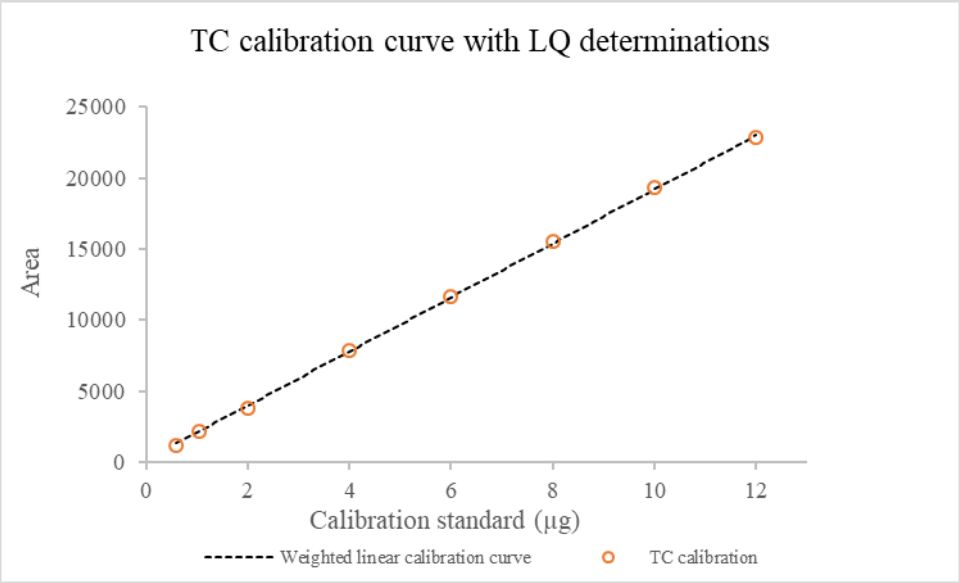

Figure 24 – TC calibration curve for linearity test after inclusion of the LQ.

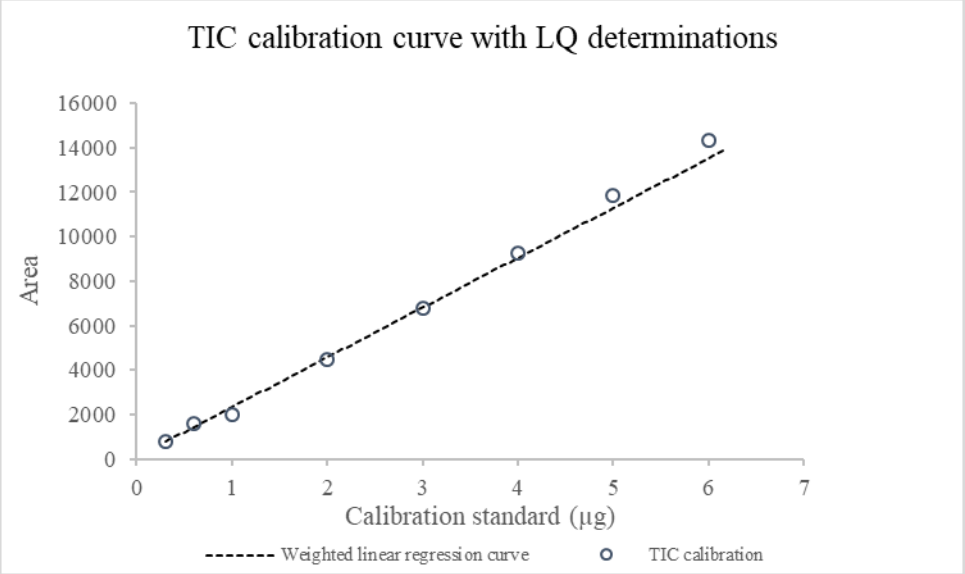

Figure 25 – TIC calibration curve for linearity test after inclusion of the LQ.

Both TC (**Figure 24**) and TIC (**Figure 25**) calibration curves present reasonable adjustments since the respective *r* values (**Table 24**) are in accordance with the acceptance criteria set by the laboratory ($r \geq 0.999$). In addition, accuracy of the LQ standards of both calibration curves was evaluated again, being the values shown in **Table 25**.

Table 25 – Accuracy assessment of the LQ standards when performing the respective calibration curves.

Parameter (µg)	Standard (µg)	Weight (µg)	*Er* (%)	CV (%)
TC	0.60	0.52	-13.2	1.4
		0.53	-11.3	
		0.52	-13.4	
	1.05	1.06	1.1	0.2
		1.06	1.2	
		1.06	0.7	
TIC	0.30	0.30	-0.2	0.2
		0.30	0.1	
		0.30	0.1	
	0.60	0.66	9.2	2.5
		0.68	13.0	
		0.65	7.7	

Note: the three weight readings result from three volume injections that were performed from the same vial, not replicates of each standard.

As before, the precision of the weight values, evaluated by the CV, is satisfactory. values are satisfactory. As for accuracy, it improved for TIC standards regarding the first test. Concerning TC standards, for 1.05 µg, the *Er* value is less than 10 %, i.e., it meets the acceptance criteria defined. However, for standard 0.6 µg this value exceeds the criterion, although not substantially.

3.1.7. Daily calibration and effluent analysis

The deviation of the TC calibration from the default calibration persisted and, therefore, it was continually reported to the equipment suppliers, but little relevance was given to the information passed by the laboratory, and, the situation remained.

In order to continue the work, in RAIZ laboratory, the analysis of real samples began. Consequently, before real samples analysis, TC and TIC calibration curves were performed, together with calibration CSs (TIC – 0.3, 1 and 6 µg; TC – 0.6, 2 and 12 µg).

Firstly, the analysed samples corresponded to untreated influent and treated effluent, whose TOC values measured by the DM are shown in **Table 26** and **27**, respectively. **Figure 26** shows a graphical example of the TC calibration, namely that of 31st January, along with the samples analysed on that same day.

Regarding TIC, mostly of the assessed values correspond to a very low concentration, because the samples that arrived at the RAIZ laboratory, were already acidified (pH < 2), which resulted into a very low concentration of this parameter. Thus, according to **section 2.2.3. – A**, the TOC result of the analysed samples is equal to TC content.

Taking into account the divergence, between the calibration curve performed at the RAIZ and the factory calibration, TOC value of the samples was also evaluated through the default calibration (TOC_a) (**Tables 26** and **27**).

Table 26 – TOC values obtained for treated effluent samples.

Sample	Date	*f*	TC_w (mg/L)	TIC_w (mg/L)	TOC_w (mg/L)	TOC_a (mg/L)
1	10/jan	20	233.6	< LQ	233.6	225.5
2	10/jan	20	284.6	< LQ	284.6	245.4
3	17/jan	20	221.6	< LQ	221.6	192.8
4	17/jan	20	285.4	< LQ	285.4	246.1
5	24/jan	20	308.7	< LQ	308.7	275.9
6	24/jan	20	323.2	< LQ	323.2	288.5
7	31/jan	10	285.9	< LQ	285.9	254.7
8	31/jan	10	270.2	< LQ	270.2	241.0

Note: subscript w – value assessed through RAIZ calibration curve; subscript a – value assessed by the default calibration.

Table 27 – TOC values obtained for untreated influent samples.

Sample	Date	*f*	TC_w (mg/L)	TIC_w (mg/L)	TOC_w (mg/L)	TOC_a (mg/L)
1	10/jan	50	580.1	< LQ	580.1	502.7
2	10/jan	50	880.6	< LQ	880.6	755.9
3	17/jan	50	474.3	2.5	471.9	412.5
4	17/jan	50	622.0	2.7	619.3	535.9
5	24/jan	50	619.1	< LQ	619.1	557.0
6	24/jan	50	717.8	< LQ	717.8	642.8
7	31/jan	20	777.3	< LQ	777.3	690.4
8	31/jan	20	598.5	< LQ	598.5	533.0

Note: subscript w – value assessed through RAIZ calibration curve; subscript a – value assessed by the default calibration.

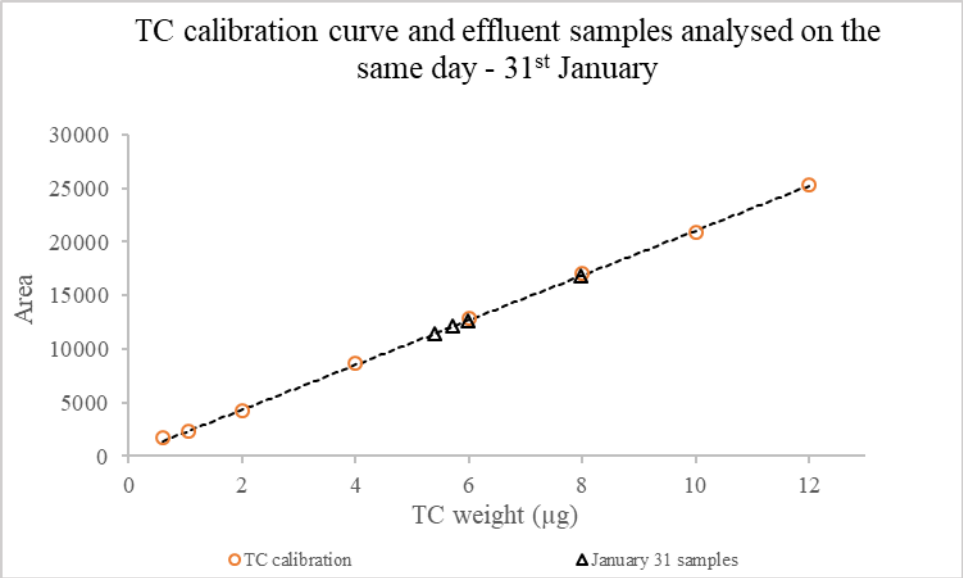

Figure 26 – TC calibration curve and samples analysed on the same day.

The difference between TOC values assessed by RAIZ calibration curves and the default calibration is significant. Therefore, to assess the differences between the slope values of the calibration curves of each analysis day and the slope of the default calibration curve, a *t*-test was again performed. The values of the slopes, as well as the variables used in the *t*-test are in **Table 28**.

Table 28 – Slope values of the daily TC and TIC calibration curves for effluent analysis and variables used to perform the *t*-test.

	Date	b_{RAIZ}	$\bar{b}_{RAIZ}$	s	$b_{default}$	t_{exp}	$t_{critical}$
TC	10/jan	2000.2	2035.1	49.2	2374.3	13.78	3.18
	17/jan	1987.3					
	24/jan	2063.5					
	31/jan	2089.5					
TIC	10/jan	2397.2	2399.6	132.1	2459.5	0.91	3.18
	17/jan	2574.4					
	24/jan	2254.7					
	31/jan	2372.0					

For TC, significant differences between the slope of the RAIZ calibration curves and the factory were verified ($t_{exp} > t_{critical}$), which is noticeable observing the values. Regarding TIC, the differences between the slopes were not considered statistically significant since $t_{exp} > t_{critical}$.

The difference between TOC values measured by the RAIZ calibration curves and the analyser is due to the proved deviation for TC. However, by this time, this issue was not yet solved and more samples were analysed with the equipment to show that the situation remained.

3.1.8. Daily calibration and water analysis

RAIZ laboratory receives water samples from the surrounding areas of the wastewater discharge points of the P&P industry to monitor the values of numerous parameters for these matrices, being TOC one of the assessed parameters.

Regarding this, several water samples were analysed in Vario TOC Select. **Table 29** shows the TOC values obtained from both the calibration curves performed at RAIZ (TOC_{WLR}) and the analyser calibration (TOC_a), for each of the water (w – **Table 29**) and leachate (lc – **Table 29**) samples. Additionally, the studied samples come from three different P&P mills and are, therefore, grouped according to their origin, namely A, B or C.

Table 29 – TOC values obtained by the daily calibration, default calibration and history for the analysed samples from water and leachate matrices.

Origin	Sample	Matrix	Date	*f*	TC_{WLR} (mg/L)	TIC_{WLR} (mg/L)	TOC_{WLR} (mg/L)	TOC_a (mg/L)	TOC_h (mg/L)
A	1	w	7/feb	-	44.1	25.2	18.9	10.5	-
	2	w	27/feb	2	6.5	< LQ	6.5	7.9	-
	3	lc	7/feb	5	114.8	91.9	22.9	0.9	180
	4	w	7/feb	-	31.8	23.1	8.7	2.4	3.2
	5	w	11/feb	-	37.1	25.1	12.0	3.9	4.7
	6	w	13/feb	10	185.3	161.5	23.8	-	8.1
	7	w	13/feb	10	77.8	60.4	17.4	4.8	4.6
	8	w	11/feb	-	38.6	27.8	10.7	2.2	2.8
	9	w	7/feb	-	12.3	6.9	5.4	3.3	4.1
	10	w	7/feb	-	12.9	7.0	5.9	3.9	2.6
B	1	w	19/feb	5	61.2	46.9	14.4	5.1	4.5
	2	w	19/feb	5	36.4	31.6	4.8	1.2	1.3
	3	w	19/feb	5	35.6	25.4	10.2	6.9	6.6
	4	lc	19/feb	100	3370.4	2113.6	1256.9	586.3	735
C	1	w	25/feb	-	7.3	5.3	2.0	0.9	0.75
	2	w	27/feb	100	1081.6	715.2	366.5	214.3	186
	3	w	25/feb	-	30.4	22.7	7.7	2.1	2.3
	4	w	27/feb	10	74.4	58.8	15.5	5.6	3.7
	5	lc	27/feb	200	4942.4	4198.0	744.4	90.5	244

Note: subscript WLR – values obtained with the weighted linear regression; subscript h – previous values from 2018; subscript a – values obtained with the analyser calibration.

Although each numbered sample corresponds to one of the two matrices (water and leachate), each sample also corresponds to a geographically fixed collecting point, being the TOC concentration of each point monitored several times a year. During the present work, it was possible to have access

to some of the previous values. And, it was verified that for some samples, TOC values do not vary significantly from year to year.

Regarding the values obtained in the present work (**Table 29**), TIC values are greater than the TOC values for most samples. According to ISO 20236:2018 for TOC determination (28), the DM should be used in the analysis of samples with higher TOC content than TIC content, or at least of similar size. Therefore, taking into account the TIC values of the analysed water and leachate samples, in future analyses of this type of sample compositions, the DM may not be the most suitable method for determining the TOC content in the samples (28).

Also, from the obtained results it can be concluded that the TOC_{WLR} and TOC_a of each sample showed a high discrepancy. In view of this, samples A-9 and A-10 were sent to an accredited external laboratory for TOC analysis. The provided results for each sample were 3.4 mg/L and 3.2 mg/L, respectively. These values are practically consistent with the analyser TOC values (TOC_a) for these samples, confirming once again the problem that existed with TC calibration.

Taking into account the differences, the slopes of the daily calibration curves of both parameters were compared with the factory calibration, once again by a statistical *t*-test. **Figures 27** and **28** refer to this comparison for TC and TIC, respectively. **Table 29** shows the slope of the calibration curves of the sample analysis days, as well as the variables necessary to carry out the statistical test.

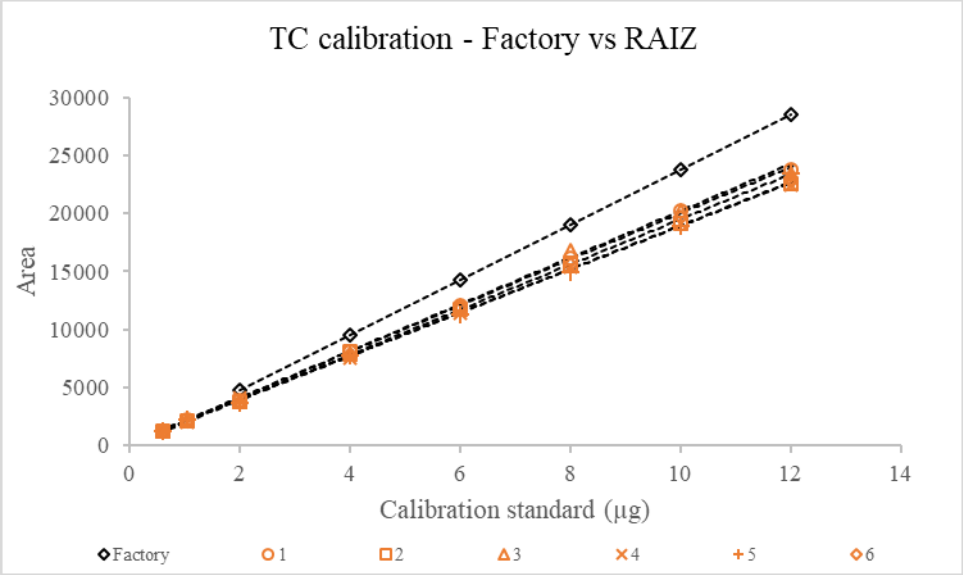

Figure 27 – Comparison of daily TC calibration curves, for analysis of water and leachate samples, with default calibration.

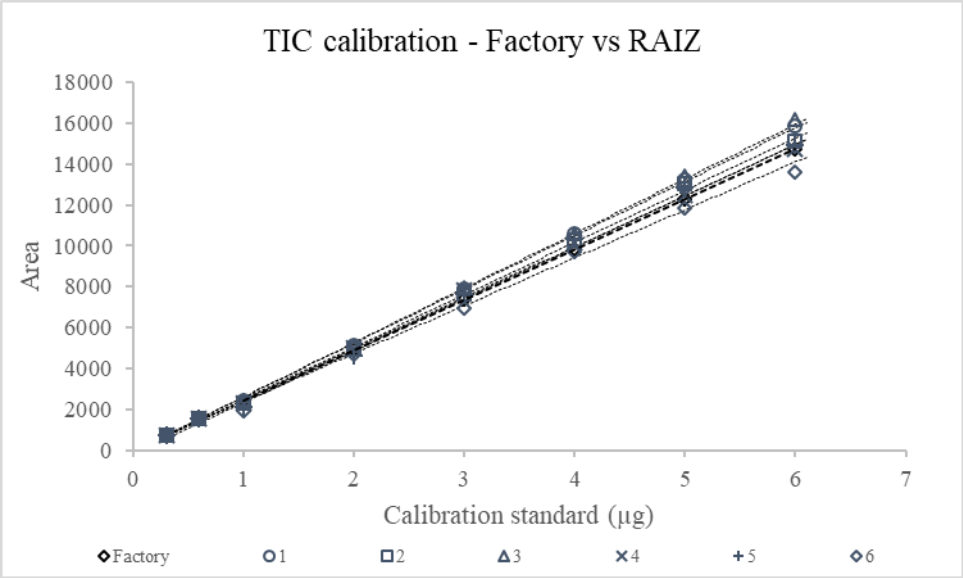

Figure 28 – Comparison of daily TIC calibration curves, for analysis of water and leachate samples, with default calibration.

Regarding the TIC parameter, the result of the statistical *t*-test, $t_{exp} < t_{critical}$ (**Table 30**), indicates that the differences between the two slope values (RAIZ and factory) are not significant statistically, which is also evidenced by **Figure 28**, where the curves are overlapped.

Table 30 – Slope values of the daily TC and TIC calibration curves for water analysis and variables used to perform the *t*-test.

	No.	Date	b_{RAIZ}	$\overline{b}_{RAIZ}$	S	$b_{default}$	t_{exp}	$t_{critical}$
TC	1	07/Feb	1988.8	1942.6	61.5	2374.3	17.21	2.57
	2	11/Feb	1866.1					
	3	13/Feb	2016.9					
	4	19/Feb	1872.5					
	5	25/Feb	1947.2					
	6	27/Feb	1963.8					
TIC	1	07/Feb	2631.8	2536.4	114.4	2459.5	1.65	2.57
	2	11/Feb	2554.8					
	3	13/Feb	2666.5					
	4	19/Feb	2524.9					
	5	25/Feb	2497.0					
	6	27/Feb	2343.1					

In **Figure 27**, the TC calibration curves show a systematic deviation from the factory curve, which was also statistically proven, since $t_{exp} > t_{critical}$. This result indicates that significant differences between the factory slope and the average slope of RAIZ curves are verified, which had already been confirmed before. The systematic deviation of TC calibration curves justifies the difference between

the TOC values obtained by the TC and TIC calibrations of the equipment and the ones assessed by interpolation of the TC and TIC areas in the calibration curves performed in each analysis day.

As mentioned, RAIZ laboratory had previous analysis values (TOC_h) for some of the samples, being the values from 2018 in **Table 29**. Although TOC values may vary for some of the samples, for most they remain stable over time. In view of this, it could be concluded that the obtained values with the analyser calibration show greater agreement with the previous values, confirming once again that the Vario TOC Select analyser could not be working properly. The situation was repeatedly reported to the analyser technicians, but little relevance was given to the situation and the problem remained.

Since it was necessary to continue the present work and it was not possible to have confidence in the equipment results regarding the DM, validation of the direct TOC analysis method (NPOC) was initiated.

3.1.9. New calibration of TC and TIC parameters

After the insistence of the RAIZ laboratory, technicians from the equipment supplier company came to the laboratory facilities in the beginning of July. The analyser was tested by the analysis of the mixed TC/TIC standard and a calibration of both parameters was performed.

Once again, what had been happening since the beginning of the work on the equipment (October 2018) was confirmed. The reference value of the areas for each standard in the set factory calibration was not equal to the areas for the same standards when calibration was performed on RAIZ. That is, the same carbon values generated a lower signal.

Thus, it was concluded that the analyser calibration was no longer valid. Therefore, the equipment was calibrated according to the new conditions. **Figures 29** and **30** show the old and new calibration curves for each TC and TIC, respectively. In order to confirm the statistical significance of the differences between the area values of each calibration for each calibration standard, a paired *t*-test was performed. In **Table 31** are shown the *t*-test results and the signal reference values for each of the standards, in each calibration curve.

As can be seen, for both TC and TIC, $t_{exp} > t_{critical}$, which supports the observed differences between the two area values of each calibration curve, for each standard. So far, the results of the statistical tests performed for TIC had not shown significant differences from the factory values. However, it is possible that, over time, the calibration of this parameter has also ceased to be effective, showing a greater difference from the initial factory values.

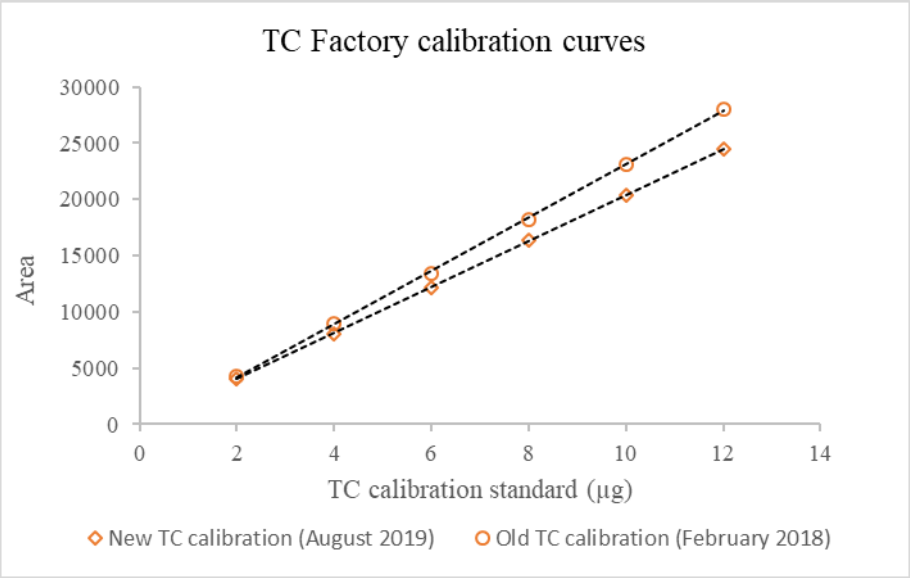

Figure 29 – New and old default calibration curves of the TC parameter.

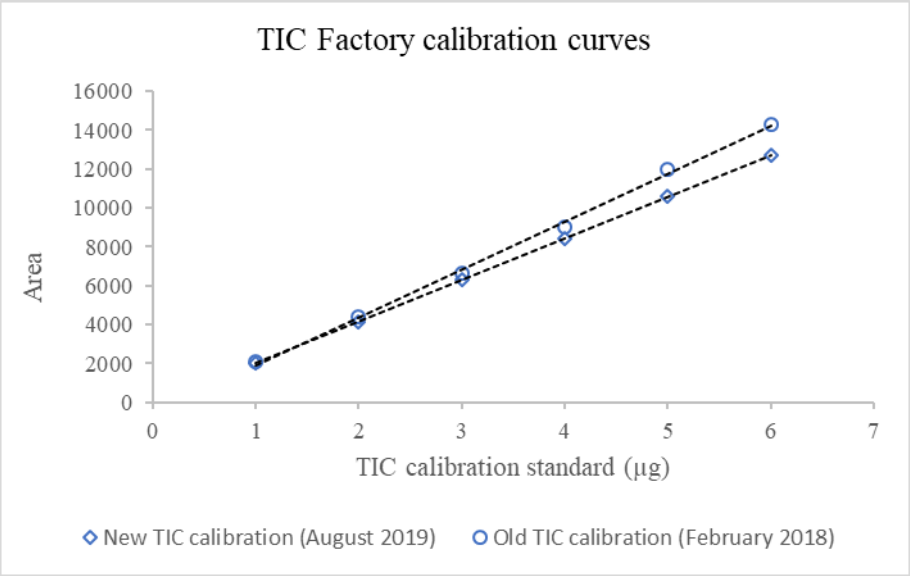

Figure 30 – New and old default calibration curves of the TIC parameter.

In addition, the validation of the new TC and TIC calibrations was performed. Validation is required as it is the documented assurance that the equipment is operating accurately, giving confidence in its analytical measurements. It is usually done by checking equipment performance against traceable CSs. Traceability is a property of a standard or measurement result that can be related to national or international standards through successive comparisons, being the uncertainties involved estimated (52).

Therefore, the new calibration of both parameters was verified on November 27th 2019, by a technician from the equipment supplier company, using a traceable reference material, namely a

20/10 mg/L TC/TIC (± 2 %) certified standard purchased from Reagecon (53). This allowed having confidence in the results given by the equipment, correlating them to a reference.

Table 31 – Signal reference values for each of the standards in the new and old calibration.

	Standard (µg)	**Area$_O$**	**Area$_N$**	$\bar{d}$	***S***	t_{exp}	$t_{critical}$
TC	2	4314.3	4060.0	1755.8	1232.1	3.49	2.57
	4	8927.7	8056.7				
	6	13363.7	12142.0				
	8	18189.0	16343.3				
	10	23133.7	20319.3				
	12	28045.7	24518.3				
	ECC	**Area** = 2047.6 **TCweight** + 0.1					
TIC	1	2096.0	2038.3	691.3	636.8	2.66	2.57
	2	4390.3	4161.3				
	3	6613.0	6303.7				
	4	9005.3	8413.3				
	5	11974.5	10575.7				
	6	14289.7	12729.0				
	ECC	**Area** = 2138.1 **TICweight** + 0.1					

Note: subscript O – area values according to the old calibration.; subscript N – area values according to the new calibration.

3.2.Direct method validation by Vario TOC Select analyser

3.2.1. Work range

The working range was assessed by the performance of the HV test. After performing the Grubbs test to identify possible outliers, the first 10 values free from outliers were used to perform the HV test. The results are shown in **Table 32**. In **Table A6** (**Appendix A**) are shown the area values and the corresponding NPOC weight of the ten replicates of each of the extreme NPOC standards (0.30 µg and 3.0 µg).

Table 32 – Variables used to perform the HV test for NPOC.

Work range (µg)	**X_i (µg)**	**S_i^2**	**PG**	$F_{critical}$
TC [0.3 – 3]	0.30	618.8	1.15	5.35
	3.00	538.6		

According to ISO 8466 – 1:1990, PG test value was compared with the tabulated value of the Snedecor/Fisher distribution ($F_{critical}$), for a confidence level of 99 % and nine DF ($f_1 = f_2 = 9$). It was concluded that PG < $F_{critical}$, which means that there are no statistically significant differences

between variances and therefore there is homoscedasticity (40). Thus, based on the obtained result, the working range verified for the NPOC method is adequate.

Additionally, the experimental values of the ten replicates for each standard to carry out the HV test were plotted to observe its dispersion around their theoretical value, as shown in **Figure 31**.

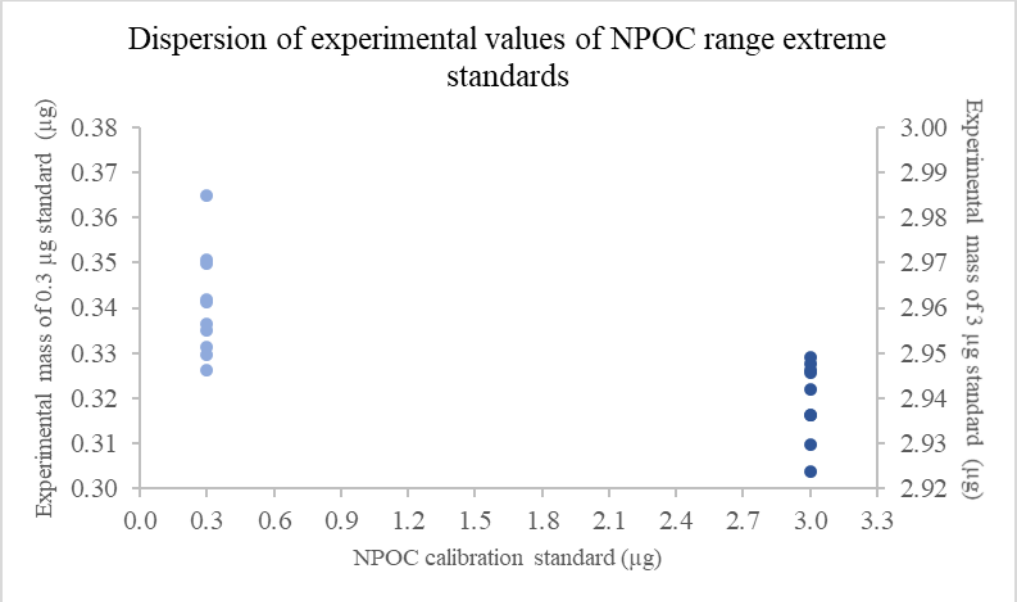

Figure 31 – Graphical representation of the experimental weight values dispersion used in the NPOC HV test.

As can be seen, the dispersion of the ten weight values for each of the calibration standards is not significantly different. This is in agreement with the calculated variance values (**Table 32**), as these are also very close, contrary to what was verified for TC and TIC work ranges. This similarity justifies the positive result of the statistical test, i.e., the homogeneity between the variances of the NPOC work range extremes.

3.2.2. Linearity and sensitivity

As mentioned in section **2.5.2.**, this parameter was assessed by performing several calibration curves. Then, the collected data in the calibration was used to perform the statistical linearity test for each calibration curve. To achieve this, the corresponding first- and second-degree functions were calculated. **Table 33** shows the resultant parameters related to the first-degree function, as well as those required to perform the *F*-test. The second-degree function parameters, calculated according to ISO 8466 – 2, are given in **Table A7** of **Appendix A**.

The difference of the variances (DS^2) and the variance associated with the second-degree calibration function (S_y^2) were used to calculate PG value, according to **Equation 17**, in order to perform the *F*-test. As it is shown in **Table 33**, all the calculated PG values are under the $F_{critical}$ value (10.13) for 95 % confidence and a DF number corresponding to 1, for the numerator, and 3, for the denominator. Consequently, considering that the result of the statistical test is PG < $F_{critical}$ for all the

performed calibrations, it was concluded that the second-degree function does not lead to a significantly better adjustment, so the first order function was chosen.

Table 33 – Coefficients and variables used to assess the sensitivity and linearity of the NPOC calibration curve.

No.	Date	*b*	*a*	*r*	$S_{y/x}$	S_y^2	DS^2	PG	$b_{deviation}$ (%)
1	31/May	2079.9	103.4	0.99985	42.1	44.8	31.1	0.70	2.4
2	5/Jun	2015.7	-5.3	0.99990	33.4	31.6	40.5	1.28	5.4
3	7/Jun	2007.5	37.3	0.99989	34.8	36.5	27.9	0.77	5.8
4*	14/Jun	1864.7	9.2	0.99906	95.2	101.0	72.0	0.71	12.5
5	17/Jun	2035.7	-6.9	0.99995	22.2	20.0	30.9	1.54	4.5
6	18/Jun	2018.2	14.4	0.99993	28.3	29.9	22.0	0.73	5.3
7	19/Jun	2030.1	-38.2	0.99997	19.0	20.3	13.5	0.66	4.7
8*	21/Jun	2253.2	125.3	0.98275	499.8	470.8	615.9	1.31	-5.7
9	24/Jun	2006.3	27.3	0.99996	21.1	23.4	11.9	0.51	5.9
10	28/Jun	2002.5	54.3	0.99964	63.2	61.4	70.5	1.15	6.1
11	4/Jul	2039.2	-14.1	0.99990	34.3	34.0	35.8	1.05	4.3
Mean		2026.1	19.1	0.99989	33.2	33.5	31.6	0.93	4.9
Max		2079.9	103.4	0.99997	63.2	61.4	70.5	1.54	6.1
Min		2002.5	-38.2	0.99964	19.0	20.0	11.9	0.51	2.4
S		24.1	42.5	0.00010	13.6	13.2	17.4	0.34	1.1

Note: calibrations 4 and 8 (*) were not taken into account in the calculations of bottom parameters, as they corresponded to anomalous calibrations curves, because problems were verified during their execution.

Additionally, besides the linearity statistical test, calibration data was plotted along with the respective linear regression line. The graphical representation of calibration data helps to highlight any nonlinearity. **Figure 32** shows the calibration curves performed (**Table 33**), which supports the linearity shown by the statistical test.

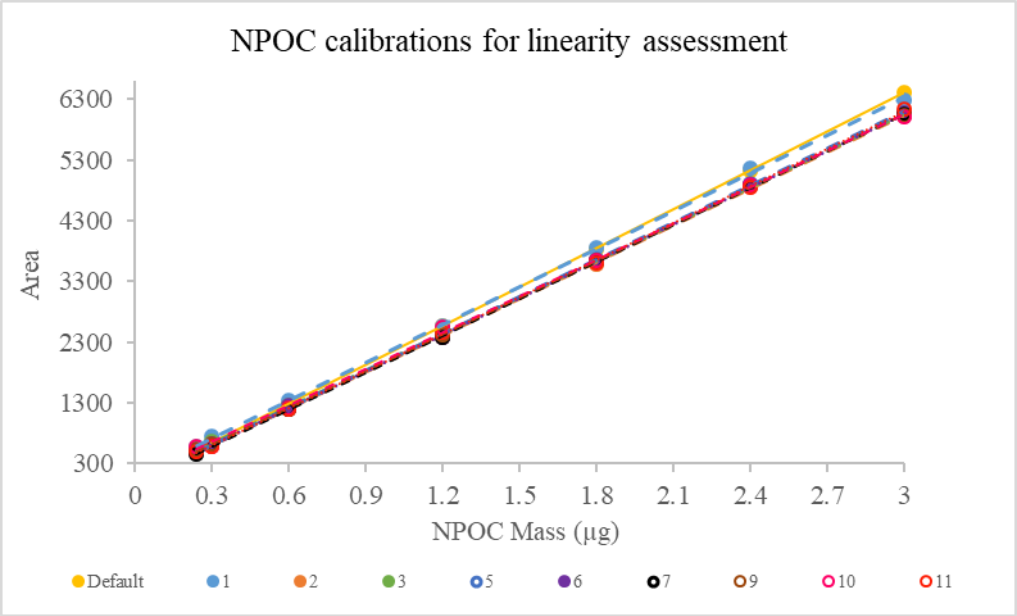

Figure 32 – NPOC calibration curves used for linearity assessment. Note: calibrations no. 4 and 8 are not represented due to problems during their execution.

The correlation coefficient was also calculated in order to assess the quality of the analytical calibration (**Table 33**). Thus, $r \geq 0.999$ was taken as the acceptance criterion of the various curves and all met this criterion, showing that the experimental points fit well the calibration curve. The no. 8 calibration curve is an exception, which was not taken into account for reasons that will be then explained. Although the coefficient of the calibration curve no. 4 met the previous criterion, the concentration values of the standards were abnormally low. Thus, it was also not taken into consideration either (**Table 33**).

Another calibration curve control parameter corresponds to the slope, in order to check the method sensitivity. The method follows a first order model; thus, the sensitivity is constant over the entire working range and equal to the slope of the calibration curve. Although the variation between the several slope values is not very significant, the slope does not remain unchanged over time, undergoing variations. This may represent a greater sensitivity of the method to external factors, which may be a disadvantage. On the other hand, higher sensitivity can detect small amounts of sample, which is an advantage.

In the no. 8 calibration curve, the concentration recorded by the analyser regarding the 2.4 µg calibration standard was found to be abnormally high, namely 10 mg/L, when it should correspond to 8 mg/L. This may have been due to an error while preparing the calibration standards, or possible equipment oscillations. Due to this, the slope of the calibration curve performed is higher, because this point goes out of calibration making the corresponding correlation coefficient not reveal good quality of calibration line adjustment.

Finally, the percentage deviation of the slope ($b_{deviation}$) of each calibration curve performed against the calibration curve present in the analyser and performed by the manufacturer was also calculated (**Table 33**). This was performed in order to assess the factory calibration and an acceptance criterion of a maximum deviation of 5 % was established. As can be seen, half of the calibration curves considered meet the acceptance criterion, i.e., have a $b_{deviation}$ value of less than 5 %. Although the deviation of the remaining curves is greater than the acceptance criterion, it does not exceed it very significantly. Besides, the average value of deviations is less than 5 % (4.9 % - **Table 33**).

The higher slope deviation of the curve no. 4 from the default calibration curve (12.5 % - **Table 33**) is probably due to the maintenance of the ash finger (**Appendix C – Vario TOC Select Analyzer Operation/Maintenance Procedure**) performed on that same day. In order to replace the old component with the new one, it was necessary to open the combustion tube and handle its components, since the ash finger is inside the tube. Handling may have led to slight contamination inside the combustion tube that may not have been completely removed at the time of calibration, although the conditioning of the new tube has been performed (**Appendix C** – **Vario TOC Select Analyzer Operation/Maintenance Procedure**).

3.2.3. Analytical limits

In the present validation work, more attention has been given to LQ, since the purpose of using the analyser is to accurately quantify the analyte.

As the NPOC method follows a linear calibration, the analytical limits were calculated by the slope and the residual standard deviation of the calibration curves. For this purpose, from two calibration curves, the theoretical LD and LQ were calculated according to **Equations 29** and **31**. The values are shown in **Table 34**.

Table 34 – Variables and coefficients of the calibration curves for the calculation of the NPOC analytical limits.

Date	*b*	*a*	$S_{y/x}$	LD (µg)	LQ (µg)
Default	2131.7	8.8	39.0	0.060	0.183
6/05	2056.6	-20.3	31.7	0.051	0.154
			Mean	0.056	0.169

To begin with, the slope of the calibration curve performed on May 6th meets the established acceptance criteria of 5 % for the slope deviation. The average of the LQs obtained (**Table 34)** corresponds to 0.17 µg. Therefore, in the first HV test, two determinations were chosen based on the average theoretical LQ, and also tested, besides the low and high NPOC calibration standards (0.3 and 3 µg).

Due to the available volumetric glassware and to the concentration, the first determination tested was 0.18 µg. For the sake of convenience, it was also chosen to test the 0.24 µg level if no

homoscedasticity or lack of accuracy were verified with the 0.18 µg standard. **Table 35** shows the obtained results.

Table 35 – Variables used to perform HV test to NPOC work range with the LQ weight determinations.

Work range	X_i (µg)	S_i^2	PG	$F_{critical}$
0.18 – 3.0	0.18	251.8	2.14	5.35
	3.0	538.6		
0.24 – 3.0	0.24	1253.8	2.33	5.35
	3.0	538.6		

As it can be observed, the condition of homogeneity of variances was verified, considering both the variance associated with the determinations 0.18 µg and 0.24 µg. However, from the calculated *Er* (0.18 µg – 23.4 %; 0.24 µg – 17.6 %) value (**Table A6** – **Appendix A**), the obtained accuracy was low for the two tested standards, since the experimental weight was quite distant from the theoretical value. Thus, it was decided to include in the calibration curve for testing only the mass level 0.24 µg, although the accuracy obtained is not the best either.

With HV verified, the next goal was to test the LQ performance. For this purpose, in each working session, besides the calibration curve, the calibration CSs, corresponding to the 0.24 µg LQ standard and to the initial working range thresholds (0.30 µg and 3.0 µg), were also analysed, being the results shown in **Table 36**.

Table 36 – Weight and *Er* values of CSs analysed in each work session.

Date	0.24 (µg)	*Er* (%)	0.30 (µg)	*Er* (%)	3.0 (µg)	*Er* (%)
31/05	0.277	15.5	0.35	16.8	2.88	3.9
5/06	0.227	5.6	0.29	4.2	2.93	2.3
7/06	0.254	5.9	0.32	7.1	2.93	2.4
17/06	0.250	4.2	0.32	8.2	2.97	1.0
18/06	0.212	11.6	0.27	11.2	2.97	1.1
19/06	0.245	2.0	0.31	4.5	2.96	1.3
24/06	0.243	1.4	0.30	1.1	2.96	1.2
28/06	0.268	11.7	0.26	12.6	2.93	2.2
4/07	0.260	8.4	0.32	6.9	2.94	2.0
Mean	0.248	7.4	0.30	8.1	2.94	1.9
S	0.020		0.029		0.028	
CV	8.06		9.51		0.94	

The acceptance criterion defined for the CSs was 10 % of their theoretical value. For the highest standard (3.0 µg), the *Er* value verified for all days was less than 10 %, so the defined acceptance

criterion was confirmed. Regarding the two lowest standards (0.24 µg and 0.30 µg), it was concluded that, for days 31, 18 and 28, *Er* has exceeded the set value. Still, for two of them, *Er* was not very different from the established value (≈ 11 %).

The LQ should also be tested regarding precision, in order to assess whether it is satisfactory. This was carried out by calculating the CV value. According to IUPAC indications, this value should not exceed 10 % (37), which has been confirmed in the present work for the three CSs tested (**Table 36**).

3.2.4. Precision

3.2.4.1.Repeatability

The repeatability assessment was performed by measuring at the lower end of the working range (0.24 µg – 3.0 µg), as the working zone extremes are more likely to vary. **Table 37** are displayed the repeatability results or both types of sample matrices.

Table 37 – Repeatability assessment of the NPOC method for the treated and untreated effluent matrices.

Replicate no.	Treated effluent		Untreated influent	
	NPOC (µg)	$\|x_i - x_{i-1}\|$ (µg)	NPOC (µg)	$\|x_i - x_{i-1}\|$ (µg)
1	0.23	-	0.68	-
2	0.26	0.030	0.72	0.043
3	0.24	0.021	0.68	0.047
4	0.23	0.005	0.62	0.055
5	0.23	0.000	0.66	0.041
6	0.27	0.035	0.66	0.007
7	0.25	0.015	0.67	0.016
8	0.24	0.015	0.71	0.042
9	0.24	0.006	0.69	0.023
10	0.30	0.059	0.68	0.007
$\bar{X}$	0.25	-	0.68	-
S	0.022	-	0.029	-
Δr	0.071	-	0.093	-
CV*r* (%)	8.9	-	4.3	-

For a confidence level of 95 % and nine DF, the repeatability limit (Δr) was calculated according to **Equation 34**. Both values represent the maximum allowable value for the absolute difference between two independent measurements of the same sample for the respective matrix. These results indicate that, in the same analysis day, the difference between two duplicates of the lower standard

should be less or equal to 0.071 µg and 0.093 µg for the treated and untreated effluent samples, respectively.

Beyond the Δr value, the CV*r* is a widely used indicator to traduce the repeatability of a method, because it allows verifying the extent of variability in relation to the mean value. According to EN 1484:1997 for the TOC analysis, the repeatability variation coefficient must be less than 10 % (27). In this case, both values meet the criteria (< 10 %), corresponding to sufficiently low CV*r*. The CV*r* value corresponding to the treated effluent is higher, which may be related to the greater deviation associated with that zone of the calibration curve since the obtained NPOC weight falls in the zone of the last standard (0.24 µg), or with the smallest amount of analyte present in the sample.

3.2.4.2.Intermediate precision

IP consisted of assessing precision under identical samples in an extended period of time. This dispersion was assessed weekly using CCs, namely range (*R*) CCs (37). By this tool, precision can be controlled since range is the difference between the highest and the lowest value in a set of analyses.

Therefore, IP was assessed by range CCs for two types of sample compositions, namely untreated influent and treated effluent samples. The samples were analysed in duplicate, which corresponds to a small subgroup size ($n = 2$). Since the use of CCs was part of the validation of a new method in the RAIZ laboratory, there were no history values that could be used to pre-set the control limits. Consequently, the control limits were estimated from the analysed samples and the calculation formulas from **Table 4**, for a number of observations in the same subgroup of 2 ($n = 2$).

Figure 33 shows the range (*R*) chart for the tested eight samples of treated effluent, analysed in duplicate. Range (*R*) CCs reveal the variation within the subgroup, in order to detect oscillations in the process and before the mean control chart. As shown in the graph, all points corresponding to the relative ranges (*R* %) between duplicates of the analysed samples are within the estimated control limits, indicating that the process is under control.

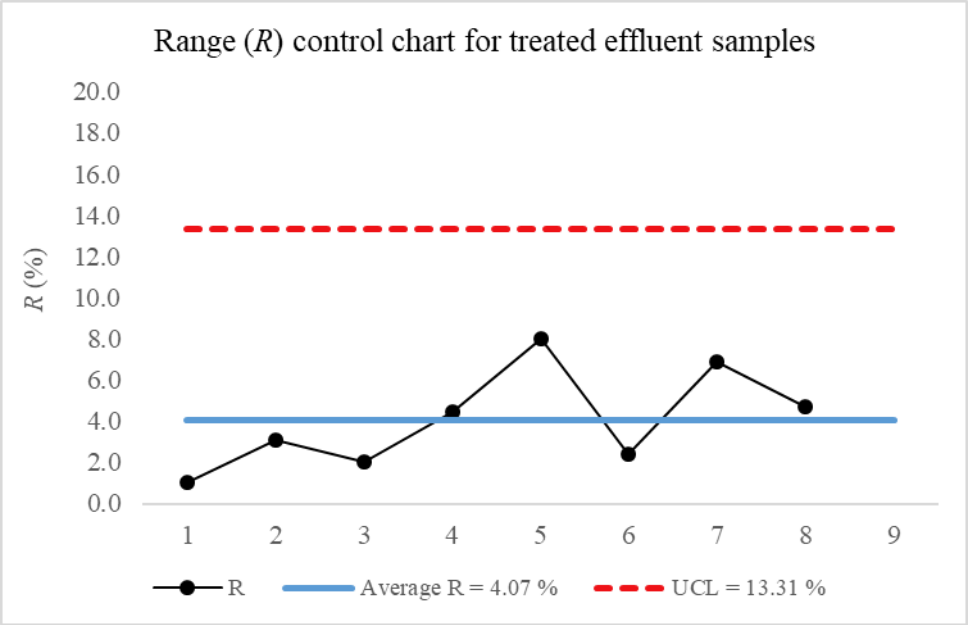

Figure 33 – Range CC for the assessment of IP in treated effluent samples.

Moreover, IP was also evaluated in untreated influent samples. In **Figure 34**, the range CC for untreated influent is shown. Like it was verified for the treated effluent (**Figure 33**), the range CC indicates that the process is under control. Additionally, most points are below the average range value, like in **Figure 33**. However, the range (*R*) between some duplicates of untreated influent is greater than that found in the treated effluent. The analysed samples are very particulate and the duplicates correspond to two individual portions of the same sample. So, it is possible that some particulate matter might have contributed to a greater difference between the range of the duplicates *R*, although the analysis was carried out with homogenization. Nevertheless, based on this chart, the process is under control.

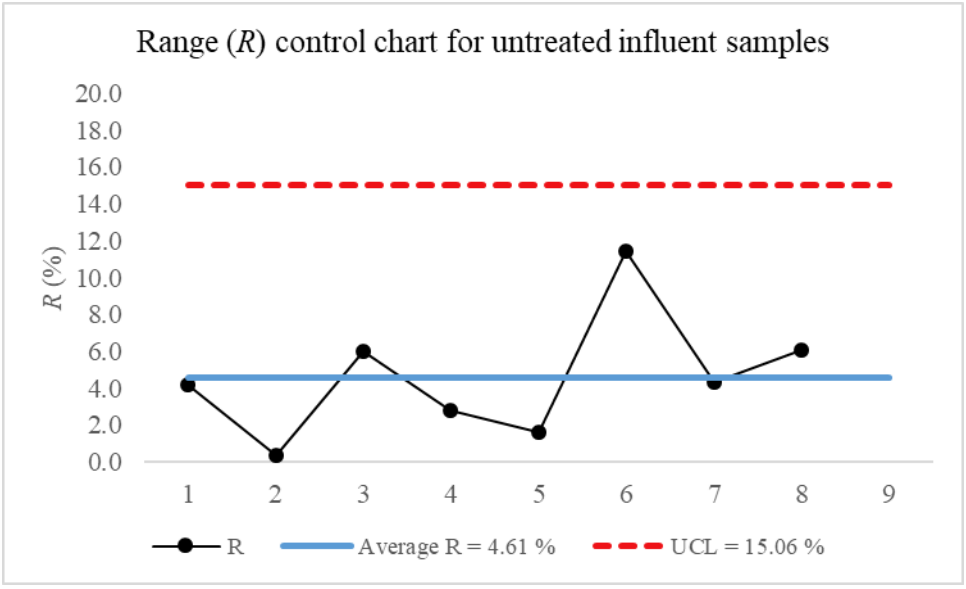

Figure 34 – Range CC for the assessment of IP in untreated influent samples.

As mentioned in **section 1.12.**, CCs are most often prepared and studied in pairs, one for location and other for spread analysis. In this case, the most suitable location CC would be the average CC, since range and average CCs are used when sample subgroups are small, as is the case (n = 2).

However, an average CC has little value in the present work. The NPOC concentration in the influent and, thus, in the effluent depends on the process conditions and the changes it may undergo. Although the samples represented in each CC correspond to samples from the same matrix, variations in the process may cause variations in the NPOC concentration of the effluent that is analysed in the RAIZ laboratory. Consequently, an average CC has no great informative value. Moreover, the aim is merely to assess possible differences between sample duplicates, and in this context, an average CC is not useful.

In addition, the number of subgroups used to construct the control chart is reduced. Therefore, if there is interest in establishing a CC for location analysis in the future, more samples need to be tested to better establish the control limits of the average CC to be used.

3.2.4.3. Reproducibility

Reproducibility is the precision measurement that assesses the dispersion of the results of a method when the same sample is analysed by different analysts, in different laboratories or different equipment. Therefore, it is often evaluated by interlaboratory studies, as it was in the present work.

The performance of the participant laboratories was evaluated by calculating the Z-score parameter (37), according to **Equation 40**. In **Figure 35** is shown the graphical representation of the Z-score values of the 45 laboratories that participated in the TOC laboratory intercomparison assay. Only the results obtained that fall between -3 and 3 are represented.

The assigned TOC value attributed to the sample concentration was 91.9 mg/L. This corresponds to the consensus value derived from the finally accepted results and is expressed by means of the robust mean. The TOC concentration obtained by RAIZ for the interlaboratory sample was 92.8 mg/L, which corresponded to a Z-score of 0.12, a very good result. Additionally, RAIZ result is further reinforced when compared with those of other participating laboratories (**Figure 35**).

This, besides being a very good result in terms of ability, also demonstrates the good functioning of the analyser regarding NPOC method and, being Z-score also an accuracy measure, the obtained result indicates that the NPOC method is highly accurate.

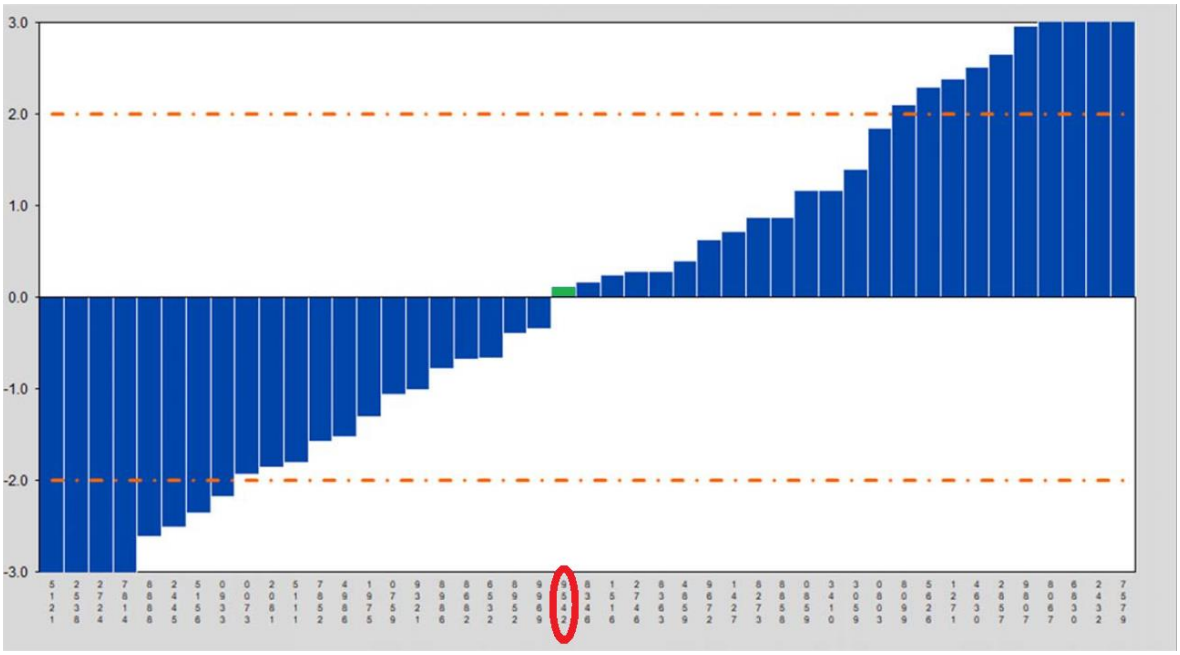

Figure 35 – Graphical representation of the Z-score values of the participating laboratories. The *xx* axis corresponds to the numeric code assigned to each laboratory. The number surrounded by red was the number assigned to RAIZ and the green column represents the result of RAIZ.

Besides the Z-score, the standard deviation for proficiency assignment (σ_{pt}) was calculated. This value was assessed by Horwitz function modified by Thompson, as referred by the laboratory that has organized the interlaboratory test.

The final TOC concentration value of the sample (91.9 mg/L), and the σ_{pt} was 7.5. Thus, **Equation 35** was used to assess the CV_R, according to the following expression.

$$CV_R = \frac{7.5}{91.9} \times 100\ \% = 8.2\ \%$$

The standard ISO 20236:2018 (28), for TOC determination by combustion at high temperature, reports CV_R values for wastewater obtained in an interlaboratory, in the order of 11.2 %. Thus, the value obtained in the interlaboratory is quite acceptable. Nevertheless, it should be noted that the CV_R value results from only one interlaboratory test and more participations are required in order to

obtain a more representative value. In addition, the concentration value obtained (91.9 mg/L) corresponds to a different level from the concentration value mentioned in the standard ISO 20236:2018 (28) (22.9 mg/L). However, since the coefficient of variation measures the dispersion of the results, this question may not be so relevant.

Reproducibility is an important parameter when the goal is to implement a new analytical method. However, in the case of the present work, as it is a method validation, it is intended to evaluate a method that will be performed in the same laboratory. Thus, reproducibility is not so relevant since the final objective is to carry out the method in that same laboratory, in the same equipment and under conditions that vary little over time.

3.2.5. Uncertainty associated with the trueness of the NPOC method

As it was mentioned, trueness is a component of the error that evaluates the possible influence of systematic errors in a method. Although several ways can be used to assess trueness, in the present work and taking into account the available resources, the method bias was assessed by spiking studies.

3.2.5.1. Uncertainty associated with standard spiking solution concentration

The standard solution used to perform the recovery trials was prepared in the RAIZ laboratory. Although uncertainty associated with trueness is related to the empirical approaches, in the present work also the bottom-up approach was used to calculate the uncertainty associated with the solution used to perform the spikes in the samples. The preparation of this solution encompasses two major uncertainty components, namely:

A. **uncertainty associated with carbon mass**, comprising several uncertainties that will be mentioned later;
B. **uncertainty associated with the stock solution concentration**.

These two major components added uncertainty to the final spiking solution, consequently will be addressed next in the performed calculations.

A. Uncertainty component associated with carbon mass

The spiking solution was prepared from the stock calibration solution (**2.2.1.2.**). Since the NPOC parameter is a measurement of the carbon concentration in the samples, the uncertainty of the solutions involved throughout the process should take into account the uncertainty associated with carbon concentration and not to the potassium hydrogen phthalate ($C_8H_5O_4K$) concentration.

Therefore, the first step was to calculate, as follows, the carbon weight (m_{carbon}) present in the reagent ($C_8H_5O_4K$) portion used to prepare the stock solution:

$$m_{carbon} = 2.1258\ g\ C_8H_5KO_4 \times \frac{1\ mol\ C_8H_5KO_4}{204.2212\ g\ C_8H_5KO_4} \times \frac{8\ mol\ C}{1\ mol\ C_8H_5KO_4} \times \frac{12.0107\ g\ C}{1\ mol\ C} = 1.00018\ g\ C$$

Given the above expression to assess the carbon mass present in the reagent, its value depends on four components, namely:

- the weighted reagent ($C_8H_5O_4K$);
- the carbon atomic weight;
- the molecular weight of the reagent ($C_8H_5O_4K$);
- the purity of the reagent ($C_8H_5O_4K$).

Therefore, the uncertainty associated with carbon mass depends on the uncertainty associated with each of the aforementioned components, that will be taken into account in the following calculations.

Uncertainty associated with the weighted $C_8H_5O_4K$

In order to perform this calculation, the relevant weightings were:

- beaker and $C_8H_5O_4K$: 51.2858 g;
- beaker: 49.1600 g;
- $C_8H_5O_4K$: 2.1258 g.

As it can be observed, the difference between the tare mass and gross mass (beaker and reagent) was not significant and the two were in the same range of the scale, the standard uncertainty associated with $C_8H_5O_4K$ weight, $u(m_{C_8H_5O_4K})$, was calculated by **Equation 52**. Thus, taking into account that, in the 50.0000 g area of the scale, the uncertainty and k value correspond to 0.000061 g and 2.00, respectively, the final value of $u(m_{C_8H_5O_4K})$ was assessed as followed.

$$u(m_{C_8H_5O_4K}) = \sqrt{2 \times \left(\frac{0.000061}{2.00}\right)^2} = 4.31335 \times 10^{-5}\ g$$

As can be seen, the assessed uncertainty value is very small, yet it has been accounted for in the overall calculations.

Uncertainty associated with the carbon atomic weight

According to the IUPAC table, where the uncertainties associated with the atomic weights of each chemical element are described (47), the uncertainty of carbon atomic weight is ± 0.0008 g/mol (45). Consequently, to calculate its standard uncertainty, $u(AW_C)$, a rectangular distribution was

considered and **Equation 53** was used, being the final carbon atomic standard uncertainty value assessed according to the following formula.

$$u(\mathrm{AW}_C) = \frac{0.0008}{\sqrt{3}}$$

Uncertainty associated with the $C_8H_5O_4K$ molecular weight

In order to estimate this uncertainty component, the standard uncertainty calculation performed in the previous section for carbon was completed for each one of the chemical elements that comprise the $C_8H_5O_4K$ molecule, based in the IUPAC table for the atomic weights (47). Then, the standard uncertainty value of each element was multiplied by the respective number of atoms being the results shown in **Table 38**. Also, in **Table 38** are displayed the atomic weights of each of the elements, taking into account their number of atoms, in order to estimate the molecular weight associated with $C_8H_5O_4K$.

Table 38 – Atomic weights and uncertainties for the constituent elements $C_8H_5O_4K$.

Element	Number of Atoms	Atomic weight (g/mol)	Standard uncertainty (g/mol)
C	8	12.0107 × 8 = 96.0856	0.00046 × 8 = 0.0037
H	5	1.00794 × 5 = 5.0397	0.000040 × 5 = 0.00020
O	4	15.9994 × 4 = 63.9976	0.00017 × 4 = 0.00068
K	1	39.0983 × 1= 39.0983	0.000058 × 1= 0.000058

Thus, the standard uncertainty associated with $C_8H_5O_4K$ molecular weight, $u(MM_{\mathrm{C_8H_5O_4K}})$, was calculated, based on **Equation 49**, i.e., by the square root of the sum of the squares of each single atom contribution.

$$u(MM_{\mathrm{C_8H_5O_4K}}) = \sqrt{0.0037^2 + 0.00020^2 + 0.00068^2 + 0.000058^2} = 0.0037\ g/mol$$

Uncertainty associated with the $C_8H_5O_4K$ purity

The used potassium hydrogen phthalate was purchased from Merck and, according to the analysis certificate, its purity and associated uncertainty are 100.00 % ± 0.05 %, respectively. Thus, standard uncertainty associated with $C_8H_5O_4K$ purity, $u(pur_{\mathrm{C_8H_5O_4K}})$, was calculated, according to **Equation 54**, as having a rectangular distribution, as follows.

$$\mathrm{u}(pur_{\mathrm{C_8H_5O_4K}}) = \frac{0.0005}{\sqrt{3}}$$

Finally, taking into account the above components and their uncertainty contribution, the final standard uncertainty associated to carbon weight ($u(\mathrm{m}_{carbon})$), was calculated according to **Equation 50**, since the carbon weight value (m_{carbon}) resulted from the multiplication and division of several quantities.

$$u(\mathrm{m}_{carbon}) = \mathrm{m}_{carbon} \times \sqrt{\left(\frac{u(m_{\mathrm{C_8H_5O_4K}})}{m_{\mathrm{C_8H_5O_4K}}}\right)^2 + \left(\frac{u(\mathrm{AW}_C)}{\mathrm{AW}_C}\right)^2 + \left(\frac{u(MM_{\mathrm{C_8H_5O_4K}})}{MM_{\mathrm{C_8H_5O_4K}}}\right)^2 + \left(\frac{u(pur_{\mathrm{C_8H_5O_4K}})}{pur_{\mathrm{C_8H_5O_4K}}}\right)^2}$$

Thus, by substituting the values of the various quantities and its uncertainties, the value of $u(m_{carbon})$ (0.000293 g) was obtained as shown below.

$$u(\mathrm{m}_{carbon}) = 1.0001 \times \sqrt{\left(\frac{4.31335 \times 10^{-5}}{2.1258}\right)^2 + \left(\frac{0.0008/\sqrt{3}}{12.0107}\right)^2 + \left(\frac{0.0038}{204.2212}^2\right) + \left(\frac{0.0005/\sqrt{3}}{1.000}\right)^2} \leftrightarrow$$

$$u(\mathrm{m}_{carbon}) = 0.000293\ g$$

B. Uncertainty associated with the stock solution concentration

The uncertainty previously calculated in **section A.** was used in the next step to assess the uncertainty associated with the stock standard solution (**2.2.1.2.**), from which the spiking solution was prepared.

Regarding the stock solution concentration, taking into account that the carbon mass (**section A.**) corresponded to 1000.18 mg C, the final carbon concentration in the solution was 1000.18 mg/L, once the solution final volume was 1000.0 mL.

Thus, given that the stock solution concentration value has resulted from the carbon weight and the solution final volume (volume of the volumetric flask), in the calculation of its standard uncertainty, two components were considered, namely the standard uncertainty associated with the carbon mass ($u(\mathrm{m}_{carbon})$), and the standard uncertainty associated with the volumetric flask $u(v_{flask})$, in which the solution was prepared. Regarding the latter, the manufacturer provides details on the maximum volume deviation as 0.4 mL. Thus, the standard uncertainty associated with the stock solution was calculated according to **Equation 50**, being translated by the following expression.

$$u(C_{Stock}) = C_{Stock} \times \sqrt{\left(\frac{u(\mathrm{m}_{carbon})}{\mathrm{m}_{carbon}}\right)^2 + \left(\frac{u(v_{flask})}{v_{flask}}\right)^2}$$

Consequently, by substituting the values of the various quantities and the respective uncertainties, the value of $u(C_{stock})$ (0.373 mg/L) was calculated below.

$$u(C_{Stock}) = 1000.18 \times \sqrt{\left(\frac{0.000293}{1.00018}\right)^2 + \left(\frac{(0.4/\sqrt{3})}{1000}\right)^2} \leftrightarrow$$

$$u(C_{Stock}) = 0.373\ mg/L$$

The final solution used for spiking the samples was prepared by dilution from the stock solution. Thus, to calculate the uncertainty associated to the final solution concentration, were taken into account the uncertainties associated with the 100.0 mL volumetric flask, $u(v_2)$, where dilution was performed, the uncertainty associated to the 10.00 mL volumetric pipette $u(v_1)$, used to carry out the dilution, and the stock solution uncertainty, from where the aliquot was pipetted. According to the manufacturer's instructions, the maximum deviation reported for the volumes of the volumetric flask and pipette is (100.0 ± 0.1) mL and (10.00 ± 0.02) mL, respectively. Therefore, the standard uncertainty associated with the spike solution was assessed, by **Equation 50**, as the following expression.

$$u\left(C_{Spike\ solution}\right) = C_{Spike\ solution} \times \sqrt{\left(\frac{u(C_{Stock})}{C_{Stock}}\right)^2 + \left(\frac{u(v_1)}{v_1}\right)^2 + \left(\frac{u(v_2)}{v_2}\right)^2}$$

By substituting the values of the various quantities and the respective uncertainties, the value of $u(C_{spike\ solution})$ is 0.134 mg/L, as demonstrated below.

$$u\left(C_{Spike\ solution}\right) = 100.00 \times \sqrt{\left(\frac{0.373}{1000.18}\right)^2 + \left(\frac{(0.02/\sqrt{3})}{10}\right)^2 + \left(\frac{(0.1/\sqrt{3})}{100}\right)^2} \leftrightarrow$$

$$u\left(C_{Spike\ solution}\right) = 100.00\ \times 0.001344\ mg/L$$

$$u\left(C_{Spike\ solution}\right) = 0.134\ \ mg/L$$

For the calculation of the combined uncertainty, the several standard uncertainty components contributing to its calculation shall be accounted for as relative standard uncertainties, so that the method in question is applicable at various concentration levels (43). Regarding this, the previously calculated uncertainty was transformed into relative standard uncertainty by dividing it by the measurand value (solution concentration), as shown below.

$$u'\left(C_{Spike\ solution}\right) = \frac{0.134}{100.02} * 100\ \% = 0.134\ \%$$

Consequently, this value was used in the calculation of the uncertainty associated with the trueness of the NPOC method.

3.2.5.2. Standard uncertainty associated with the added analyte concentration

The uncertainty associated with the NPOC method was calculated for two types of matrices, namely untreated influent and treated effluent samples. The added volume of the spiking solution was different for each matrix, namely 5 mL and 4 mL for influent and effluent, respectively. Therefore, in order to calculate the uncertainty associated with the added analyte concentration, u'_{add}, (**Equation 64**), first the relative standard uncertainty associated with the added volume (u_v) was assessed.

For that purpose, **Equation 66** was used. The systematic uncertainty component of the added volume ($u_{v,\,sys}$) was assessed by the tolerance of both volumetric pipettes (5 mL and 4 mL), namely ± 0.015 mL and, a rectangular distribution was considered to treat this tolerance. The volume component associated with random errors ($u_{v,\,rep}$) for each of the volumetric pipettes was quantified in the laboratory, under repeatability conditions, and corresponds to a variation coefficient (0.0016 mL – 5 mL volumetric pipette; 0.0018 mL – 4 mL volumetric pipette) that is shown in the u_v formula in **Table 39**. The values of the ten measurements used to assess the repeatability component for each volumetric pipette are given in **Table A8** (**Appendix A**). In **Table 39** is summarized the u_v calculation procedure.

Then, taking into account the relative standard uncertainty associated with the spiking solution concentration, u'_{conc} (**3.2.5.1.**), the final u'_{add} value was estimated (**Table 39**).

Table 39 – Calculation of u'_{add} and its uncertainty components.

Uncertainty components	Untreated influent	Treated effluent
u_v	$\sqrt{(\frac{(0.015/\sqrt{3})}{5.000})^2 + 0.0016^2} = 0.00233$ $= 0.233\ \%$	$\sqrt{(\frac{(0.015/\sqrt{3})}{4.000})^2 + 0.0018^2} = 0.00284$ $= 0.284\ \%$
u'_{conc}	0.134 %	0.134 %
u'_{add}	$\sqrt{(0.232)^2 + 0.134^2} = 0.269\ \%$	$\sqrt{(0.284)^2 + 0.134^2} = 0.314\ \%$

Standard uncertainty associated with the trueness of the NPOC method

Finally, the uncertainty associated with trueness ($u'_{trueness}$) was assessed by performing measurements on spiked samples. For that, according to the literature, six samples of each matrix were analysed, in duplicate, with and without spike (43). In order to calculate b_{rms} value, the recovery deviation, b_i', was calculated considering full recovery (**Equation 62**). Then, through the sum of the square of b_i, b_{rms} value was assessed by **Equation 61**. Below is an example of the calculations performed to assess b_{rms} value.

Calculation example

Regarding the first sample of untreated effluent (**Table 40**), its recovery value corresponded to 95.5 %. Thus, the b_i' value was assessed by the following calculation (**Equation 62**).

$$b_i' = \frac{95.5\ \% - 100\ \%}{100\ \%} = -0.045$$

This step was performed for the remain five samples of this matrix (**Table 40**). Then, the sum of the squares of all resulting b_i' values (0.059) was used to calculate b_{rms} (**Equation 61**), as shown below.

$$b_{rms} = \sqrt{\frac{0.059}{6}} \times 100\ \% = 9.91\ \%$$

Finally, the uncertainty associated with the method trueness ($u'_{trueness}$) was calculated through **Equation 60**. In **Table 40** are shown the values of the variables necessary for calculating $u'_{trueness}$.

Table 40 – Variable values used to assess trueness uncertainty for influent and effluent samples.

Matrix	No.	N_i (%)	b_i'	$(b_i')^2$	b_{rms} (%)	u'_{add} (%)	$u'_{trueness}$ (%)
Untreated influent	1	95.5	-0.045	0.002	**9.91**	**0.269**	**9.92**
	2	81.6	-0.184	0.034			
	3	100.9	0.009	0.000			
	4	90.3	-0.097	0.009			
	5	100.0	0.000	0.000			
	6	111.7	0.117	0.014			
Treated effluent	1	89.7	-0.103	0.011	**4.84**	**0.314**	**4.85**
	2	104.3	0.043	0.002			
	3	102.3	0.023	0.001			
	4	100.5	0.005	0.000			
	5	97.9	-0.021	0.000			
	6	97.5	-0.025	0.001			

As it can be observed, the value of the uncertainty associated with the trueness for untreated influent samples (9.92 %) is higher than the same value determined for the effluent samples (4.85 %). Regarding values in **Table 40**, it is clear that b_{rms} value of the differences in the recovery percentage is higher for influent than for effluent, i.e., the recovery percentages in the untreated influent samples differ more from the reference value (100 %).

This may be due to the fact that the samples, prior to the treatment, have more suspended solids and possibly compounds, which may interfere with the analysis – matrix effect. This effect is therefore reflected in greater uncertainty associated with trueness for this type of sample composition.

3.2.6. Uncertainty associated with the precision of the NPOC method

The uncertainty component associated with precision was calculated by combining the data from sample replicates and CSs. The combination of these two elements represents a complementary approach and, in most cases, a widely and adequate characterization of the real dispersion involved in a particular test method.

Regarding the CSs, on the same days as the calibration standards were read for day curve construction (**Table 36**), the three CSs were analysed, that is, the LQ (0.24 µg) and the two CSs considering the initial calibration curve (0.30 and 3.0 µg) and their values are shown in **Table 41**.

Table 41 – Values of the CSs used to evaluate the uncertainty associated with NPOC method precision.

Date	0.24 µg CS	0.30 µg CS	3.0 µg CS
1	0.277	0.35	2.88
2	0.227	0.29	2.93
3	0.254	0.32	2.93
5	0.250	0.32	2.97
6	0.212	0.27	2.97
7	0.245	0.31	2.96
9	0.243	0.30	2.96
10	0.268	0.26	2.93
11	0.260	0.32	2.94
$\overline{\overline{X}}$ (µg)	0.248	0.30	2.94
s	0.020	0.029	0.028
RSD (%)	8.06	9.51	0.94
$u_{precision,CS,rel}$ (%)		**9.51**	

Note: calibrations 4 and 8 were removed due to problems in their execution.

The relative standard deviation (RSD) was calculated for each of the CSs and, as can be seen, the 0.3 µg CS has the greatest variation, although this is not very different from the uncertainty associated with the 0.24 µg CS. The calculation of uncertainties should be performed taking into account the worst-case scenario, i.e., the largest variation so that the uncertainty is never underestimated. Thus, it was assumed that the uncertainty component from CSs was the greatest of the RSDs, which is equivalent to uncertainty (**Equation 55**).

Regarding the samples, once again two matrices were analysed, influent and effluent, and each sample was analysed in duplicate. Additionally, all the samples were analysed under repeatability conditions, as all the differences between the duplicates of each sample were below the Δr calculated in section **3.2.4.1.**, for both influent (0.093 µg) and effluent (0.071 µg) samples, as well as the calculated CV values are below the CV*r* values. **Tables A9** and **A10** of **Appendix A** show the calculated differences between duplicates and CV values for effluent and influent, respectively.

To estimate the uncertainty component of the samples, **Equation 58** was used, which is equal to the absolute standard deviation of all sample duplicates ($S_{precision}$), by matrix. But first, this value was also transformed into its relative standard deviation (*RSD*), by dividing it by the absolute mean of the concentration of the samples ($\bar{\bar{X}}$). In **Table 42** are presented the variables used in the calculation of the uncertainty component associated with the samples.

Afterwards, the uncertainty associated with the CSs was combined with the uncertainty of the sample duplicates using **Equation 57**. The results are presented in **Table 43**.

Table 42 – Variables used in the assessment of precision uncertainty component associated with samples.

Matrix	$\sum_{j=1}^{t}(y_{j1}-y_{j2})^2$	$S_{precision}$ **(mg/L)**	$\bar{\bar{X}}$ **(mg/L)**	***RSD***	$u_{preci,samp,rel}$ **(%)**
Effluent	544.2	5.8	179.3	0.033	3.3
Influent	4781.9	18.5	542.7	0.034	3.4

Table 43 – Values of uncertainty associated with precision for influent and effluent samples.

Matrix	$u_{preci,samp,rel}$ **(%)**	$u_{precision,CS,rel}$ **(%)**	$u_{precision,rel}$ **(%)**
Effluent	3.3	9.5	10.1
Influent	3.4		10.1

3.2.7. Combined and expanded uncertainty of the NPOC method

After estimating the uncertainties associated with trueness and accuracy, the next step was to estimate the combined standard uncertainty. This was calculated by combining the previous uncertainties, according to **Equation 67**, in their relative form. Then, expanded uncertainty was calculated by multiplying the combined uncertainty by an expansion factor (*k*). Since a number of tests greater than six were used to estimate the uncertainty value, *k* value takes the value of two for a confidence level of 95 % (43). In **Table 44** are shown the final values for both the combined and expanded uncertainties.

Table 44 – Combined and expanded uncertainty values of the NPOC method for influent and effluent.

Matrix	$u'_{trueness}$ (%)	$u'_{precision}$ (%)	u_c (%)	U (%)
Effluent	4.85	10.1	11.2	22.3
Influent	9.92	10.1	14.2	28.3

As can be seen, the expanded uncertainty value associated with the treated effluent is significantly lower than the value obtained for the untreated influent. The uncertainty component responsible for this difference is the method bias, as the uncertainty associated with precision is not significantly different for both types of samples (**Table 43**).

The uncertainty associated with trueness was determined by recovery tests on real samples (**section 3.2.5.**). Thus, the difference in the uncertainty value between the two sample types is due to possible matrix effects that interfered with the recovery of the added amount of analyte to the samples. Untreated influent samples have a higher amount of suspended solids and possibly unremoved compounds that may interfere with NPOC analysis, hence the greater associated uncertainty.

Until the equipment was purchased and its results were reliable, RAIZ laboratory hired an external company to evaluate the TOC concentration in its samples. The company in question is accredited for TOC analysis and its TOC determination method has an associated expanded uncertainty of ± 20.0 %. This value, although lower than the values determined in the present work, is similar to the *U* value determined for treated effluent (22.3 % - **Table 44**).

The calculated uncertainty value shall be verified to ensure that it has been correctly estimated (43). One way to assess whether uncertainty has been well estimated is through standard error, *En* (43). This evaluation parameter was calculated following the RAIZ participation in the interlaboratory test, through **Equation 41**. For this, the reference value considered was the concentration value attributed to the interlaboratory sample (91.9 mg/L) and the experimental value the obtained by RAIZ (92.8 mg/L) for the same sample. In addition, the uncertainty value associated with the interlaboratory result (2.8 %) was also used. The *En* value was calculated as shown below.

$$E_n = \frac{|92.8 - 91.9|}{\sqrt{22.3^2 + 2.8^2}} = 0.04 < 1$$

As can be seen, the value of *En* (0.04) is smaller than 1, indicating that the expanded uncertainty for treated effluent samples was well assessed (43). It is not possible to confirm the uncertainty value for untreated influent since the interlaboratory sample corresponds to treated wastewater and, therefore, the comparison with untreated effluent is not significant.

3.3.TNb calibration according to surface water matrix

Due to the numerous problems already mentioned, that were verified throughout the present work, it was not possible to exploit TNb analysis as originally planned.

RAIZ has several research areas and, therefore, the laboratory receives a high diversity of samples for analysis, coming not only from the P&P industry process, but also related to the forestry and environmental areas. One of the sample matrices corresponds to surface waters. Although the analyser had an already established calibration for TNb analysis (**Table 5**), it was newly calibrated with respect to the surface water matrix.

As mentioned in **2.1.2.1.**, TNb method is an extension of other methods. Consequently, the analyser software does not provide any method for the individual analysis of this parameter. Once TNb default calibration was performed together with NPOC, during the performance of the new calibration, that feature was maintained.

Additionally, TNb calibration by Vario TOC Select analyser has to be performed per sample composition regarding the nitrate and ammonium proportion. Therefore, the composition of the sample had to be known in advance, in order to prepare all the solutions according to the nitrogen species composition of the analysed surface waters. The samples used to establish the ratio had been previously analysed in RAIZ, being the concentration values of each species and of total nitrogen (TNb) shown in **Table 45**.

Table 45 – Prior concentration of TNb and nitrogen species in water samples.

Sample	NO_3^- (mg/L)	NH_4^+ (mg/L)	TNb (mg/L)
A	1.4	0.3	< 1.0 (LQ)
B	1.2	< 0.15 (LQ)	< 1.0 (LQ)

Regarding sample B, the exact concentration of NH_4^+ was not available, the only information was that it was less than 0.15 mg/L, the LQ value for the method with which the assessment was performed. Therefore, in order to perform the calculation of TNb value, the concentration assumed was 0.15 mg/L, since, in the worst case, this corresponded to the maximum concentration for the parameter.

As both species do not have only nitrogen, it was necessary to verify the nitrogen percentage in each one. So then follows the calculation example performed for NH_4^+.

Calculation example

Taking into account the molar masses of hydrogen (Mr=1.0078 g/mol) and nitrogen (Mr=14.0067 g/mol), and taking the molar mass of NH_4^+ (Mr=14.0067 + 4 ×1.0078 = 18.04 g/mol) as 100 %, N fraction in the ammonium molecule is assessed as follows.

$$\% N_{NH_4^+} = \frac{14.0067 \times 100\ \%}{18.04} = 77.65\ \%$$

The same was performed for NO_3^-, being the calculated value of 22.59 %. Therefore, the nitrogen concentrations in each nitrogen species for both samples shown in **Table 46** were assessed by applying the above-calculated percentages to the NO_3^- and NH_4^+ concentrations in each sample.

Table 46 – Concentration of TNb, NO_3^- and NH_4^+ calculated according to the percentages of nitrogen in each species.

Sample	NO_3^- (mg/L)	$N_{NO_3^-}$ (mg/L)	$N_{NO_3^-}$ (%)	NH_4^+ (mg/L)	$N_{NH_4^+}$ (mg/L)	$N_{NH_4^+}$ (%)	TNb (mg/L)
A	1.4	0.32	57.6	0.30	0.23	42.4	0.55
B	1.2	0.27	69.8	0.15	0.12	30.2	0.39

Taking the TNb concentration as 100 % and knowing the TNb concentration from each species for each sample, the ratio of the two compounds, essential for the preparation of the stock solution (**2.3.1.1.**), was calculated. The results are also shown in **Table 46**.

The fractions obtained for each sample are not equal, yet the difference is not significant. Thus, a compromise between both samples for the preparation of the stock solution was chosen, namely 65 % NO_3^- and 35 % NH_4^+. Thus, the weight values of each of the reagents mentioned in section **2.3.1.1.** were calculated according to this ratio.

Therefore, in order to carry out the new TNb calibration, the standard solutions were prepared according to **2.3.1.2.** and the procedure to be performed in the analyser software is described in **Appendix D**, namely the Vario TOC Select calibration/operation procedure in liquid samples produced during this thesis for the use in the RAIZ laboratory.

Figure 36 shows the new TNb calibration curve of the calibration performed in RAIZ. In **Table 47** the signal values for each of the standards are presented, as well as its equation (ECC).

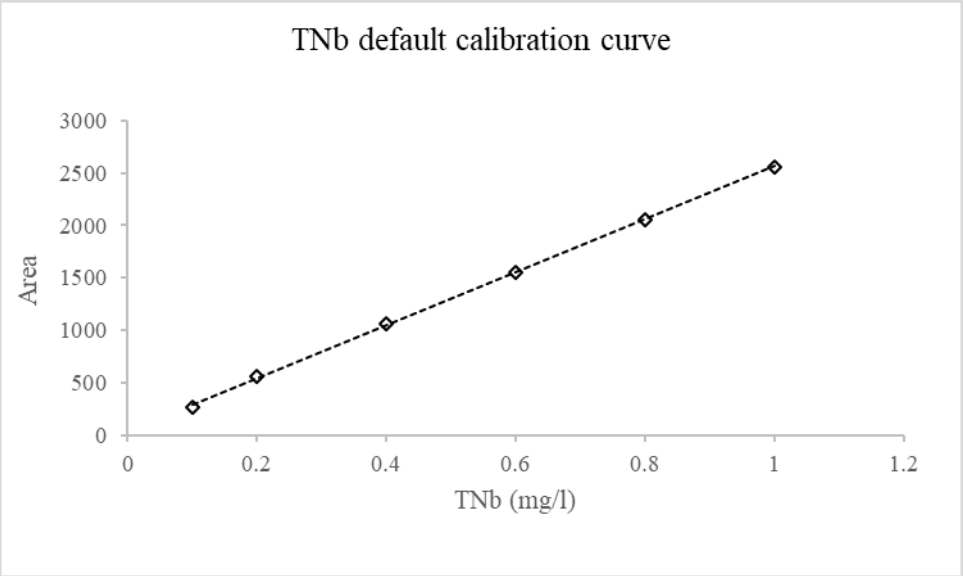

Figure 36 – TNb calibration curve according to the composition of the water samples.

By observing the graph and the *r* value (0.99986), it can be concluded that the TNb function for the interpolation of an area value in weight (µg) is linearly calibrated, presenting good linearity.

Table 47 – TNb standards and the respective signal value and concentration standard.

Standard concentration (mg/L)	**Standard (µg)**	**Area**
1	0.1	260
2	0.2	555
4	0.4	1057
6	0.6	1554
8	0.8	2050
10	1.0	2564
CCE	**Area** = 2525.06 **Weight** – 0.04	

To complete the process, samples A and B were analysed in the equipment and the respective concentration values are shown in **Table 48**. By the high-temperature combustion method, it is not possible to distinguish between the two nitrogen species analysed, consequently is only possible to compare the TNb concentration.

Regarding sample B, it can be seen that the determined TNb_v value is not so different from the initially calculated value (0.39 mg/L – **Table 46**) even though it has been assessed assuming a concentration of NH_4^+. Still, it is in agreement with the result already obtained by the RAIZ laboratory (< 1 mg/L).

Table 48 – TNb concentration of samples assessed by Vario TOC Select analyser.

Sample	TNb (mg/L)	TNb_v (mg/L)
A	<1.0 LQ	0.80
B	<1.0 LQ	0.42

Note: TNb_v – concentration assessed by Vario TOC Select.

Regarding sample A, the obtained TNb_v value is not in agreement with the initially calculated value (0.55 mg/L – **Table 46**). However, it must be noticed that the initial value calculation was performed from low concentration values and, therefore, with much-associated uncertainty. Still, once again is in agreement with the TNb value previously obtained by RAIZ.

3.4. COD/TOC relationship in the P&P industry effluents

In the present work, two different methods were performed to assess TOC value. Therefore, the ratio between the two parameters was evaluated by comparing COD value, either with the TOC value determined by the DM or with the NPOC value, evaluated by the direct method. Additionally, was tried to establish correlations for two types of sample composition, namely untreated influent and treated effluent.

3.4.1. COD/TOC relationship – difference method

The relationship between the TOC and COD parameters was evaluated by the analysis of 8 samples for each sample composition. **Figures 37** and **38** show the relations established for the mean data from the analysed effluent and influent samples. The COD values were represented with the corresponding uncertainty, that was assessed by taking into account the uncertainty associated with the analysis method already established in RAIZ (± 20.8 %). The same was not carried out for TOC values since, due to the problems found in the present work, it was not possible to finalize the validation of the method.

Tables A11 and **A12** of **Appendix A** show the values of both parameters used to establish the two correlations for untreated influent and treated effluent, respectively. Additionally, regression analysis was also performed to obtain predictive equations, for COD from TOC, for both types of wastewater, which are shown in **Table 49**.

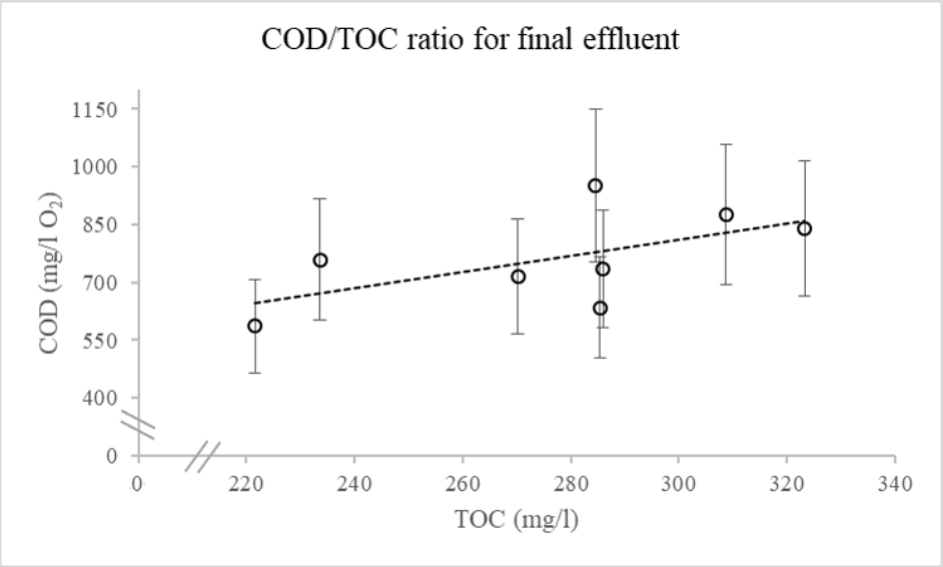

Figure 37 – Relationship between COD and TOC parameters for treated effluent. The error bars shown correspond to the expanded uncertainty associated with the COD method.

The correlation coefficient (*r*) was used to assess the quality of the correlation between the two water quality parameters and, as it can be seen from the obtained values (**Table 48**), it is very poor for both types of sample composition. Even so, for the effluent matrix (**Figure 37**), the data dispersion was more significant, resembling a set of points, rather than presenting a linear behaviour, which is translated by the low *r* value (0.5915).

Regarding influent samples (**Figure 38**), the established correlation was better (r = 0.8835). However, as it was verified for the effluent (**Figure 37**), there are points with similar TOC values and different COD values. Nevertheless, COD values have high associated uncertainty and, on the other hand, the several problems verified with the DM may have affected the accurate determination of TOC values.

The resultant value of the COD:TOC ratio for the effluent (2.1) was not very different from the value reported by Ekstrand et al. 2013 (2.4), for treated effluent from the kraft process and with the same bleaching process (alkaline treatment, ECF) of the samples in the present work (12).

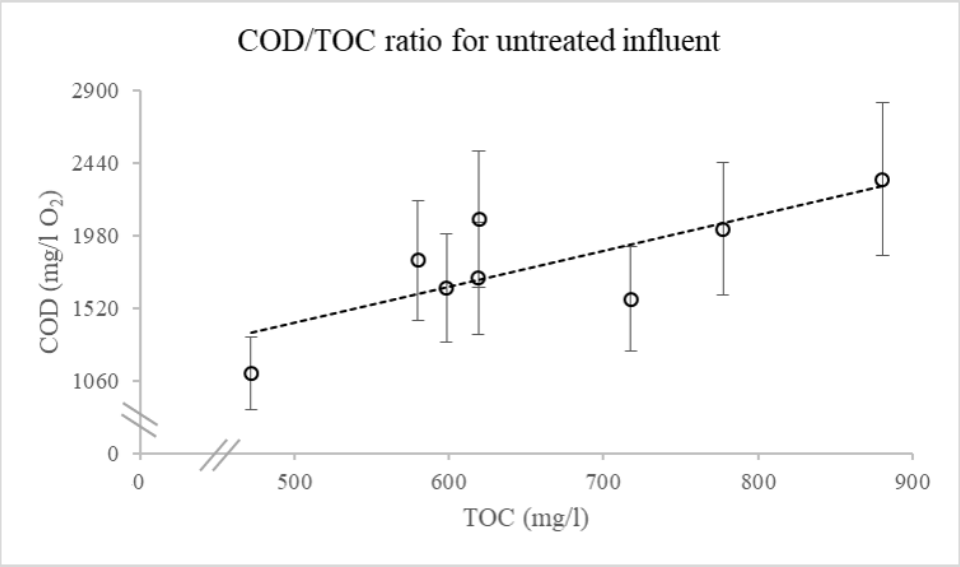

Figure 38 – Relationship between COD and TOC parameters for untreated influent. The error bars shown correspond to the expanded uncertainty associated with the COD method.

Table 49 – Correlation COD and TOC using regression analysis for influent and final effluent.

Relationship	*r*	*b*	*a*	Regression equation
COD:TOC influent	0.8835	2.3	289.9	**COD** = 2.3**TOC** + 289.9
COD:TOC effluent	0.5915	2.1	181.7	**COD** = 2.1**TOC** + 181.7

Additionally, Dubber et al. 2010 (26) studied the COD:TOC relationship for municipal wastewater. Although the quality of the correlations (influent – r = 0.959; effluent – r = 0.820) was greater than in the present study, the verified influent r value was also superior (0.8835) than the effluent. Still, in order to establish a more significant and representative COD:TOC correlation for both wastewater types, more samples should be analysed.

3.4.2. COD/NPOC relationship – direct method

In order to study the correlation between COD and NPOC parameters, nine influent and ten effluent samples were analysed in duplicate, and under IP conditions. **Figures 39** and **40** show the mean values of the analysed samples for both types of sample composition. In **Tables A13** and **A14** of **Appendix A** are shown the values used to establish the correlations for both matrices. The values of both parameters were represented with their associated uncertainty, estimated taking into account the overall uncertainty of each method. In this case, the uncertainty associated with the NPOC method, which was assessed in the present work (**Table 44**), was used.

The ordinary linear regression is the most used in Analytical Chemistry, being implemented by the least square deviation method. In this case, it is assumed that only one of the variables, namely the one associated to the signal (y), is subject to error, being the error associated with variable x

considered negligible. However, there are situations where both variables are subject to significant errors. This is the case of the present work, where both COD (*y*) and NPOC (*x*) parameters have an associated uncertainty. In these cases, least squares regression with errors in both variables, specifically orthogonal distance regression (ODR) must be applied. In OLS method, the main goal is to minimize residual errors in the *y*-direction, whereas in ODR both errors in *x*- and *y*-directions are minimized (54).

Consequently, the ODR model was used to calculate the fitting regression line between the two water quality parameters, being the obtained predictive equations for COD from NPOC, for both types of wastewater, shown in **Table 50**.

The correlation coefficient values for both influent (r = 0.99745) and effluent (r = 0.98860) samples indicate a good and better correlation than it was found for the previous correlations established through the analysis by the DM. However, for both sample types, some inconsistent points were verified, namely the fact that some NPOC values have similar COD values and vice versa, as it happened for the previous values. However, the same seems to occur in already established correlations present in the literature (26,55).

It should be understood that the two parameters do not necessarily have the same target. While TOC is an evaluation of non-purgeable organic carbon, COD is primarily a measure of chemically oxidizable organics, measuring the amount of oxygen equivalent to the organic matter present in the wastewater (24,56). Besides, it should be considered that part of the TIC fraction, that might be present in the samples, may not have been completely removed by acidification and purging and was erroneously accounted for as NPOC (56).

The effluent samples used to establish the correlation between the two water quality parameters come from the bleached kraft pulp production process, where chloride is used as a bleaching agent. As already mentioned (**section 2.4.4.**), chloride is an interferent in COD analysis and its presence was verified throughout this work in both sample types (**Figure 15**), being more significant in untreated effluent samples. The chloride test has some subjectivity as it depends entirely on the analyst's perceptiveness. Additionally, even diluting samples when necessary, the addition of mercury(II) sulfate reduces this interference but does not totally eliminate it (49). This might also have led to inaccuracies in the determined COD values and, thus, affecting the established correlations.

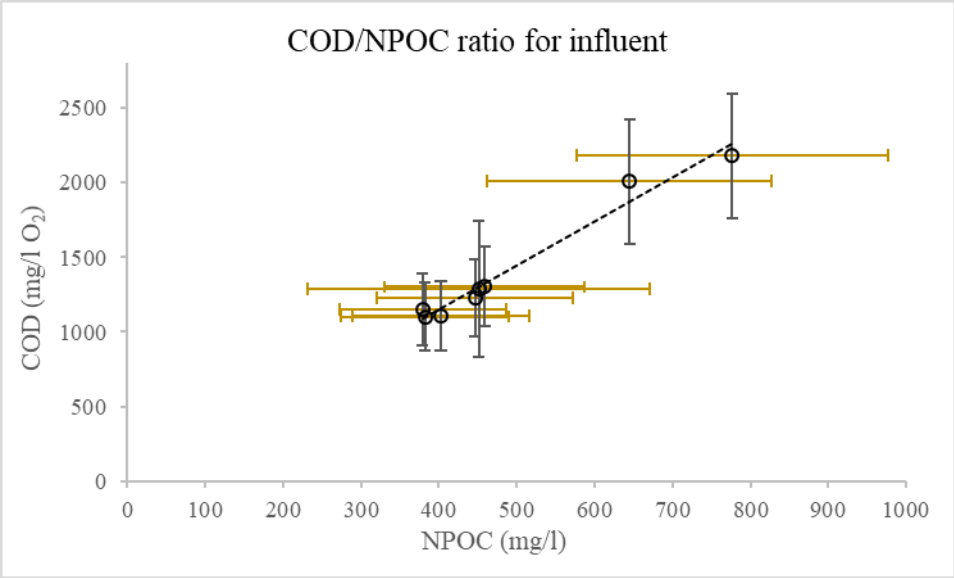

Figure 39 – Relationship between COD and NPOC parameters for untreated influent. The error bars shown correspond to the expanded uncertainty associated with the COD and NPOC methods.

Moreover, as can be seen in both **Figures 39** and **40** of the two correlations, the uncertainty range associated with each point for both parameters is quite significant, which may also justify the fact that some NPOC values have similar COD values and vice versa.

The establishment of correlations between these two parameters is in the interest of RAIZ, and the P&P industry in general. However, insufficiently information has been reported about the possibility of replacing the COD with TOC (or NPOC), largely due to the difficulties in establishing a good relationship (55). Also, the BOD test continues to be widely used as a parameter for evaluating organic matter in water, being preferred to other tests, despite its various limitations (i.e. assay time, etc.) (57). Therefore, many of the correlations established in literature are intended to replace BOD by other parameters (COD, TOC, etc.) (58).

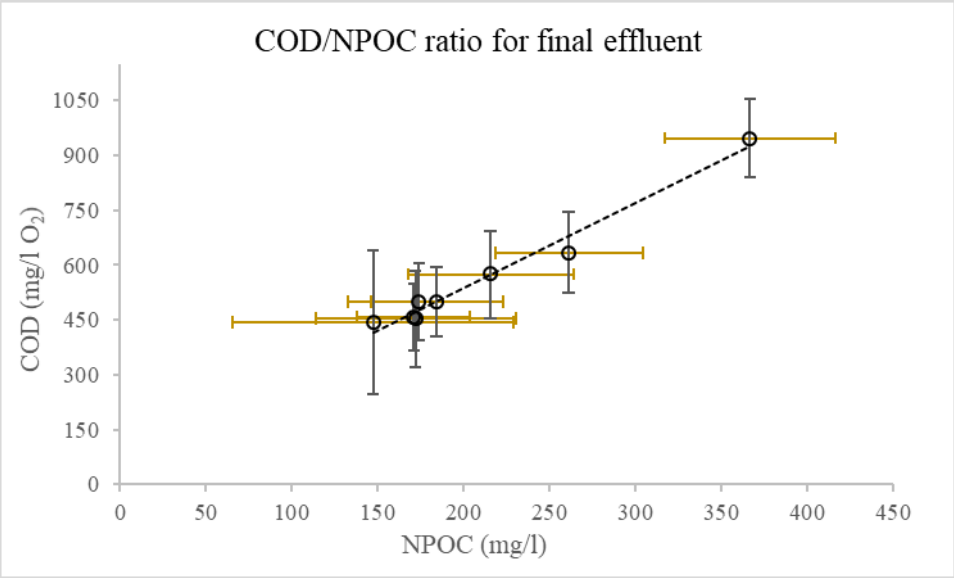

Figure 40 – Relationship between COD and NPOC parameters for treated effluent. The error bars shown correspond to the expanded uncertainty associated with the COD and NPOC methods.

Table 50 – Correlation COD and NPOC using regression analysis for influent and final effluent.

Relationship	*r*	*b*	*a*	Regression equation
COD:NPOC influent	0.99745	3.02	-68.79	**COD** = 3.02**NPOC** – 68.79
COD:NPOC effluent	0.98860	2.36	64.01	**COD** = 2.36**NPOC** + 64.01

Regarding the assessed COD:NPOC ratio for effluent, namely 2.36, is very close to the value of 2.4 reported by Ekstrand et al. 2013 (12), for treated effluent from the same production process as the samples in this study. Regarding the COD:NPOC ratio for influent, the assessed value in this study (3.02) resembles the reported value by Dubber et al. 2010 (3.0) (26).

In addition to the difficulties presented above, the establishment of a factor reflecting the relationship between these two parameters is complicated, since it requires that the wastewater and its mixture of substances do not change in their composition. Wastewater from the P&P industry consists of several types of compounds and there is still little information about the organic composition of these effluents (15). Therefore, a better characterization should be done in order to facilitate the understanding of the factors that may affect the relationship between these two parameters, in order to accelerate the full implementation of the TOC as a water quality parameter.

Moreover, in order to improve and make the established COD:TOC/NPOC correlations more representative of each of the analysed matrices, more samples should be analysed to confirm the assessed values and to avoid possible errors.

4. Conclusions and future work

The current study consisted on the validation and implementation of the direct method (NPOC) for TOC analysis, by the HTC methodology. It followed the acquisition by the RAIZ laboratory of a TOC analyser from *Elementar* company (Vario TOC Select analyser).

Validation was performed through the evaluation of the working range, linearity, sensitivity, analytical limits, with special attention to the limit of quantification, and precision. The uncertainty associated with the NPOC method was also estimated taking into consideration the two major uncertainty components, precision and trueness. The implementation was accomplished by the proposal of a procedure for operation and maintenance of Vario TOC Select analyser, developed in the present work, being this presented in Appendix C of this report.

In addition, since the implementation of the TOC parameter must be accompanied by a correlation factor with COD, it was also carried out a study to establish a COD/TOC relationship for the P&P industry effluents. As secondary work, conditioned by time, the validation of the TNb analysis method, was also an objective of the present study.

The validation of the direct method (NPOC) began by testing the factory working range (0.3 µg – 3.0 µg NPOC), having been confirmed the homoscedasticity condition of the data. The NPOC calibration curve, of the area as a function of NPOC weight, showed good linearity, proven statistically by Mandels' test and by the correlation coefficient (r = 0.99989).

Regarding sensitivity, despite some variation, the obtained slopes of the calibration curves were satisfactory over the evaluation time, since, the average slope deviation (4.9 %) from the factory slope was within the acceptance criteria established during this work, i.e., 5 % deviation from the factory slope.

The LQ of 0.24 µg was tested over time by the analysis of CSs and its accuracy and precision were evaluated. To assess the accuracy, 10 % of the LQ theoretical value was established as the acceptance criterion and most of the values met the established criterion. Precision was assessed by the CV calculation, whose value did not exceed 10 %, the limit value according to the IUPAC. Therefore, the LQ value proved to be appropriate for the NPOC method.

Three precision measurements were evaluated for the NPOC method, namely repeatability, intermediate precision (IP) and reproducibility. The repeatability and IP of the NPOC method were assessed for untreated and treated effluent samples. Concerning repeatability, it proved to be acceptable for both matrices, since CVr values were below 10 %, as advised in EN 1484:1997 for TOC analysis. The obtained values were 4.3%, for untreated influent, and 8.9 %, for treated effluent. Besides, the samples analysed throughout the work met the repeatability limits that were defined for untreated (Δr = 0.093 µg) and treated effluent (Δr = 0.071 µg). As for the IP, the range CCs showed

that the process was under control for both types of samples, being the range of the duplicates of all samples within the established control limits. However, IP was evaluated for a relatively small number of samples. Therefore, in order to confirm the control limits and make more representative the results, in the future, more samples of each matrix must be analysed.

The present work enabled the participation in a TOC interlaboratory trial, in which 44 other laboratories were involved. The sample analysed was the same for all the participants and corresponded to an effluent sample from an urban wastewater treatment plant with a final TOC concentration value of 91.9 mg/L. The result obtained by RAIZ was 92.8 mg/L, which was the best result of all the TOC values obtained in the interlaboratory test, corresponded to a Z-score of 0.12. This result also demonstrates the good functioning of the analyser regarding the NPOC method.

In the interlaboratory trial was also possible to evaluate reproducibility, CV_R=8.2 %. However, having this value been estimated from only one interlaboratory trial, in the future, are required more participations in interlaboratory tests to properly estimate the assessed value.

The estimation of the expanded uncertainty associated with the NPOC method was also calculated for the untreated and treated effluent matrices, being the calculated values of 28.3 % and 22.3 %, respectively. For this, the two main uncertainty components, namely precision and trueness, were estimated from CSs and spiked samples. Also, the bottom-up approach was first used to calculate the uncertainty associated with the spiking solution ($u'(C_{\text{spike solution}})$ = 0.134 %).

Initially, it was intended to perform the validation of the DM for TOC analysis. The heteroscedasticity observed between the endpoints of the calibration curves of TC (2 µg – 12 µg) and TIC (1 µg – 6 µg) led to the application of the weighted linear regression (WLR). However, the same standard solution (20/10 mg/L TC/TIC), i.e., the same amount of carbon, has systematically generated an area value smaller than the factory area values for the same standard. This difference was found to be more significant for TC because the difference between the two the signal values (factory calibration and RAIZ calibration) increased as the amount of carbon increased as well. The area values for the TC standards in the factory calibration were never verified at RAIZ during the time the DM was tested. After several attempts to solve the problem, it was found that the analyser problem was that the initial factory calibration was not operational since the beginning of the work with the equipment. Therefore, a new calibration was carried out, regarding TC and TIC, and properly validated.

In addition to this problem, it was only at the end of the present work that the technicians of the analyser supplier company clarified the real way of subtracting the equipment blank values, and this was another factor that made working with the DM difficult.

The COD:TOC/NPOC ratio was calculated in the present work for untreated and treated effluent. The quality of the established correlations was evaluated by the correlation coefficient (*r*).

The COD:NPOC ratio was the most relevant, presenting ratio values of 3.02, for untreated influent, and 2.36, for treated effluent, similar to some already reported in the literature (3.0 and 2.4). It can be concluded that predictive equations can be established between COD and TOC parameters, but more samples must be analysed in order to increase the representativeness of the relationships.

As mentioned in the present study, the COD:NPOC correlation may be affected by several factors. In order to identify the extent of influence of the various interferents to improve the established correlations, it is of great importance to know the composition of the matrices analysed. The wastewater from the P&P industry has a high number of compounds, whose presence is dependent on the process. If these compounds are known in the matrix whose correlation is to be obtained, it will be possible to estimate a theoretical correlation factor between the two parameters which, when compared to the one experimentally obtained, may help to mislead the influence of the various interferers. Therefore, as future work, it is in the interest of *The Navigator Company*, and even of the P&P industry in general, to conduct a survey of the composition of its effluents, so that the use of the TOC parameter can definitely become a reality as COD and BOD substitute. Besides, greater confidence in the correlation established for a given matrix would help to implement TOC technology online for real-time monitoring, facilitating immediate action in case of any abnormality in the process. Also, it would avoid the cost of sample transportation.

Regarding the TNb method, the initially proposed work, namely the method validation, could not be performed due to time issues. However, the equipment was calibrated according to the surface water matrix, whose samples are very recurrent in the RAIZ laboratory. Thus, if the method is to be used in the future, it will be necessary to validate it in order to have confidence in its results. A drawback of the TNb method is the fact that calibration by Vario TOC select analyser has to be performed per sample composition regarding the nitrate and ammonium proportion. Therefore, it is necessary that the proportion of nitrogen species does not vary between samples of the same matrix or that their concentration is known beforehand, which is not very practical.

Finally, the present work, although it corresponded to an area not explored during the first year of the Master's degree in Industrial and Environmental Biotechnology, it allowed to contact and develop capacities in the area of analysis methods, which are used in so many other, where biotechnology is no exception. It also allowed developing critical thinking in results analysis, as well as to improve the capacity to overcome the numerous obstacles that emerged in the real context of a company.

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Appendix A – Tables

Table A1 shows the analyser signal (area) and the corresponding weight values of the ten replicates used to perform the HV test for the TC and TIC work ranges.

Table 51 – Area and weight values of TC and TIC used in the HV tests for assessing the respective work ranges.

Replicate	2 µg standard		12 µg standard		1 µg standard		6 µg standard	
no.	Area	TC (µg)	Area	TC (µg)	Area	TIC (µg)	Area	TIC (µg)
1	3791	1.9	23042	10.0	2471	1.2	13004	5.5
2	3821	1.9	23121	10.0	2485	1.2	13085	5.5
3	3822	1.9	23140	10.0	2485	1.2	13193	5.6
4	3824	1.9	23190	10.0	2515	1.3	13275	5.6
5	3844	1.9	23240	10.1	2543	1.3	13301	5.6
6	3851	1.9	23243	10.1	2570	1.3	13332	5.6
7	3853	1.9	23248	10.1	2575	1.3	13334	5.6
8	3854	1.9	23261	10.1	2620	1.3	13335	5.7
9	3860	1.9	23264	10.1	2644	1.3	13724	5.8
10	3868	1.9	23293	10.1	2695	1.3	13862	5.9
Mean	3838.8	1.9	23204.2	10.0	2560.3	1.3	13344.5	5.7
S	23.60	0.01	79.55	0.03	75.05	0.03	263.55	0.11
CV (%)	-	0.53	-	0.33	-	2.40	-	1.90
***Er* (%)**	-	-6.0	-	-16.4	-	26.9	-	-5.8

Table A2 shows the analyser signal (area) and the corresponding weight values used to perform the HV test for both TC and TIC half working ranges.

Table 52 – Area and weight values of TC and TIC used in the HV test with half working ranges.

Replicate	7.5 µg standard		3.5 µg standard	
no.	Area	TC (µg)	Area	TIC (µg)
1	14719	6.46	8256.5	3.6
2	14749	6.47	8263.5	3.6
3	14802	6.50	8385.5	3.6
4	14814	6.50	8509.5	3.7
5	14856	6.52	8550.5	3.7
6	14866	6.52	8599.5	3.7
7	14872	6.53	8629.5	3.7
8	14872	6.53	8799.5	3.8
9	14894	6.54	8860.5	3.8
10	14896	6.54	8862.5	3.8

Mean	-	6.51	-	3.71
S	-	0.03	-	0.09
CV (%)	-	0.4	-	2.5
***Er* (%)**	-	-13.2	-	6.1

Table A3 shows the representative areas of each of the weight determinations used to calculate the corresponding mass from the coefficients of the calibration curves of both TC and TIC used in the *t*-test.

Table 53 – Representative area values used in the statistical *t*-test.

Parameter	Weight (µg)	Area
TIC	1.0	2100
	3.5	8200
	6.0	14300
TC	2.0	4000
	7.0	14300
	12.0	24200

Tables A4 and **A5** show the slope and intercept values, the coefficients of the calibration curve (CC) used to carry out the averages *t*-test (**section 3.1.4.**), as well as all calculated and necessary values to perform the test for TIC and TC, respectively.

Table 54 – Values used in the averages statistical *t*-test for TIC.

With $NaHCO_3$					**Without $NaHCO_3$**				
Coefficients CC		**Weight TIC (µg)**			**Coefficients CC**		**Weight TIC (µg)**		
b	***a***	**1.0**	**3.5**	**6.0**	***b***	***a***	**1.0**	**3.5**	**6.0**
2275.8	-331.0	1.1	3.7	6.4	2399.7	-387.6	1.0	3.6	6.1
2447.3	-326.0	1.0	3.5	6.0	2347.9	-285.3	1.0	3.6	6.2
2447.3	-302.9	1.0	3.5	6.0	2582.4	-321.2	0.9	3.3	5.7
2477.7	-216.1	0.9	3.4	5.9	2684.9	-319.2	0.9	3.2	5.4
					2628.1	-412.5	1.0	3.3	5.6
					2483.1	-317.9	1.0	3.4	5.9
Mean		1.0	3.5	6.1	**Mean**		1.0	3.4	5.8
S		0.06	0.15	0.25	***S***		0.05	0.18	0.30
S^2		0.003	0.024	0.064	S^2		0.003	0.031	0.093
FD		3	3	3	**FD**		5	5	5

Table 55 – Values used in the averages statistical *t*-test for TC.

With $NaHCO_3$					Without $NaHCO_3$				
Coefficients CC		**Weight TC (µg)**			**Coefficients CC**		**Weight TC (µg)**		
b	***a***	**2.0**	**7.0**	**12.0**	***b***	***a***	**2.0**	**7.0**	**12.0**
2023.9	-0.4	2.0	7.1	12.0	2058.6	44.7	1.9	6.9	11.7
2022.2	425.6	1.8	6.9	11.8	2020.2	-87.1	2.0	7.1	12.0
2051.6	279.8	1.8	6.8	11.7	2073.3	-162.5	2.0	7.0	11.8
1952.3	71.4	2.0	7.3	12.4					
1994.2	123.0	1.9	7.1	12.1					
Mean		1.9	7.0	12.0	**Mean**		2.0	7.0	11.8
S		0.11	0.19	0.28	***S***		0.05	0.10	0.16
S^2		0.011	0.035	0.076	S^2		0.003	0.010	0.026
FD		4	4	4	**FD**		2	2	2

In **Table A6** are shown the analyser signal (area) and the corresponding weight values of the ten replicates used to perform the HV test for the NPOC work range, before and after insertion of the LQ standards into the calibration curve.

Table 56 – Area and weight values of NPOC used in the HV test for assessing the working range.

Replicate no.	0.18 µg standard		0.24 µg standard		0.30 µg standard		3.0 µg standard	
	Area	**NPOC (µg)**	**Area**	**NPOC (µg)**	**Area**	**NPOC (µg)**	**Area**	**NPOC (µg)**
1	465.3	0.21	538.3	0.25	704.3	0.33	6227.3	2.92
2	468.3	0.22	571.3	0.26	711.3	0.33	6241.3	2.92
3	470.3	0.22	581.3	0.27	715.3	0.33	6254.3	2.93
4	470.3	0.22	611.3	0.28	723.3	0.34	6268.3	2.94
5	476.3	0.22	622.3	0.29	726.3	0.34	6268.3	2.94
6	478.3	0.22	625.3	0.29	736.3	0.34	6280.3	2.94
7	484.3	0.22	634.3	0.29	737.3	0.34	6288.3	2.95
8	496.3	0.23	638.3	0.30	754.3	0.35	6289.3	2.95
9	502.3	0.23	638.3	0.30	756.3	0.35	6292.3	2.95
10	511.3	0.24	645.3	0.30	786.3	0.36	6295.3	2.95
Mean	482.3	0.22	610.6	0.28	735.1	0.34	6270.5	2.94
S	15.9	0.01	35.4	0.02	24.88	0.01	23.21	0.01
CV (%)	-	3.4	-	5.9	-	3.4	-	0.4
***Er* (%)**	-	23.4	-	17.6	-	13.6	-	-2.1

Table A7 shows the coefficients of the first- and second-degree functions, calculated according to ISO 8466, parts 1 and 2, respectively, for the performance of the linearity statistical test to evaluate the NPOC calibration curve.

Table 57 – Variables and coefficients of the first- and second-degree functions to perform NPOC linearity statistical.

No.	*b*	*a*	*r*	$S_{y/x}$	*a*	*b*	*c*	Sy^2	DS^2	*PG*
1	2079.9	103.4	0.99985	42.1	83.4	2125.5	-14.8	44.8	31.1	0.70
2	2015.7	-5.3	0.99990	33.4	22.6	1952.5	20.4	31.6	40.5	1.28
3	2007.5	37.3	0.99989	34.8	56.1	1964.9	13.8	36.5	27.9	0.77
4*	1864.7	9.2	0.99906	95.2	56.3	1757.9	34.6	101.0	72.0	0.71
5	2035.7	-6.9	0.99995	22.2	-27.4	2082.3	-15.1	20.0	30.9	1.54
6	2018.2	14.4	0.99993	28.3	28.9	1985.2	10.7	29.9	22.0	0.73
7	2030.1	-38.2	0.99997	19.0	-29.8	2011.0	6.2	20.3	13.5	0.66
8*	2253.2	125.3	0.98275	499.8	-296.2	3210.5	-309.9	470.8	615.9	1.31
9	2006.3	27.3	0.99996	21.1	31.6	1996.6	3.14	23.4	11.9	0.51
10	2002.5	54.3	0.99964	63.2	5.3	2113.9	-36.1	61.4	70.5	1.15
11	2039.2	-14.1	0.99990	34.3	10.9	1982.3	18.4	34.0	35.8	1.05
Mean	2026.1	19.1	0.99989	33.2	20.2	2023.8	0.7	33.5	31.6	0.93
Max	2079.9	103.4	0.99997	63.2	83.4	2125.5	20.4	61.4	70.5	1.54
Min	2002.5	-38.2	0.99964	19.0	-29.8	1952.5	-36.1	20.0	11.9	0.51
S	24.1	42.5	0.00010	13.6	36.3	65.7	18.9	13.2	17.4	0.34

Note: calibrations 4 and 8 (*) were not taken into account in the calculations of bottom parameters, as they corresponded to anomalous calibrations curves.

The volume component associated with the repeatability of the glassware used for carrying out the recovery tests was determined by repeated weightings of the water used to prepare the solutions. The values of the ten measurements are given in **Table A8**.

Table 58 – Values of the measurements used to assess the volume repeatability component of the volumetric material used to estimate the uncertainty associated with the trueness.

Weighing	**1000 mL VF**	**100 mL VF**	**10 mL VP**	**5 mL VP**	**4 mL VP**
no.	Mass (g)	Mass (g)	Mass (g)	Mass (g)	Mass (g)
1	996.77	99.08	9.99	4.98	4.00
2	996.60	99.14	9.98	4.99	4.00
3	996.50	99.03	9.98	5.00	4.01
4	996.44	99.14	9.96	5.00	3.99
5	996.49	99.20	9.97	5.00	3.99
6	996.49	99.18	9.97	5.01	4.01
7	996.51	99.09	9.99	4.99	3.99
8	996.68	99.10	9.97	4.99	4.00
9	996.54	99.10	9.97	4.99	4.00
10	996.53	99.16	9.96	4.98	4.00
Mean (g)	**996.56**	**99.12**	**9.98**	**4.99**	**4.00**
***S* (g)**	**0.101**	**0.051**	**0.011**	**0.008**	**0.007**
CV (%)	**0.0001=0.010 %**	**0.0005=0.05 %**	**0.0011=0.11 %**	**0.0016=0.16 %**	**0.0018 = 0.18 %**

Note: VF – volumetric flask; VP – volumetric pipette.

The uncertainty associated with the precision of the NPOC method was assessed from samples and CSs. To ensure that the analysis of the samples was performed under repeatability conditions, the difference between the duplicates of each sample was calculated and confirmed to be lower than the Δr calculated in section **3.2.4.1.** (**Table 37**). Similarly, CV*r* was calculated and compared with the values determined in **3.2.4.1.** (**Table 37**). The calculated values are shown in **Tables A9** and **A10** for treated and untreated effluent, respectively.

Table 59 – Repeatability confirmation between duplicates of the treated effluent samples used to determine uncertainty associated with the precision of the NPOC method.

Sample no.	$NPOC_A$ (µg)	$NPOC_B$ (µg)	$\lvert X_B-X_A \rvert$ (µg)	Δr (µg)	Mean (µg)	S (µg)	CV (%)	CV_r (%)
1	0.35	0.39	0.046	0.071	0.37	0.033	8.8	8.9
2	0.38	0.39	0.012		0.38	0.008	2.2	
3	0.35	0.34	0.007		0.34	0.005	1.4	
4	0.58	0.60	0.026		0.59	0.018	3.1	
5	0.71	0.77	0.059		0.74	0.042	5.6	
6	0.69	0.68	0.017		0.68	0.012	1.7	
7	0.74	0.69	0.049		0.71	0.034	4.8	
8	0.71	0.68	0.033		0.70	0.023	3.3	

Note: the subscripts A and B correspond to the two duplicates performed from each sample.

Table 60 – Repeatability confirmation between duplicates of the untreated influent samples used to determine uncertainty associated with the precision of the NPOC method.

Sample no.	$NPOC_A$ (µg)	$NPOC_B$ (µg)	$\lvert X_i-X_{i-1} \rvert$ (µg)	Δr (µg)	Mean (µg)	S (µg)	CV (%)	CV_r (%)
1	0.78	0.75	0.032	0.093	0.76	0.023	3.0	4.3
2	1.03	1.03	0.004		1.03	0.003	0.3	
3	0.62	0.59	0.036		0.61	0.026	4.2	
4	1.11	1.14	0.032		1.13	0.023	2.0	
5	1.25	1.23	0.020		1.24	0.014	1.1	
6	0.68	0.76	0.083		0.72	0.059	8.1	
7	0.95	0.89	0.056		0.92	0.039	4.3	

Note: the subscripts A and B correspond to the two duplicates performed from each sample.

In order to establish the correlation between TOC and COD for both types of sample matrices that were analysed, the values of **Tables A11** and **A12** were used.

Table 61 – COD and TOC values used to carry out regression analysis for untreated influent.

Sample	Date	f_{TOC}	TOC_w (mg/L)	f_{COD}	COD (mg/L O_2)
1	10/jan	50	580.1	10	1823.3
2	10/jan	50	880.6	10	2338.4
3	17/jan	50	471.9	20	1109.5
4	17/jan	50	619.3	40	2086.9
5	24/jan	50	619.1	50	1712.9
6	24/jan	50	717.8	50	1579.5
7	31/jan	20	777.3	50	2024.2
8	31/jan	20	598.5	50	1649.3

Table 62 – COD and TOC values used to carry out regression analysis for treated effluent.

Sample	Date	f_{TOC}	TOC_w (mg/L)	f_{COD}	COD (mg/L O_2)
1	10/jan	20	233.6	10	759.3
2	10/jan	20	284.6	10	952.5
3	17/jan	20	221.6	10	586.5
4	17/jan	20	285.4	10	634.8
5	24/jan	20	308.7	20	875.7
6	24/jan	20	323.2	20	840.2
7	31/jan	10	285.9	20	736.0
8	31/jan	10	270.2	20	715.6

Moreover, **Tables A13** and **A14** show the values used to establish the COD:NPOC relationship for influent and effluent, respectively.

Table 63 – COD and NPOC values used to carry out regression analysis for untreated influent.

Sample	Date	NPOC (mg/L)	COD (mg/L O_2)
1	05/jun	382.4	1100.6
2	07/jun	644.9	2009.5
3	07/jun	446.2	1227.7
4	17/jun	379.2	1150.5
5	17/jun	402.4	1106.3
6	18/jun	705.4	2003.9
7	19/jun	776.8	2181.8
8	24/jun	451.6	1286.8
9	04/jul	458.7	1303.0

Table 64 – COD and NPOC values used to carry out regression analysis for treated effluent.

Sample	Date	NPOC (mg/L)	COD (mg/L O_2)
1	05/jun	215.6	574.5
2	07/jun	192.5	531.6
3	07/jun	223.2	512.8
4	07/jun	261.0	633.6
5	07/jun	366.6	946.9
6	17/jun	171.8	453.6
7	18/jun	147.3	443.3
8	19/jun	184.2	499.9
9	24/jun	170.9	457.0
10	04/jul	174.0	499.9

Appendix B – Figures

In **Figure B1** is shown the view of the Excel spreadsheet created to calculate the several parameters to assess the equation of the weighted linear calibration curve of the analysis day.

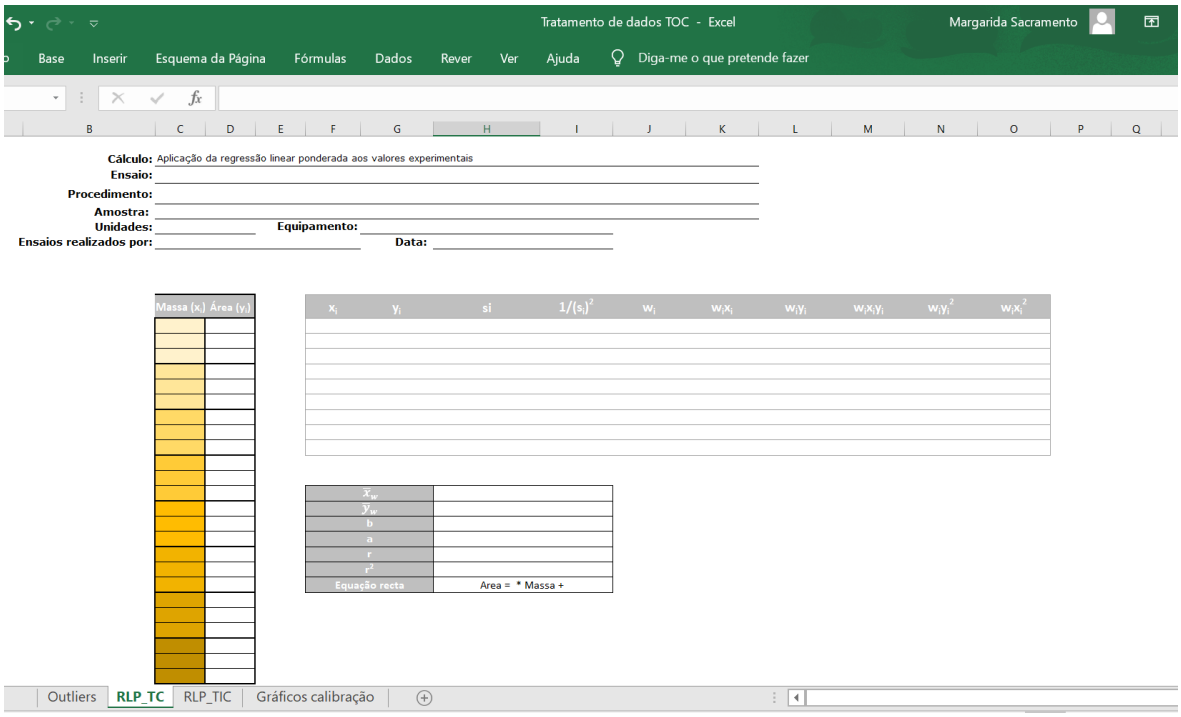


Figure 41 – View of the Excel spreadsheet for the calculation of the daily weighted calibration curves used in TOC determination through DM.

Appendix C – Procedure of operation and maintenance of Vario TOC Select analyser

This appendix presents the proposed operation and maintenance procedure of the Vario TOC Select analyser, which was elaborated during the present work. Once it was done in the context of internal use in RAIZ, it is written in Portuguese.

1. OBJECTIVO E ÂMBITO

Este procedimento especifica o modo de operação do equipamento vario TOC select, analisador de carbono orgânico total (TOC) da empresa Elementar.

2. DESCRIÇÃO

2.1. Definições do método

Para o presente método algumas definições são aplicáveis:

- **Carbono total (CT ou TC, em inglês)** – soma de todo o carbono ligado a compostos orgânicos e inorgânicos presentes na água, incluindo o carbono elementar.
- **Carbono orgânico total (COT ou TOC, em inglês)** – somatório de todo o carbono presente na água, ligado a compostos orgânicos dissolvidos ou em suspensão. Cianato, carbono elementar e tiocianato também são contabilizados.
- **Carbono inorgânico total (CIT ou TIC, em inglês)** – carbonatos, hidrogenocarbonatos, monóxido e dióxido de carbono (CO_2) presentes na água.
- **Carbono orgânico dissolvido (COD)** – totalidade do carbono ligado a compostos orgânicos presentes em água que têm a capacidade de passar por uma membrana com um tamanho de poro de 0,45 µm. Cianato e tiocianato também são quantificados.
- **Carbono orgânico purgável (POC, em inglês)** – carbono orgânico volátil nas condições do método.
- **Carbono orgânico não purgável (NPOC, em inglês)** - carbono orgânico não volátil nas condições do método.

2.2. Princípio geral do método

A determinação do parâmetro TOC pode ser realizada de duas formas, direta ou indiretamente. Uma vez que as amostras de água, para além de carbono orgânico, contêm também CO_2 e carbonatos (fração TIC), a determinação de TOC, pelo método direto, tem que ser realizada após a eliminação de TIC. Neste método, a amostra é acidificada e purgada com um gás livre de CO_2 e compostos orgânicos (e.g. O_2, como previsto no analisador de TOC do RAIZ). Desta forma, o que é contabilizado no final é apenas o TOC.

A determinação de TOC pode ser também realizada indiretamente. Para isso, é necessária a determinação individual de TC e TIC, sendo o TOC obtido pela diferença entre estes dois parâmetros (TC-TIC=TOC). O método da diferença é normalmente usado quando as amostras são ricas em compostos orgânicos voláteis (e.g. benzeno, tolueno, …), evitando-se perdas destes compostos por *stripping* (perda de componentes mais voláteis de uma mistura líquida por contacto com um gás). Este método também se adequa a amostras em que a fração inorgânica é menor ou, pelo menos, semelhante à orgânica.

2.3. Descrição geral das funções do Vario TOC Select

No equipamento Vario TOC Select a deteção do carbono orgânico ou inorgânico faz-se por meio do CO_2. No caso do carbono total (TC), este é gerado por combustão total da amostra a 850 °C; já no TIC, o CO_2 é gerado por acidificação da amostra e posterior purga pelo O_2. No caso da determinação direta de TOC, a oxidação do carbono presente faz-se por combustão, tal como para o TC.

Para ambos os parâmetros referidos anteriormente, a quantidade de CO_2 gerada é lida num detetor infravermelho não dispersivo (NDIR), sendo calculada através da área do pico que o CO_2 produz ao chegar ao detetor. Por meio da calibração estabelecida no equipamento, a área é convertida em massa de carbono e, por conseguinte, em concentração.

2.3.1. Ligar o analisador

Existem duas formas de ligar o equipamento:

A. <u>Quando o equipamento tiver sido totalmente desligado, proceder da seguinte forma:</u>

1. Ligar a fonte de alimentação do equipamento e do computador que lhe está associado;

2. Ligar o computador e respetivo monitor → esperar até que o processo tenha sido finalizado;

3. Abrir o fornecimento de O_2 na válvula de alimentação específica para o equipamento e, de seguida, ligar o equipamento pressionando o botão lateral direito (**Figura 1**);

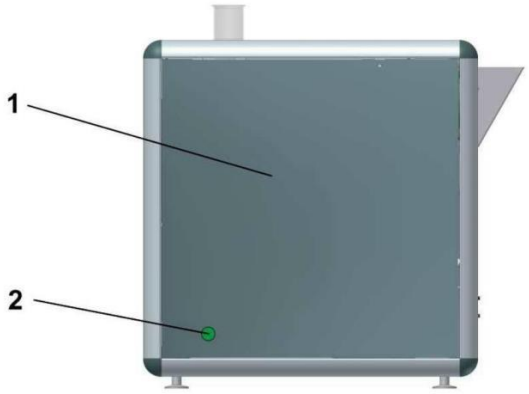


Figura 1 – Vista lateral direita do equipamento Vario TOC Select; 1 - Porta lateral direita; 2 - Botão principal.

4. Abrir o software do analisador;

5. Verificar se a ligação entre o analisador e o computador foi estabelecida. Caso não tenha sido, o software é executado em modo offline, o que pode ser confirmado no software, na área do ***status view***, tal como apresentado na **Figura 2**. Se isto se verificar, fechar o software e voltar a abrir para que se efetue a ligação.

Figura 2 - Vista do ***status view*** quando este se encontra em modo offline.

B. <u>Quando o equipamento tiver sido colocado em *standby* ("dormir"), proceder da seguinte forma:</u>

1. Abrir o fornecimento de O_2 do equipamento;

2. Abrir a janela da função ***Sleep/Wake up*** no software operacional através da seleção de ***Options*** > ***Settings*** > ***Sleep/Wake up***;

3. No botão ***wake up now*** o equipamento é reativado de forma imediata. O botão  presente na barra de ferramentas do software apresenta a mesma função.

NOTA: Se tiver sido anteriormente definida a hora (e dia se for o caso) para o ***Wake up*** do equipamento, este será reativado automaticamente à hora decidida sem que seja necessário realizar o ***Wake up*** manual.

2.3.2. Desligar o analisador

Caso o equipamento tenha uma utilização diária ou esteja parado durante um curto período de tempo (1 a 5 dias) pode ser ativada a função de ***Sleeping*** ao invés de desligar o equipamento, o que só é aconselhado em pausas superiores a 5 dias.

A. Para desligar o equipamento por curtos períodos de tempo, proceder da seguinte forma:

1. Abrir a janela da função ***Sleep/Wake up*** do software através da seleção de ***Options*** > ***Settings*** > ***Sleep/Wake up***;

2. No botão ***Sleep now*** o equipamento é desativado imediatamente;

3. Caso se queira programar a suspensão automática do analisador definir as condições (ex.: fornecimento de gás, temperatura tubo de combustão, altura da suspensão, etc.) na mesma janela e pressionar ***OK*** para fechar a janela e guardar as definições que foram alteradas. O equipamento é desligado e ativado novamente de acordo com as definições estabelecidas.

NOTA: Função ***Sleeping*** - durante intervalos de medição curtos (<5 dias) o equipamento e o computador permanecem ligados; apenas o fornecimento de gás O_2 é cortado, evitando perdas por possíveis fugas existentes.

B. Para desligar o equipamento por períodos de tempo longos (> 5 dias), proceder da seguinte forma:

1. Definir a temperatura do forno do tubo de combustão para 0 °C. Para isso, abrir a janela ***Instrument parameters*** acessível em ***Options***> ***Settings***> ***Parameters***;

2. Esperar 2-3 horas até que a temperatura do equipamento seja inferior a 55 °C;

3. Cortar o fornecimento de O_2 e, de seguida, sair do software em ***File***> ***Exit***;

4. Desligar o equipamento no botão lateral direito (**Figura 1**), os dispositivos periféricos e a fonte e alimentação. O equipamento está pronto para longas pausas.

2.3.3. Usar o software do analisador

Na **Figura 3** é apresentada a vista principal do software do equipamento com as diferentes áreas e barras funcionais.

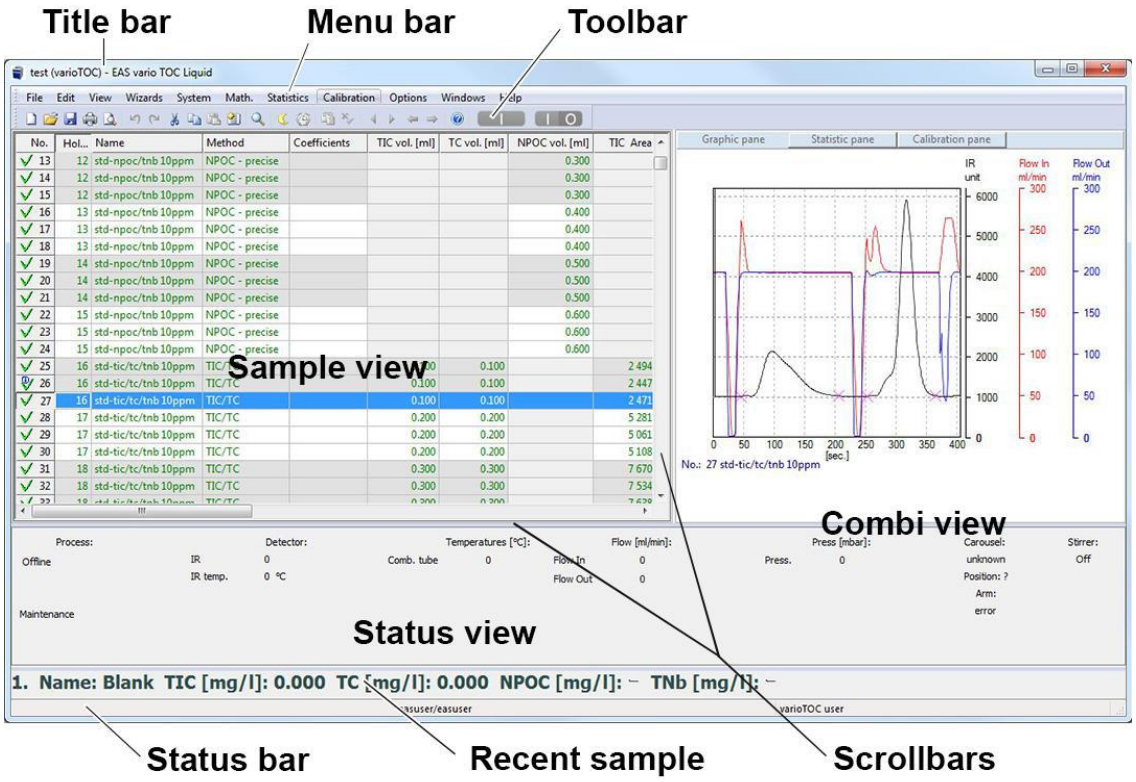


Figura 3 – Vista principal do software operacional com as várias áreas e barras funcionais.

O significado das várias áreas e barras funcionais é o seguinte:

Title bar (barra de título) – apresenta o nome do ficheiro que está a ser processado/aberto;

Menu bar (barra de menu) – onde estão acessíveis todas as funcionalidades do software;

Toolbar (barra de ferramentas) – permite o acesso rápido a funções e comandos que são requisitados com mais frequência (e.g. criar um novo documento, ativar o equipamento quando este se encontra no estado de Sleepling, etc.);

Combi view (área do gráfico) – exibe o desenvolvimento da análise da amostra atual ao longo do tempo para parâmetros como o sinal do detetor, o sinal da célula eletroquímica e o fluxo e pressão de O_2;

Status view (área de estado) – contém informação sobre o estado de operação do analisador;

Sample view (área das amostras) – assemelha-se a uma folha de cálculo tradicional e permite introduzir e obter informação de todas as amostras numa dada série. Nesta área do software é definida a sequência da análise das amostras/padrões, que deve corresponder à ordem em que estão organizadas no carrossel do equipamento. Na Tabela 1 estão descritas as funções das várias colunas desta área.

Tabela 1 – Nome das colunas de introdução de informação no software e respetiva função.

Nome da coluna	Função
Nome	Nome da amostra (e.g. ***Blank*** para os brancos).
Método	Método de análise usado na amostra correspondente.
Coeficientes	Atribuição de um conjunto de coeficientes para o cálculo realizado pelo equipamento (definir os coeficientes de ***default***).
Volume	Volume de injeção da amostra em mililitros.
Memo	Informação adicional ou características especiais da amostra a ser analisada (não é tida em conta pelo equipamento).
Fator diluição	Fator de diluição aplicado nas amostras – essencial para o desconto do branco (ponto **3.7.**).

Além disso, a informação inserida na área das amostras obedece a um código de cores, cujo significado está descrito na **Tabela 2**.

Tabela 2 - Código de cores do software e respetivo significado.

Código de cores	Significado
Fundo verde	Indica a amostra em análise no momento.
Texto vermelho	Indica a amostra com a ***stop tag***, isto é, onde a análise será interrompida.
Texto verde	Indica amostras de calibração ou de medição de rotina (e.g. brancos).
Texto preto	Identificativo de todas as outras amostras.

2.3.3.1. Realizar operações básicas no software

Exportar os dados de uma análise

É possível extrair uma análise realizada em formato Excel. Para isso, clicar na barra de menu em ***File*** > ***Export/Import*** > ***Create Excel sheet***.

Alterar o nº de injeções por vial

Para selecionar o nº de determinações por vial (e.g. triplo), ir à barra de menu, clicar em ***System*** > ***Feeding*** e selecionar o nº de determinações pretendido.

Alterar a posição do carrossel

Para mover o carrossel para outra posição, ir à barra de menu, clicar em ***System*** > ***Carousel position*** e indicar o nº da posição pretendida. Clicar em ***Ok***. O carrossel muda para a posição desejada.

2.3.4. Princípio base de funcionamento, calibração e determinação da concentração pelo analisador

Após o CO_2 resultante da oxidação das amostras entrar no detetor IR, é gerado um sinal elétrico de medição dependente do conteúdo da amostra em C e/ou N. O sinal é digitalizado e integrado, resultando num valor de área. Caso se verifique, o valor do branco é descontado e, dependendo do parâmetro em medição e do conjunto dos coeficientes selecionados, o conteúdo absoluto da amostra é calculado.

Para que haja confiança nos resultados fornecidos pelo equipamento, é necessário que esteja estabelecida uma relação entre o sinal do detetor do equipamento e o conteúdo da amostra em C e/ou N – calibração. **A Tabela 3** apresenta as gamas de calibração do equipamento para os vários parâmetros.

Tabela 3 – Parâmetros de medição e respetiva gama de calibração.

Parâmetro	Gama de calibração (µg)
TC	2 - 12
TIC	1 - 6
NPOC	0.3 - 3
TN_b	0.3 - 3

Para cada um dos parâmetros da **Tabela 3** estão estabelecidas as rectas de calibração através das quais é calculado pelo software o conteúdo em massa de cada

um dos parâmetros tendo em conta a área. As várias rectas de calibração estão definidas de acordo com a fórmula:

$$y = a + b \cdot x + c \cdot x^2 + d \cdot x^3 + e \cdot x^4$$

Y= massa do elemento (mg);

a-e = coeficientes de calibração;

x = área do pico.

A partir do valor de massa calculado e do respetivo volume de injeção, a concentração da amostra é dada pela seguinte fórmula:

$$c = \frac{a \cdot 1000000}{v}$$

c = concentração do parâmetro (mg/l);

a = conteúdo absoluto do parâmetro (mg);

v = volume de injeção (µl).

Os coeficientes usados para o cálculo em cada um dos parâmetros estão disponíveis a partir da barra de menu em ***Calibration*** > ***Coefficients***. Nessa janela, para cada um dos parâmetros, são ainda indicados os valores das áreas mínima e

máxima da calibração. Na **Figura 4** é apresentada a janela do software referente aos coeficientes de TIC.

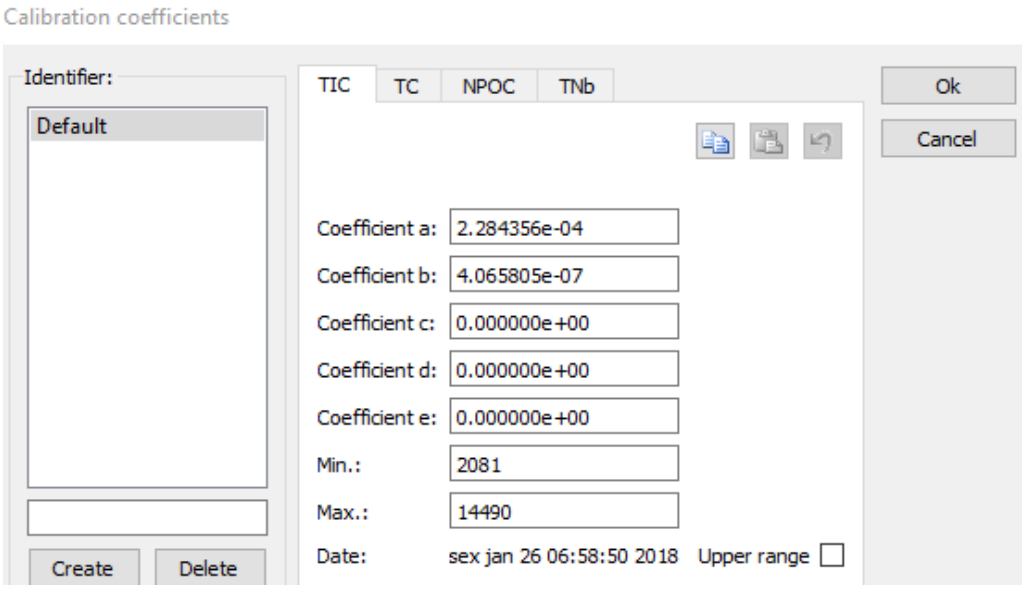


Figura 4 – Separador do parâmetro TIC na janela Calibration Coefficients.

2.3.5. Métodos de análise

Para que o equipamento leia uma amostra, quer seja de branco, padrão ou amostra, é necessário definir no software qual o método a usar. Vários métodos de análise estão pré-definidos no software. A cada método está associado a um conjunto de coeficientes que definem a reta que será usada no cálculo de interpolação da área calculada em massa do parâmetro em análise. Na Tabela 4 estão definidos os métodos mais usados, assim como a indicação do tipo de coeficientes usados na interpolação.

Mais informação relativa às definições destes e de outros métodos podem ser consultadas acedendo a ***Options*** > ***Settings*** > ***Methods*** (**Figura 5**). Na janela ***Methods*** podem ainda ser definidos novos métodos consoante as necessidades do utilizador. Para isso, clicar em ***New*** e introduzir os parâmetros do novo método no lado direito da janela. Para finalizar, clicar em ***Create*** e o nome do método aparecerá na área esquerda da janela e pode ser usado para análise de amostras.

Tabela 4 – Nome dos métodos de análise, sua descrição e coeficientes associados.

Método	Descrição	Coeficientes associados
TC_RAIZ	A amostra é diretamente injetada no forno, não se fazendo distinção entre TOC e TIC.	TC
TOC	Mera medição de TC. A amostra é diretamente injetada no forno, logo o TIC deverá ser removido antes da análise ou não ser representativo na amostra (<LQ) (método direto).	TC
*NPOC**	Mera medição de TC. A amostra é diretamente injetada no forno. Difere do método *TOC* pois inclui acidificação das amostras antes da análise.	NPOC
TIC	O TIC é purgado no *sparger* por acidificação. Método usado quando se pretende analisar pelo método da diferença (TC-TIC=TOC).	TIC
TIC/TC	O TIC é purgado no *sparger* e medido; TC é determinado por injeção direta no forno. Da diferença entre eles resulta o TOC (TC-TIC=TOC).	TIC e TC

Com o carrossel é possível acidificar as amostras de forma automática no início da análise. Colocar um vial com 10 % (m/v) HCl na posição nº 32 do carrossel, posição que está definida em ***Parameters e que é possível alterar, se necessário. A agulha desloca-se até à posição definida, retira ácido que depois injeta em cada um dos vials cujo método de análise for o NPOC. Após o último vial ser acidificado, decorre 1 minuto até

o início da análise. Caso não seja possível realizar a acidificação de forma automática, acidificadar as amostras para pH < 2 com um ácido adequado (e.g. HCl) antes da medição no devido tempo.

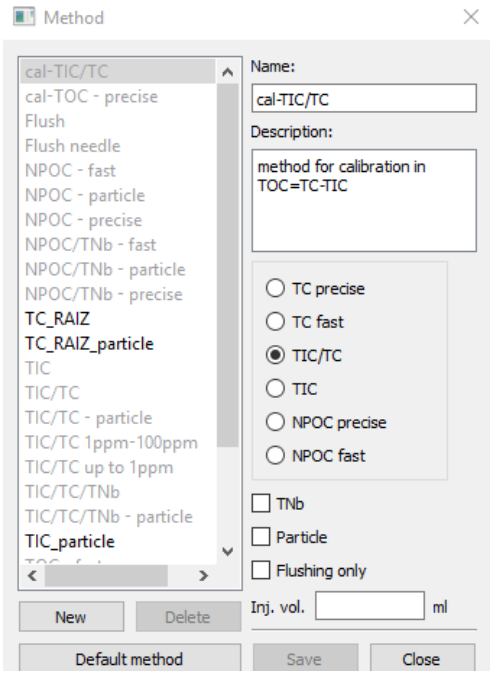


Figura 5 - Vista da janela ***Method***, onde podem ser visualizados e criados métodos de análise.

2.3.5.1. Extensões e especificações dos métodos

Na janela ***Method***, nos parâmetros de cada método, existem algumas especificações e a possibilidade de adicionar extensões que fazem variar a análise (**Figura 5**). Na **Tabela 5** são apresentadas as especificações/extensões com mais detalhe.

Tabela 5 – Especificações e extensões dos métodos de análise.

Especificação	**Extensão**	
Precise Vs *Fast*	*Particle*	Determinação TN_b
Em ambas, a amostra é injetada diretamente no forno, mas no *precise* a primeira medição corre em segundo plano (*dummy peak*) e não é avaliada nem tida em conta nos cálculos. Assim, evita-se quase completamente, que resquícios das medições anteriores possam afetar a medição da amostra seguinte, mas é mais demorada que a *fast* (15 Vs 12 minutos). **NOTA**: os métodos com a especificação *fast*	Ativa a rotação de um íman por baixo da posição da amostra em análise; por introdução de um magneto no *vial* da amostra, cria agitação e evita a sedimentação das partículas em	Determinação simultânea do azoto total. **NOTA**: este tópico será abordado mais à frente.

são úteis para realizar as leituras de limpeza entre amostras e padrões, uma vez que o seu valor de área não é tão relevante.	suspensão. **NOTA**: função controlada automaticamente.	

2.3.6. Parâmetros de análise do equipamento

Para além das definições associadas a cada um dos métodos de análise existentes no software (**Figura 5**), há ainda alguns parâmetros do equipamento que são independentes do método e estão pré-estabelecidos. São essenciais à análise e compreendem a temperatura de operação do forno, os tempos de integração, o fluxo e pressão do O_2, os parâmetros do detetor e da célula eletroquímica, a velocidade de enchimento e de injeção da seringa e o doseamento de ácido no ***sparger***. É possível consultá-los e alterá-los em ***Options*** > ***Settings*** > ***Device Parameters***.

2.3.7. Constituição, cálculo e forma de desconto do branco

A determinação do valor do branco pelo equipamento faz parte do trabalho de rotina. Este valor é calculado para desconto da área relativa à preparação das amostras (água de diluição). Como todos os padrões e diluições das amostras a analisar pelo equipamento são preparados com água millipore, as amostras de branco devem ser constituídas unicamente pela água millipore usada para preparação das amostras e padrões.

Para o cálculo do valor do branco no modo de análise de líquidos, o software identifica as amostras de branco através da palavra-chave que está definida para este tipo de amostras – ***Blank***. Esta pode ser alterada a qualquer altura em ***Options*** > ***Settings*** > ***Keywords***. Os brancos devem ter o mesmo método de análise que as amostras, uma vez que é calculado um valor de branco separado para cada método.

Após análise dos brancos, o software calcula a média das áreas dos picos dos brancos e divide pelo valor do volume de injeção (o volume de injeção deve ser igual para todos os brancos!), uma vez que as amostras podem não ter o mesmo volume de

injeção dos brancos. Desta forma, é obtida uma ***taxa do valor do branco*** de acordo com a fórmula seguinte:

$$\bar{r} = \frac{\sum b_i}{n \cdot v}$$

$\bar{r}$ = taxa do valor do branco;

b_r = áreas dos picos dos brancos;

n = nº de brancos;

v = volume de injeção dos brancos (ml);

i = índice de 1 até n.

Após o cálculo da taxa do valor do branco, as áreas das amostras são compensadas da seguinte forma:

$$a_{comp} = a - \bar{r} \cdot v$$

a_{comp} = área compensada do pico da amostra;

a = área bruta do pico da amostra;

$\bar{r}$ = taxa do valor do branco;

v = volume de injeção da amostra (ml).

A área que resulta da medição e que é inserida na coluna respetiva do software diz respeito à área bruta da amostra, ou seja, sem o desconto do valor do branco. Só o valor da concentração correspondente é que é calculado através da área compensada, tendo em conta o valor do branco. Para isso, é necessário introduzir no software o fator de diluição aplicado à amostra na coluna respetiva, como indicado na **Figura 6**. Caso não seja inserido qualquer valor referente ao fator de diluição, o software assume que a amostra não sofreu diluição e, portanto, não desconta nenhum valor referente ao branco.

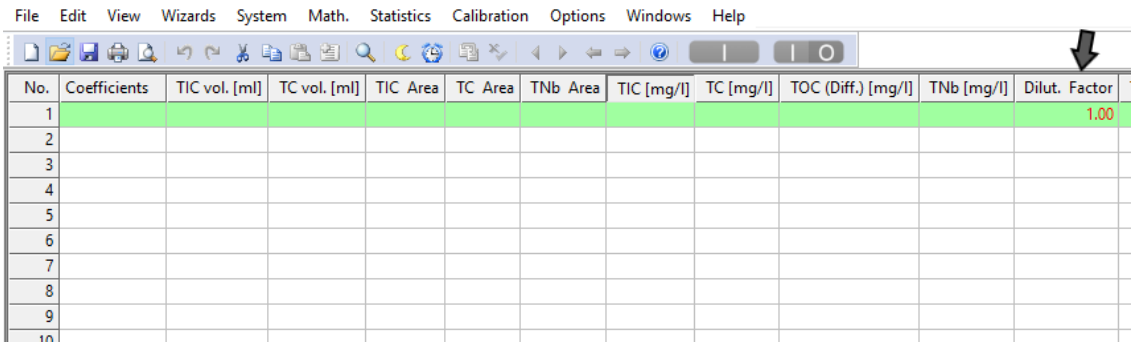


Figura 6 – Vista do software de análise com a indicação da coluna do fator de diluição.

No caso das amostras, deve ser inserido o fator de diluição na coluna respetiva para evitar realizar o cálculo de desconto do branco fora do software e, portanto, aumentar as hipóteses de acrescentar erro ao valor final da concentração. O mesmo não se aplica à leitura dos padrões, uma vez que o valor lido corresponde ao valor final. Nesse caso, e como o que seria corrigido com o valor da taxa do branco seria a concentração, deve realizar-se a correção das áreas dos padrões fora do software.

Ainda que a forma de cálculo do valor de desconto do branco seja sempre a referida acima, o número de brancos escolhidos para o cálculo é dependente do método selecionado:

- ***Sequencially*** – uma taxa de área do branco é calculada por cada grupo de amostras de branco tendo em conta o método (e.g. NPOC – precise); o valor do branco para cada amostra é introduzido na respetiva coluna até ao próximo grupo de amostras de brancos. Este é o método de cálculo atualmente selecionado no analisador do RAIZ.
- ***Entirely*** – todas as amostras de branco entram no cálculo da taxa de valor do branco; este valor é depois aplicado a todas as amostras.

A **Figura 7** corresponde à janela do software onde as opções anteriores estão acessíveis. Para aceder a esta janela ir a ***Options***> ***Settings***> ***Calculation***. Para além do número de brancos incluídos no cálculo, na mesma janela (**Figura 7**) há ainda a possibilidade de escolher se o cálculo é efetuado de forma manual ou automática:

- **Automática** – um valor do branco é calculado automaticamente para cada amostra, tendo em conta o método selecionado e a área das amostras de branco na memória do software;
- **Manual** – o cálculo do valor do branco não é realizado, mesmo que haja amostras de branco na série. Esta opção permite que o utilizador insira

manualmente o valor do branco para amostras individuais (e.g. quando já se conhece à priori o valor do branco de uma amostra).

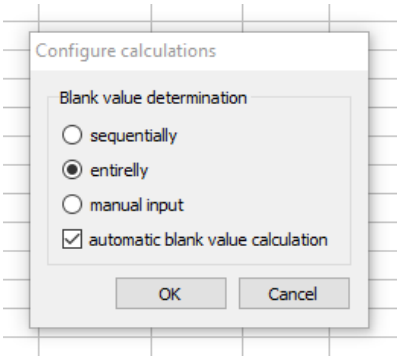


Figura 7 – Vista da janela do software onde estão acessíveis os métodos de determinação do branco. NOTA: O modo Sequentially em automático é o método selecionado atualmente no analisador de TOC do RAIZ.

Após intervalos prolongados entre medições, trabalho de manutenção ou análise de amostras muito concentradas, o primeiro valor de branco é sempre mais alto e não deverá ser considerado para o cálculo do valor da taxa do branco. Usar um nome diferente para essa amostra de forma a não ser identificada pelo software (e.g. Limpeza).

2.4. Utilização diária do Vario TOC Select

A utilização diária do analisador no modo de líquidos (atual) compreende algumas tarefas, como:

- o condicionamento do equipamento (opcional);
- determinação dos brancos do equipamento (Ponto **2.3.7.**);
- calibração consoante o método.

O condicionamento do equipamento é realizado com as soluções padrão no início de cada análise, caso se justifique. Estas amostras são de verificação das condições do equipamento, servindo para confirmar que a análise procede de forma correta, o fluxo e a pressão dos gases, etc, não interessando propriamente o valor lido pelo equipamento.

Uma análise de rotina pode processar-se da seguinte forma:

- Realizar a baseline do equipamento em ***Options*** > ***Diagnostics*** > ***Baseline recording***;
- selecionar o método a usar (e.g. NPOC-precise);

- caso haja necessidade, analisar uma amostra de condicionamento (e.g. padrão de calibração/controlo), colocá-la na posição nº 1 do amostrador automático;
- se o condicionamento do equipamento tiver sido realizado, analisar na posição nº 2 uma amostra de limpeza (*i.e.* água millipore) para evitar possíveis efeitos de arrastamento;
- do nº 3 ao nº 5, analisar 3 amostras de branco (*i.e*. millipore);
- a partir da posição nº 6 podem-se fazer correr os padrões de calibração e/ou controlo (Ponto **2.3.10.**), consoante o método da análise, e depois então fazer correr as amostras.

NOTA: a ***Hole position*** (primeira coluna do software do equipamento) é gerada automaticamente e corresponde à posição da amostra no carrossel, enquanto que a coluna do lado esquerdo numerada diz apenas respeito à ordem da análise. Ter atenção porque esta é gerada automaticamente e nem sempre a ordem pela qual é gerada está de acordo com a posição anterior.

2.4.1. Substâncias padrão e preparação das soluções stock

O equipamento está calibrado de fábrica para os vários parâmetros com base nas substâncias definidas como substâncias-padrão para cada parâmetro. As substâncias definidas são consideradas substâncias de referência para o parâmetro em questão. Para consultar as substâncias padrão que estão definidas abrir a janela ***Standard samples*** em ***Options*** > ***Settings*** > ***Standards***.

Adicionalmente, a preparação das soluções stock para efeitos de calibração do equipamento seguem a norma europeia para a determinação de TOC em água, EN 1484:1997.

IMPORTANTE: Usar água millipore fresca em cada análise para os brancos, padrões e amostras. Não usar água que esteja contida há mais de um dia no esguicho.

2.4.1.1. Solução stock para a determinação de carbono orgânico – 1000 mg/l

Dissolver, num balão volumétrico de 1000 ml 2.125 g de hidrogeno ftalato de potássio ($C_8H_5KO_4$) seco 1 h entre 105 e 120 °C (estufa) e perfazer o volume com água millipore. Esta solução é estável por 2 meses se armazenada bem fechada e no frigorífico.

2.4.1.2. Solução stock para a determinação de carbono inorgânico – 1000 mg/l

Dissolver, num balão volumétrico de 1000 ml 4.415 g de carbonato de sódio (Na_2CO_3) seco 1h à temperatura de 285 °C. Adicionar 3.500 g de hidrogeno carbonato de sódio ($NaHCO_3$) seco 2h em exsicador e perfazer o seu volume com água millipore. A solução é estável à temperatura ambiente durante duas semanas.

2.4.1.3. Calibração diária

Como referido no ponto **2.3.4.**, o equipamento possui uma gama em que está calibrado para cada um dos parâmetros. Para efeitos de controlo da calibração que está estabelecida, antes da análise de amostras são corridos os padrões de calibração e de controlo, tendo em conta o método e a forma como foi realizada a calibração de fábrica. Antes da realização da calibração, existem algumas predefinições que há que ter em conta:

- volume de injeção de brancos e amostras de limpeza: 0.6 ml;
- 1 única injeção por vial dos brancos e amostras de limpeza;
- antes da realização de uma análise, devem ser sempre corridos 3 brancos por cada método que seja usado na análise das amostras/padrões;
- amostras/padrões são sempre analisados com 3 injeções/vial.

2.4.1.4. Calibração pelo método direto – NPOC

Para o método NPOC, a calibração é realizada do seguinte modo:

1. Pipetar 2 ml da solução **2.3.9.1.** para um balão de 200 ml, perfazer o seu volume com água millipore e homogeneizar. Esta solução tem uma concentração de NPOC de 10 mg/l, correspondendo ao último padrão da curva de calibração (3 µg);
2. A partir da solução preparada no ponto anterior preparar os padrões 0.24, 0.3, 0.6, 1.2, 1.8 e 2.4 µg pipetando 2, 5, 5, 10, 15 e 20 ml para balões de 25 ml, exceto para o padrão 0.3 µg, em que é usado um balão de 50 ml. As concentrações finais de cada padrão são 0.8, 1, 2, 4, 6, 8 e 10 mg/l.
3. Preparar, a partir da solução **2.3.9.1.** de controlo, um padrão de 10 mg/l de NPOC (tal como no ponto **1.**) e por diluição preparar os padrões de controlo 0.24 e 0.3 µg pipetando os mesmos volumes que no ponto **2.**;

4. Usar 1 vial para cada uma das soluções padrão e de controlo preparadas anteriormente, assim como para cada um dos 3 brancos e para as amostras de limpeza. Colocar uma amostra de limpeza na posição nº 1 do carrossel, de seguida os brancos e depois os 7 e os 3 vials relativos aos padrões de calibração e controlo, respetivamente;
5. Preencher a folha de cálculo do software com a mesma ordem das amostras no carrossel, tal como apresentado na **Tabela 6**.

Tabela 6 – Indicações de preenchimento da folha do software para realização da curva de calibração de NPOC.

Nº vial	Nome	$V_{Injeção}$ (ml)	Nº injeções/vial	Conteúdo	Método	Coeficientes
1	Limpeza	0.6	1	Água millipore	NPOC-fast	*Default*
2-4	*Blank*	0.6	1	Água millipore	NPOC-precise	*Default*
5-12	P0.24 - 3 µg	0.3	3	0.8mg/l→0.24 µg 1mg/l→0.3 µg 2mg/l→0.6 µg 4mg/l→1.2 µg 6mg/l→ 1.8µg 8mg/l→2.4 µg 10mg/l→3 µg	NPOC-precise	*Default*
13	Limpeza	0.6	1	-	NPOC-fast	*Default*
14-16	PC0.24, PC0.3 e PC3 µg	0.3	3	0.2ml→0.6 µg 0.1ml→2 µg 0.6ml→12 µg	NPOC-precise	*Default*
15	Limpeza	0.6	1	-	NPOC-fast	*Default*

6. Quando se dá início à análise, abre-se uma janela no software que pergunta se queremos acidificar as amostras com o método NPOC, como apresentado na **Figura 8**. Neste caso, não é necessário, uma vez que se tratam de padrões que só são constituídos pela fração de carbono orgânico.

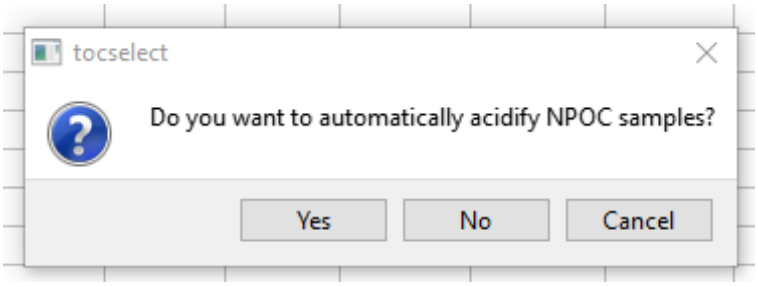


Figura 8 – Janela do método NPOC.

2.4.1.5. Calibração pelo método da diferença – TC e TIC

Para os métodos TC e TIC, a calibração é realizada do seguinte modo:

1. Pipetar 2 ml da solução **2.3.9.1.** e 2 ml da solução **2.3.9.2.** para um balão de 200 ml, perfazer o seu volume com água millipore e homogeneizar. Esta solução padrão tem uma concentração de 20 mg/l e 10 mg/l de TC e TIC, respetivamente;
2. Pipetar 30 ml do padrão TIC/TC preparado em **1.** para um balão de 200 ml, perfazer o volume com água millipore e homogeneizar. Este padrão de calibração diluído (1.5/3 mg/l de TIC/TC) é para realizar as determinações 0.6 e 1.05 µg de TC e 0.3 e 0.6 µg de TIC;
3. Preparar o padrão de controlo TIC/TC de igual forma que a solução **1.**; a partir dessa preparar o padrão de controlo diluído como em **2.**;
4. Como a calibração é realizada a partir da mesma solução usando diferentes volumes de injeção, distribuir a solução preparada em **1.** por 6 vials (padrões de TC: 2, 4, 6, 8, 10 e 12 µg) diferentes, a solução preparada em **2.** por 2 vials e encher 7 vials com água millipore para os brancos e a amostra de limpeza inicial;
5. Colocar a amostra de limpeza na posição nº 1 do carrossel, de seguida os brancos, depois os 2 vials relativos ao padrão diluído e os primeiros 6 vials com o padrão de calibração TIC/TC;
6. Preencher a folha de cálculo do software com a mesma ordem das amostras no carrossel tal como apresentado na **Tabela 7**. A partir do vial da posição nº 31 podem ser colocados os vials das amostras.

Tabela 7 – Indicações de preenchimento do software para realização das curvas de calibração de TC e TIC.

Nº vial	Nome	$V_{Injeção}$ (ml)	Nº injeções/vial	Conteúdo	Método	Coeficientes
1	Limpeza	0.6	1	Água millipore	TC-RAIZ	*Default*
2-4	*Blank*	0.6	1	Água millipore	TC-RAIZ	*Default*
5-7	*Blank*	0.6	1	Água millipore	TIC	*Default*
8-9	P0.6 - 1.05 µg	0.2 - 0.350	3	0.2ml→0.6 µg 0.350ml→1.05 µg	TC-RAIZ	*Default*
10 - 15	P2 - 12 µg	0.1-0.6	3	0.1ml→2 µg 0.2ml→4 µg 0.3ml→6 µg 0.4ml→8 µg 0.5ml→10 µg 0.6ml→12 µg	TC-RAIZ	*Default*

16	Limpeza	0.6	1	Água millipore	TC-RAIZ	*Default*
17-19	PC0.6, PC2 e PC12 µg	0.2, 0.1 e 0.6	3	0.2ml→0.6 µg 0.1ml→2 µg 0.6ml→12 µg	TC-RAIZ	*Default*
20	Limpeza	0.6	1	Água millipore	TC-RAIZ	*Default*
21-22	P0.3 – 0.6 µg	0.2 - 0.4	3	0.2ml→0.3 µg 0.4ml→0.6 µg	TIC	*Default*
23-28	P1 – 6 µg	0.1-0.6	3	0.1ml→1 µg 0.2ml→2 µg 0.3ml→3 µg 0.4ml→4 µg 0.5ml→5 µg 0.6ml→6 µg	TIC	*Default*
29	Limpeza	0.6	1	Água millipore	TIC	*Default*
30	PC0.3, PC1 e PC6 µg	0.2, 0.1 e 0.6	3	0.2ml→0.3 µg 0.1ml→1 µg 0.6ml→6 µg	TIC	*Default*
31	Limpeza	0.6	1	Água millipore	TIC	*Default*

7. De forma a que não passe muito tempo desde que são colocados no carrossel até que sejam analisados, os padrões de controlo de TC e os vials relativos à calibração e controlo de TIC devem ser colocados no carrossel à medida que forem sendo analisados os primeiros vials.

2.5. Manutenção do Vario TOC Select

Algumas partes do equipamento requerem manutenção ou substituição. Para alguns componentes existem intervalos de tempo pré-definidos para a realização dessa manutenção, que podem ser visualizados e alterados em ***Options*** > ***Maintenance*** > ***Intervals***. Esta informação também está acessível no canto inferior esquerdo na visão geral do software em ***Maintenance***. Aí é apresentada a atribuição correspondente a cada parâmetro, bem como a contagem percentual sob a forma de uma barra realçada.

O trabalho de manutenção requer sempre a preparação do equipamento relativamente à pressão do O_2.

Antes da realização do trabalho de manutenção proceder da seguinte forma:

- abrir a janela ***Replace part*** do software em ***Options*** > ***Maintenance*** > ***Replace part*** (**NOTA**: Esta função apenas corta o fornecimento de O_2 ao equipamento);
- aguardar a queda da pressão do O_2 sem pressionar nenhum botão até que apareça a mensagem ***Part can now be replaced*** e o botão ***Finish*** fique acessível (**NOTA**: a pressão foi reduzida quando os campos ***Flow*** (fluxo) e ***Pressure*** (pressão) sejam iguais a zero);

- O componente pode ser então substituído.

Após a realização do trabalho de manutenção proceder da seguinte forma:

- fechar a janela ***Replace part*** clicando em ***Finish***. O O_2 será ligado novamente de forma automática;
- verificar os valores de pressão e fluxo do O_2. Se:
  - os valores estiverem de acordo com os valores anteriores à substituição (pressão ≈ 1000 mbar; fluxo ≈ 200 ml/min) o analisador está pronto a ser usado;
  - os valores após a substituição forem significativamente diferentes dos iniciais realizar um teste de fuga (ponto **2.4.5.**) → o equipamento só estará pronto para uso novamente quando o teste for bem-sucedido.

2.5.1. Solução 10 % (m/v) de ácido fosfórico

Pipetar, para um frasco de vidro graduado de 500 ml, 35 ml (≈34.6 ml) de ácido fosfórico (H_3PO_4) 85 % (m/m). Perfazer o volume de 500 ml com água millipore e homogeneizar. Para encher o reservatório do ácido, desapertar a tampa de rosca frontal do recipiente e usar um funil pequeno. No final alterar o intervalo de manutenção respetivo.

NOTA: Esta é a solução ácida para efetuar a reação do TIC e não é de concentração rigorosa.

2.5.2. Ash finger

O ***ash finger*** do equipamento consiste num tubo de vidro, com fundo, aberto no topo e com ranhuras nas paredes. É neste componente que são acumuladas as cinzas resultantes da combustão das amostras/padrões, necessitando por isso de manutenção, pois o excesso de cinzas pode distorcer as leituras realizadas. O equipamento indica a necessidade de manutenção deste componente na janela do canto inferior esquerdo do software do equipamento.

Para realizar a mudança do ***ash finger*** proceder da seguinte forma:

1. Preparar o equipamento relativamente à pressão do O_2;
2. Retirar o tampo superior do equipamento (só levantar);
3. Retirar o carrossel. Desaparafusar os dois parafusos que o seguram e retirá-lo. O equipamento fica como apresentado na **Figura 9**;

Figura 9 – Vista da parte superior do equipamento sem o tampo e o carrossel.

4. De seguida, desapertar e retirar os dois parafusos que seguram o complexo da válvula e retirar o grampo que une o tubo de alimentação de O_2 ao tubo de combustão (**Figura 10**);

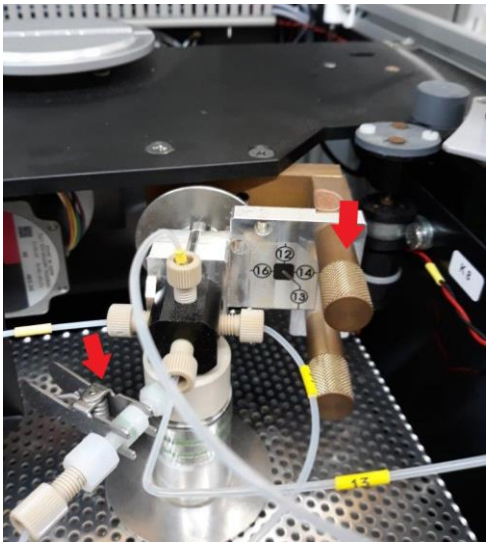


Figura 10 – Complexo da válvula de injeção.

5. Retirar o complexo da válvula e encaixá-lo na peça metálica (posição de ***parking***) como apresentado na **Figura 11**. O topo do tubo de combustão fica então aberto;

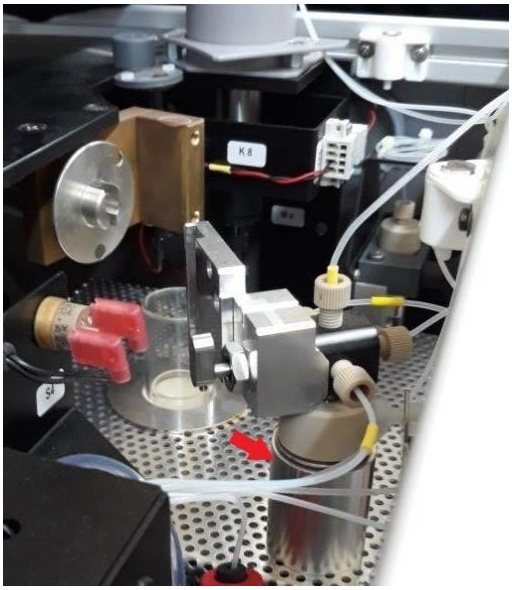


Figura 11 – Complexo da válvula na posição de parking.

6. Antes do ***ash finger*** o tubo de combustão possui no seu interior o tubo espaçador. Retirá-lo com a pinça do equipamento;
7. De seguida, o ***ash finger*** pode ser retirado com a pinça (**Figura 12**). **NOTA:** o ***ash finger*** é reutilizável caso não esteja partido;

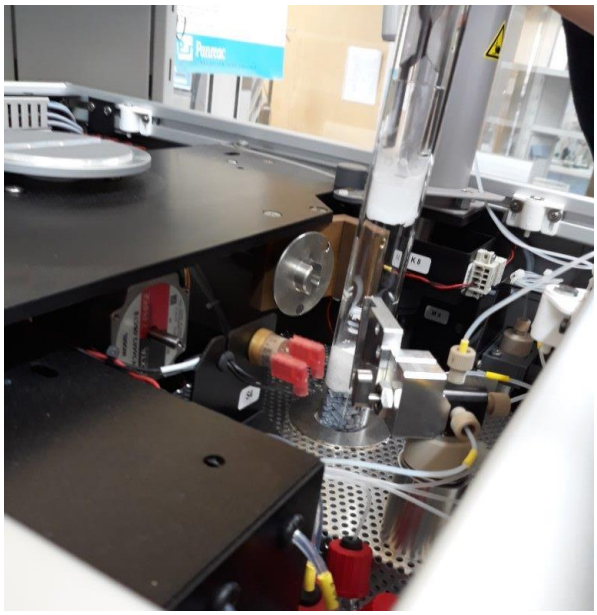


Figura 12 – *Ash finger* a ser retirado do equipamento.

8. Se o ***ash finger*** estiver partido, retirar todos os estilhaços de vidro de forma a não afetar o conteúdo do tubo de combustão (ver remoção do tubo de combustão no ponto **2.4.3.1.**). Verificar o conteúdo em reagentes do tubo. Se as chips de

quartzo ou o catalisador apresentarem desgaste, mudar o conteúdo do tubo. Se tal se verificar, proceder do seguinte modo:

8.1. Colocar o conteúdo do tubo num recipiente;

8.2. Lavar o tubo com água millipore para tentativa de remoção do possível quartzo que possa estar nas paredes;

8.3. Limpar o tubo e voltar a enchê-lo, tendo em conta as alturas das várias camadas (ponto **2.4.3.2.**);

9. Colocar o novo ***ash finger*** dentro do tubo de combustão e, de seguida, o espaçador;
10. Colocar o tubo de combustão dentro do forno e colocar novamente o grampo que une a esfera na extremidade inferior do tubo à junta tubo do condensador;
11. Encaixar o complexo da válvula na extremidade superior do tubo de combustão e com isto verificar se o enchimento total do tubo tem a altura correta (parte do complexo da válvula fica dentro do tubo de combustão). Roscar os parafusos que o suportam;
12. Voltar a colocar o amostrador no topo do equipamento, apertar os dois parafusos que o seguram (atenção à posição de cada um!) e encaixar o tampo superior do equipamento.
13. Fechar a janela ***Replace part***, clicando em ***Finish***. O O_2 será ligado novamente de forma automática. Verificar a necessidade ou não de realização de um teste de fuga pelos valores de pressão e fluxo do O_2;
14. Como este componente tem um intervalo de manutenção definido, redefinir manualmente o campo ***status*** para zero na caixa de diálogo ***Maintenance intervals***.

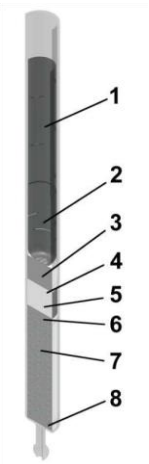
NOTA: Após a mudança do ***ash finger***, os valores dos brancos são geralmente mais elevados. Fazer correr alguns vials com água millipore, antes de realizar qualquer análise.

2.5.3. Tubo de combustão

A manutenção deste componente é essencial para a obtenção de bons resultados, uma vez que é responsável pela combustão da amostra para determinação do carbono total e orgânico. O tubo de combustão encontra-se à temperatura de 850 °C, portanto, caso a substituição do componente se faça através da função ***Replace part***, atentar à temperatura elevada em que se encontra o equipamento. Para evitar possíveis riscos, neste caso, a mudança pode ser realizada com o equipamento desligado (ponto **2.3.2. B**).

2.5.3.1. Remoção do tubo de combustão

NOTA: No caso de ter optado pela opção ***Replace part***, manter por perto do equipamento um suporte de tubos pronto para colocar os componentes quentes.



<u>Procedimento de remoção do tubo de combustão</u>:

1. Abrir a porta frontal do equipamento. Remover o carrossel e a placa de cobertura;
2. Desapertar os dois parafusos que fixam o complexo da válvula *multiway* e retirá-lo para fora do tubo de combustão de forma cuidadosa;
3. Retirar a mola que une a esfera da extremidade inferior do tubo de combustão à junta de encaixe do tubo do condensador, puxar o tubo de combustão para fora do forno e colocá-lo no suporte;
4. A remoção do tubo está concluída.

2.5.3.2. Preenchimento, instalação e condicionamento do tubo

<u>Procedimento de preenchimento do tubo de combustão</u>:

1. Inserir uma camada de lã de quartzo (8);
2. Preencher com as chips de quartzo (7);
3. Inserir uma camada de lã de quartzo (6) bem apertada;
4. Preencher com o catalisador de platina (5);
5. Inserir uma camada de lã de quartzo (4) bem apertada;
6. Preencher com chips de quartzo (3);
7. Inserir o ***ash finger*** (2) e, se necessário, o tubo de proteção (1).

<u>Instalação do novo tubo de combustão</u>:

1. Limpar as possíveis impressões digitais na parte exterior do tubo, prevenindo assim o seu desgaste. Usar acetona, por exemplo;
2. Inserir o novo tubo de combustão no forno;
3. Conectar a esfera na extremidade do tubo de combustão à junta de encaixe do tubo do condensador e colocar o grampo. **NOTA**: quando se fecha uma

junta esférica e de encaixe, deve sentir-se uma resistência elástica quando se pressiona o ***o-ring***. Caso não se verifique, deve substituir-se o ***o-ring***;

4. Encaixar a válvula *multiway* na extremidade superior do tubo de combustão e verificar se o enchimento do tubo de combustão foi realizado de forma adequada. **Atenção**: inserção inadequada da unidade de injeção pode causar a quebra do vidro do tubo;
5. Prender o complexo da válvula com os dois parafusos que o seguram;
6. Voltar a colocar a tampa superior do equipamento e o carrossel. Se este estiver ligado, realiza um reconhecimento automático do carrossel; esperar até estar concluído;
7. Mudança do tubo está concluída. Fechar a janela ***Replace part***, clicando em ***Finish***. Esperar o tempo de condicionamento do novo tubo (30 min à temperatura de trabalho);
8. Verificar se os valores de fluxo e pressão do O_2 se encontram semelhantes aos valores anteriores à mudança do tubo. Se não, efetuar um teste de fuga (ponto **2.4.5.**) antes de realizar qualquer análise.

Condicionar o tubo de combustão recém-instalado:

O condicionamento é essencial pois sem a sua realização os resultados das análises serão afetados. Serve para:

- Remover alguma humidade que possa estar no novo tubo;
- Remover contaminações.

Existem duas formas de condicionar o novo tubo instalado:

1. Fazer a lavagem dos tubos com água millipore (alternativamente com 0.8 % HCl) e um elevado volume de injeção;
2. Aquecer os tubos para remover possíveis contaminações e humidade no conteúdo do tubo.

O **condicionamento** de tubos recém-instalados passa pelas 2 formas, aquecer os tubos à temperatura de trabalho (T=850 °C no modo de líquidos) e lavar/fazer passar

um volume de injeção de água millipore elevado (ex.: durante a noite), uma vez que os brancos obtidos com o novo catalisador são normalmente elevados.

2.5.4. Tubo de absorção de água, de halogénios e tubo de filtro

Os três tipos de tubos de absorção têm a função de remover água, halogénios e evitam ainda que a sujidade se deposite em partes importantes do equipamento. Na **Figura 13** é apresentada a localização dos mesmos no equipamento. Uma vez que possuem uma estrutura semelhante, a sua manutenção vai ser abordada de forma conjunta.

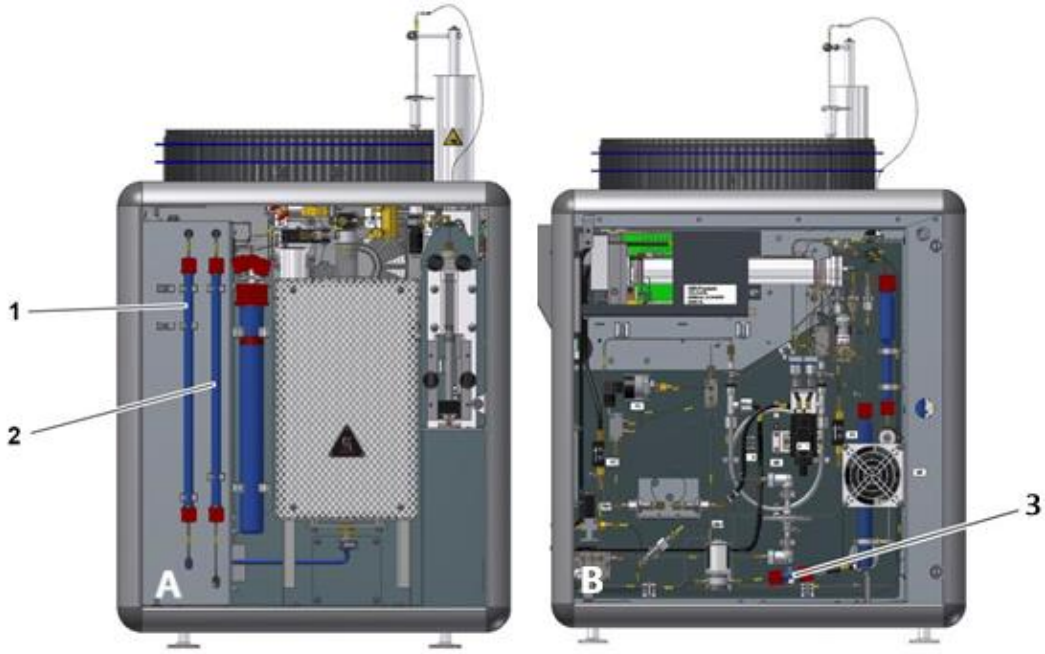


Figura 13 – Representação esquemática da localização dos tubos de absorção no analisador. A - vista frontal; B - vista lateral; 1 - tubo de absorção de halogénios; 2 - tubo de absorção de água; 3 - tubo de filtro.

2.5.4.1. Tubo de absorção de água (*Drying tube*)

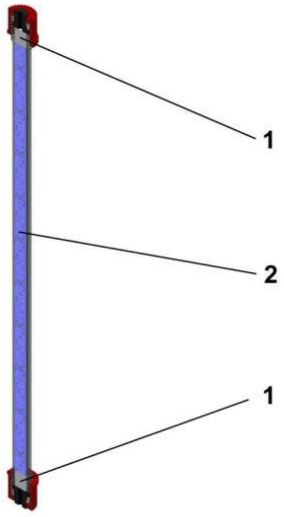


Figura 14 – Representação esquemática do tubo de absorção de água; 1 - filtro; 2 - aquatak.

Para realizar a mudança do tubo de absorção de água, proceder da seguinte forma:

1. No fundo do tubo inserir um filtro;
2. Preencher o tubo com o agente de secagem (aquatak);
3. Finalizar o preenchimento do tubo com um filtro no topo.

NOTA: O intervalo de manutenção para o tubo de absorção de água está definido e pode ser consultado no software do equipamento. No entanto, não deve ter-se só em conta este intervalo. A necessidade de manutenção do tubo de absorção de água é também realizada por observação do seu conteúdo. O aspeto normal é granular. Se este apresentar qualquer tipo de dissolução/aspeto pastoso, realizar manutenção, caso contrário poder-se-á usar o tubo para além do intervalo definido. O enchimento com o agente de secagem deve ser compacto, evitar espaços não preenchidos e colocar a sua altura o mais exata possível.

2.5.4.2. Tubo de absorção de halogénios (absorption tube)

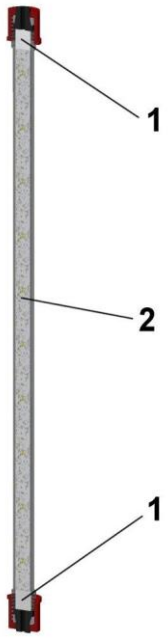


Figura 15 – Representação esquemática do tubo de absorção de halogénios; 1 – filtro; 2 - lã de bronze**.**

Para realizar a mudança do tubo de absorção de halogénios, proceder da seguinte forma:

1. No fundo do tubo inserir um filtro;
2. Preencher o tubo com a lã de bronze;
3. Finalizar o preenchimento do tubo com um filtro.

NOTA: O intervalo de manutenção para o tubo de absorção de halogénios está definido e pode ser consultado no software do equipamento. No entanto, não deve ter-se só em conta este intervalo. A necessidade de manutenção do tubo de absorção de água é também realizada por observação do seu conteúdo. O conteúdo do tubo necessita de manutenção quando a cor da lã de bronze passa a preto. Realizar apenas manutenção quando ¾ do tubo apresentarem esta cor. O enchimento com a lã de bronze deve ser compacto, evitar espaços não preenchidos e colocar a sua altura o mais exata possível.

2.5.4.3. Tubo de filtro

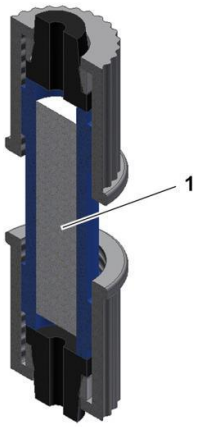


Figura 16 – Representação esquemática do tubo de filtro de absorção. 1 – Filtro.

Para realizar a mudança do tubo de filtro, proceder da seguinte forma:

1. Inserir um filtro no interior do tubo.

2.5.4.4. Remoção e instalação do tubo de absorção de água, de halogénios e do tubo de filtro

Efetuar a substituição dos tubos no modo ***Replace part***, como mencionado acima no ponto **4.**

Proceder da seguinte forma:

1. Abrir a porta frontal ou lateral consoante o tubo;
2. Soltar as tampas de rosca nas extremidades dos tubos;
3. Remover os tubos das abraçadeiras;
4. Remover o conteúdo antigo do interior dos tubos e, se necessário, lavá-los com água millipore. Deixar secar, antes da substituição do conteúdo;
5. Proceder à substituição do material de preenchimento dos tubos, de acordo com os pontos **2.4.4.1.**, **2.4.4.2.** e **2.4.4.3.**;
6. Enroscar as tampas nas extremidades dos tubos e prendê-los nas abraçadeiras novamente;
7. Fechar a janela ***Replace part*** no software do equipamento, clicando em ***Finish***. O O_2 ligar-se-á automaticamente;
8. Verificar se os valores de pressão e fluxo do O_2 estão idênticos aos valores antes da mudança do tubo. Caso contrário, realizar um ***Leak test*** (ponto **2.4.5.**). Caso o tubo substituído possua um intervalo de manutenção definido,

alterar também manualmente o estado em ***Options*** > ***Maintenance*** > ***intervals***.

2.5.5. Teste de fuga (Leak test)

O trabalho de manutenção exige a remoção do equipamento das partes em causa e, portanto, a interrupção do circuito do O_2 no equipamento. Após a reinstalação da componente à qual se realizou a manutenção, se o fluxo e a pressão não se mantiverem nos valores de referência (≈ 200 ml/min e 1000 mbar, respetivamente), deve ser realizado um teste de fuga.

Proceder da seguinte forma:

- Clicar em ***Options*** > ***Diagnostics*** > ***Leak test***. Uma janela aparece no software que pede novamente a confirmação da realização ou não do ***Leak test***;
- Como o equipamento está equipado com uma célula eletroquímica (CE), antes do início do teste, os tubos da CE devem ser removidos e ligados diretamente – fazer o bypass à EC. A **Figura 17** diz respeito à vista lateral direita do analisador onde está localizada a CE. Para fazer o bypass à CE, é necessário trocar os encaixes dos tubos 1 e 2 (**Figura 17**), ou seja:
  - Desapertar o tubo 1 e apertá-lo no encaixe da posição 2;
  - Desapertar o tubo 2 e apertá-lo no encaixe da posição 1;

Figura 17 - Vista lateral direita do analisador: A - célula eletroquímica (CE); 1 - tubo 1; 2 - tubo 2.

  - De seguida, colocar as tampas pretas ou castanhas (acessórios do kit teste de fuga) nos locais indicados pelo software;

- Clicar em ***Yes*** para dar início ao teste de fuga. O programa abre a janela ***Leak test: whole device***. O diagrama da tubagem do sistema de análise é exibido, assim como as atividades de preparação a serem realizadas (**NOTA**: mover o ponteiro do rato sobre uma área colorida do diagrama faz com que o programa exiba informações adicionais sobre o objeto correspondente);
- Após a preparação estar finalizada, clicar em ***Start*** para começar o teste de fuga a todo o sistema. A janela ***Leak test: whole device*** aparece. Na zona esquerda, a seta indica o estado atual do teste de fuga;
- No final, o resultado do teste de fuga é exibido na parte inferior. Se:
  - o símbolo  se tornar visível, o resultado do teste é negativo – não há fuga no sistema;
  - o símbolo  se tornar visível, o resultado do teste é positivo – há fuga no sistema;
- Clicar em ***Close*** para fechar a janela.

<u>Em caso de fuga, esta deve ser localizada. Proceder da seguinte forma:</u>

- Fechar a válvula de retenção na conexão com o tubo nº 10. Se após um certo tempo de estabilização, o fluxo do O_2 tiver um valor de cerca de 0-5 ml/min, o caminho do gás encontra-se livre de fuga. Estabelecer as conexões originais novamente;
- Fechar a saída do tubo de combustão por meio de uma "bandeja cega". Se, após um certo tempo de estabilização, o fluxo do O_2 tiver um valor de cerca de 0-5 ml/min, o caminho do gás encontra-se livre de fuga. Estabelecer as conexões originais novamente;
- Fechar a saída do condensador com uma tampa. Se, após um certo tempo de estabilização, o fluxo do O_2 tiver um valor de cerca de 0-5 ml/min, o caminho do gás encontra-se livre de fuga. Estabelecer as conexões originais novamente;
- Fechar a saída do tubo de halogénios no tubo nº 21 com uma tampa. Se, após um certo tempo de estabilização, o fluxo do O_2 tiver um valor de cerca de 0-5 ml/min, o caminho do gás encontra-se livre de fuga. Estabelecer as conexões originais novamente;

- Fechar a saída da membrana de secagem de gás para o tubo nº 23 por meio de uma tampa. Se, após um certo tempo de estabilização, o fluxo do O_2 tiver um valor de cerca de 0-5 ml/min, o caminho do gás encontra-se livre de fuga. Estabelecer as conexões originais novamente;
- Fechar a saída do tubo de secagem no tubo nº 24 com uma tampa. Se, após um certo tempo de estabilização, o fluxo do O_2 tiver um valor de cerca de 0-5 ml/min, o caminho do gás encontra-se livre de fuga. Estabelecer as conexões originais novamente.

Se, em alguma das etapas mencionadas anteriormente, o fluxo de O_2 corresponder a um valor acima dos 5 ml/min, pode assumir-se a existência de uma fuga na zona correspondente.

2.6. Lavagem de material a utilizar no Vario TOC Select

Os *vials* (tubos de vidro onde são colocadas as amostras) do equipamento devem ser submersos por algumas horas (ideal durante a noite) numa solução 20 % HNO_3. De seguida, devem ser lavados com água corrente (aprox. 10 vezes) e ser passados outras 3 vezes com água millipore. No caso dos *vials* serem simultaneamente usados para a análise de TNb, usar outro ácido que não interfira com a análise nem de carbono nem de azoto.

Adicionalmente, toda o material de vidro usado para a preparação de soluções a analisar pelo equipamento deve ser lavada da mesma forma.

Appendix D – Procedure of TNb calibration and operation with Vario TOC Select

In this appendix the procedure proposal of TNb calibration and operation, in liquid samples, with the Vario TOC Select analyser is presented. This procedure was elaborated during the present work and, once it was done in the context of internal use in RAIZ, it is written in Portuguese.

1. OBJECTIVO E ÂMBITO

Este documento especifica o procedimento de calibração do analisador Vario TOC Select, relativamente ao parâmetro azoto total (TNb), em amostras líquidas.

2. DESCRIÇÃO

2.1. Método de determinação de TNb

2.1.1. Definições do método

Azoto total (TNb, em inglês) – soma do azoto presente na água ou na matéria em suspensão, ligado orgânica e inorganicamente, medido nas condições deste método.

2.1.2. Princípio geral e interferências do método

A determinação do parâmetro TNb pelo analisador Vario TOC Select é realizada por combustão a elevada temperatura (850 °C) da amostra. O azoto presente na amostra é oxidado a óxido nítrico (NO), que é posteriormente lido numa célula eletroquímica (EC). O sinal é digitalizado e integrado, resultando num valor de área que, de acordo com a calibração estabelecida para o parâmetro, é transformada em massa de azoto total.

O analisador tem como principal objetivo a análise de carbono orgânico total (TOC), não tendo nenhum método que permita a análise do azoto de forma individual, sendo este sempre analisado em conjunto com o carbono. Em amostras com um conteúdo elevado de TOC, isto representa um problema, uma vez que pode levar à determinação de resultados mais baixos para o azoto. Dependendo das condições de análise, o carbono orgânico presente na amostra é oxidado, não só a dióxido de carbono (CO_2) mas também a monóxido de carbono (CO). O CO é um agente redutor e, portanto, reage com o NO, formando-se CO_2 e N_2. O N_2 não é detetado pela célula eletroquímica e o CO_2 é detetado no detetor de infravermelho. Assim, elevadas concentrações de TOC nas amostras levam à deteção por defeito do azoto presente.

Para ultrapassar esta interferência, torna-se necessário mimetizar, na medida do possível, a composição das espécies de azoto presentes nas matrizes das amostras aquando da elaboração da solução padrão para calibração. Para isso é preciso ter em consideração que:

- o azoto sob a forma de nitrato (NO_3^-) tem uma recuperação na ordem dos 100 %.
- o azoto sob a forma de amónio (NH_4^+) tem uma recuperação na ordem dos 90 %.

A Elementar aconselha ainda o uso de NH_4Cl, para contabilizar a fração de amónio, e $NaNO_3$, para representar a fração de nitratos. A solução padrão de calibração deverá ser composta por estes dois reagentes, cuja proporção deverá ser semelhante à proporção dos mesmos na matriz das amostras a analisar. Caso a composição das amostras seja desconhecida, recomenda-se a preparação de uma solução tendo em conta 50 % de amónio-N e 50 % de nitrato-N.

Tendo em conta os pressupostos anteriores, a calibração de TNb irá variar consoante a matriz das amostras em análise.

2.2. Calibração de TNb pelo analisador

Como foi mencionado, a calibração de azoto do analisador deve ter em conta as frações de NH_4^+ e NO_3^- das amostras a analisar. Além disso, deve ser ainda realizada a partir do mesmo volume de injeção e diferentes soluções padrão.

No presente documento, o procedimento de calibração exemplificado diz respeito à matriz águas superficiais. No entanto, caso haja necessidade de calibrar o analisador para outra matriz, as ações aqui descritas a realizar no software são as mesmas, podendo variar apenas parâmetros como o volume de injeção ou a concentração e composição das soluções stock e padrão.

No caso da matriz águas superficiais, a proporção verificada foi de 65 % de nitratos e 35 % de amónio. Neste caso específico, a calibração é linear, uma vez que todas as soluções-padrão estão abaixo dos 15 mg/l. Contudo, acima desta concentração, a curva de calibração deverá ter uma forma quadrática. No entanto, antes de aplicar uma função de segundo grau à calibração de TNb nestas condições, confirmar sempre que esta se adequa ao conjunto de pontos.

NOTA: Como substância de controlo da calibração, a Elementar aconselha o uso de ácido nicotínico (uma solução de 50 mg/l deverá ter uma recuperação de 100 % ± 5 %).

2.2.1. Reagentes e soluções padrão de calibração

2.2.1.1. Solução stock de NPOC – 1000 mg/l

Dissolver, num balão volumétrico de 1000 ml, 2.125 g de hidrogeno ftalato de potássio ($C_8H_5KO_4$) seco 1 h entre 105 e 120 °C (estufa) e perfazer o volume do balão com água millipore. Esta solução é estável por 2 meses se armazenada bem fechada e no frigorífico.

2.2.1.2. Solução stock de TNb – 500 mg/l

Como foi mencionado, a determinação de azoto deve ter em conta as frações de amónio e nitratos das amostras a analisar. No caso da matriz águas superficiais, a proporção verificada é de 65 % de nitratos e 35 % de amónio. Logo, para preparar uma solução de 500 mg/l em TNb, devem ser pesadas as seguintes quantidades:

- 0,667 g de NH_4Cl;
- 1,973 g de $NaNO_3$.

Caso a pureza dos reagentes não seja de 100 %, realizar o cálculo de compensação. Antes de pesar os reagentes, secá-los à temperatura de (105 ± 5) °C por 1 h e guardar os reagentes em exsicador até à sua pesagem. Dissolver cada um deles individualmente num copo, colocar num balão de 1000 ml, perfazer o volume do balão com água millipore e homogeneizar a solução. Esta solução pode ser armazenada por um período de 4 semanas.

2.2.1.3. Soluções padrão de calibração de TNb

A calibração de TNb é realizada em conjunto com a de NPOC, logo os padrões de calibração são preparados também tendo em conta a fração de NPOC. Para isso, pipetar 2 ml da solução **3.1.1.** para um balão de 200 ml. De seguida, pipetar 4 ml da solução **3.1.2.** para o balão de 200 ml, perfazer o seu volume e homogeneizar. Esta solução padrão tem uma concentração de 10 mg/l de NPOC e 10 mg/l de TNb e corresponde ao último padrão da curva de calibração. A partir desta solução preparar também os padrões 1, 2, 4, 6 e 8 mg/l de TNb e NPOC pipetando 5, 5, 10, 15 e 20 ml para balões de 50, 25, 25, 25 e 25, respetivamente, perfazer o seu volume com água millipore e homogeneizar.

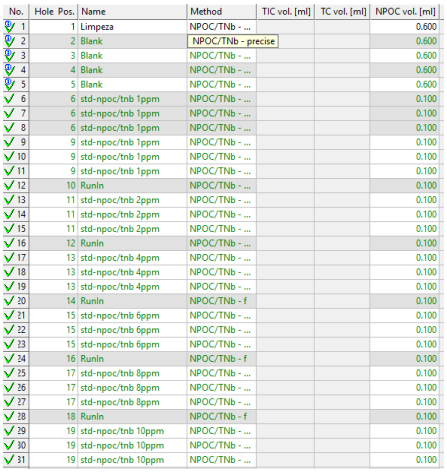
2.2.2. Procedimento de calibração

Como mencionado, a análise de TNb pelo analisador Vario TOC select não é independente da análise de carbono. No software do analisador estão previstos vários

métodos que incluem a extensão de TNb. Uma vez que a calibração de fábrica para o TNb foi realizada em conjunto com o parâmetro NPOC, a calibração de TNb realizada no RAIZ foi efetuada também em conjunto com este parâmetro, mas de forma diferente.

Para o parâmetro TNb, a calibração para análise de águas superficiais, é realizada da seguinte forma:

1. Ter a garantia de que o equipamento se encontra limpo antes da análise, de forma às áreas dos brancos não serem afetadas por resquícios de amostra lidas anteriormente (se necessário fazer correr água millipore até que os valores das áreas sejam próximos das áreas habituais dos brancos);
2. Abrir um novo documento no software;
3. Colocar quatro vials com água millipore para realizar quatro brancos (no mínimo);
4. De seguida, colocar as soluções padrão preparadas em **3.1.3.** em vials e colocá-los no carrossel por ordem crescente de concentração;
5. Preencher a folha do software de acordo com a **Figura 1**. Como a calibração de TNb vai ser realizada associada ao parâmetro NPOC, preencher a coluna do volume de injeção de NPOC com 0.1 ml para todos os padrões (não há coluna de volume para o TNb uma vez que é apenas uma extensão) e usar como método de análise o **NPOC/TNb – precise**. Entre cada solução realizar um ***Runin*** com a solução seguinte para ter a certeza que se evitam possíveis efeitos de arrastamento da solução padrão anterior. **NOTA**: é essencial que seja dado às amostras o nome definido nas substâncias standard (amostras de fundo verde), para que o software as possa identificar. Caso contrário não conseguirá realizar-se a calibração.



No.	Hole Pos.	Name	Method	TIC vol. [ml]	TC vol. [ml]	NPOC vol. [ml]
1	1	Limpeza	NPOC/TNb - ...			0.600
2	2	Blank	NPOC/TNb - precise			0.600
3	3	Blank	NPOC/TNb - ...			0.600
4	4	Blank	NPOC/TNb - ...			0.600
5	5	Blank	NPOC/TNb - ...			0.600
6	6	std-npoc/tnb 1ppm	NPOC/TNb - ...			0.100
7	6	std-npoc/tnb 1ppm	NPOC/TNb - ...			0.100
8	6	std-npoc/tnb 1ppm	NPOC/TNb - ...			0.100
9	9	std-npoc/tnb 1ppm	NPOC/TNb - ...			0.100
10	9	std-npoc/tnb 1ppm	NPOC/TNb - ...			0.100
11	9	std-npoc/tnb 1ppm	NPOC/TNb - ...			0.100
12	10	RunIn	NPOC/TNb - ...			0.100
13	11	std-npoc/tnb 2ppm	NPOC/TNb - ...			0.100
14	11	std-npoc/tnb 2ppm	NPOC/TNb - ...			0.100
15	11	std-npoc/tnb 2ppm	NPOC/TNb - ...			0.100
16	12	RunIn	NPOC/TNb - ...			0.100
17	13	std-npoc/tnb 4ppm	NPOC/TNb - ...			0.100
18	13	std-npoc/tnb 4ppm	NPOC/TNb - ...			0.100
19	13	std-npoc/tnb 4ppm	NPOC/TNb - ...			0.100
20	14	RunIn	NPOC/TNb - f			0.100
21	15	std-npoc/tnb 6ppm	NPOC/TNb - ...			0.100
22	15	std-npoc/tnb 6ppm	NPOC/TNb - ...			0.100
23	15	std-npoc/tnb 6ppm	NPOC/TNb - ...			0.100
24	16	RunIn	NPOC/TNb - f			0.100
25	17	std-npoc/tnb 8ppm	NPOC/TNb - ...			0.100
26	17	std-npoc/tnb 8ppm	NPOC/TNb - ...			0.100
27	17	std-npoc/tnb 8ppm	NPOC/TNb - ...			0.100
28	18	RunIn	NPOC/TNb - f			0.100
29	19	std-npoc/tnb 10ppm	NPOC/TNb - ...			0.100
30	19	std-npoc/tnb 10ppm	NPOC/TNb - ...			0.100
31	19	std-npoc/tnb 10ppm	NPOC/TNb - ...			0.100

Figura 18 – Vista do software com a zona das amostras preenchida de acordo com o procedimento de calibração de TNb. **NOTA**: a repetição do primeiro padrão é um equívoco, não realizar.

6. Iniciar a análise e não realizar acidificação dos padrões;

7. No final da análise ir a ***Calibration*** > ***Calibrate*****.** Abre-se uma janela que informa que os brancos serão calculados de forma sequencial, tal como apresentado na **Figura 2**;

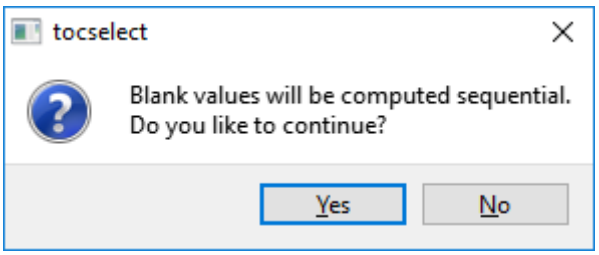


Figura 19 – Vista de janela do software com a indicação da forma de cálculo dos brancos.

8. Clicando em ***Yes*** na janela da **Figura 2**, abre-se a janela ***Configure calibration*** como apresentado na **Figura 3**. Nesta janela é possível definir se queremos que a calibração seja dividida em uma ou duas gamas de calibração, e ainda o grau do polinómio da função que traduz cada uma das gamas (no caso de serem duas). Premindo ***OK*** no separador do primeiro parâmetro, o software prossegue para o segundo para que também sejam definidas as condições para o segundo parâmetro.

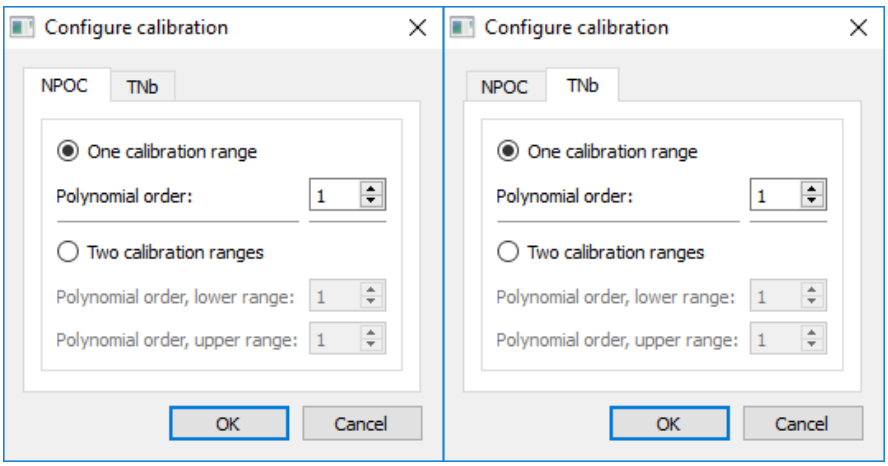


Figura 20 – Vista da janela Configure calibration com os dois separadores relativos a cada um dos parâmetros.

9. Selecionando ***OK*** no segundo separador da janela ***Configure calibration*** aparece na área do gráfico do software, o primeiro gráfico da calibração relativo ao parâmetro NPOC. Aqui, são visualizados os pontos de cada uma das medições em cada padrão. Como apresentado na **Figura 4**, clicando sobre os pontos temos acesso à informação disponível em cada ponto, assim como os coeficientes gerados para aquele parâmetro a partir da análise/calibração. Na barra de ferramentas do software, ficam ativos os primeiros ícones da **Tabela 1**, que permitem também percorrer os vários pontos do gráfico, caso o acesso com o rato, por sobreposição, seja difícil.

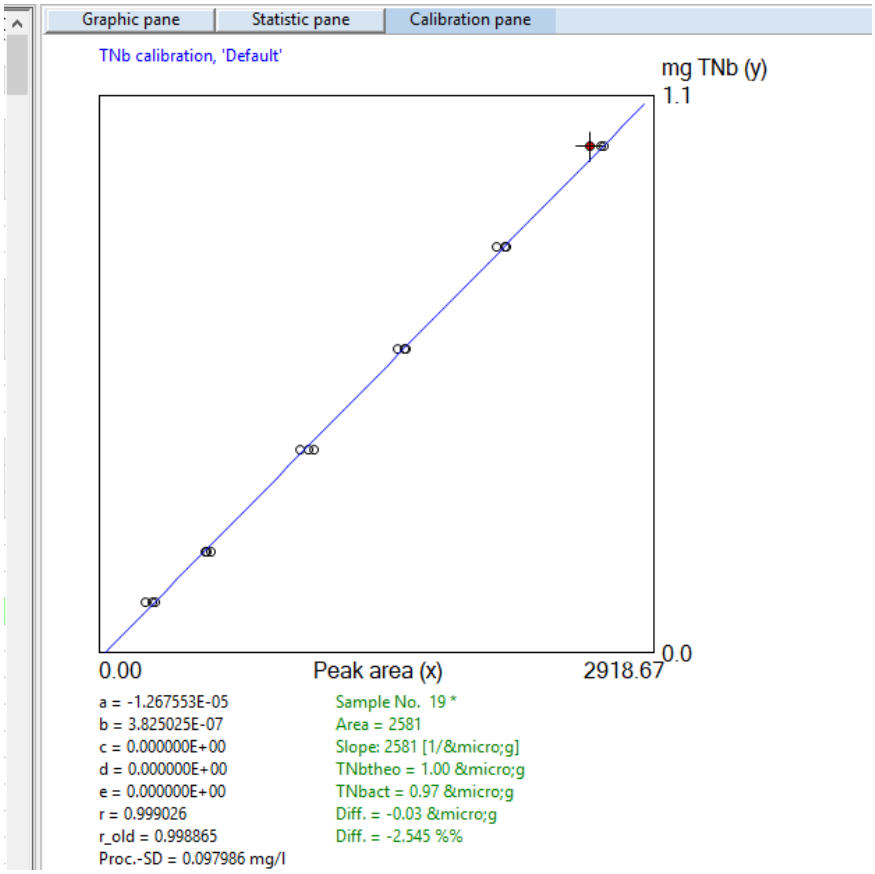


Figura 21 – Vista da área do gráfico após a calibração de TNb. **NOTA**: o gráfico é apenas um exemplo, não correspondendo ao gráfico da calibração em vigor para o parâmetro TNb.

Tabela 8 – Ícones da barra de ferramentas do software usados durante a calibração e respetiva função.

Ícone	Significado
	Percorrer os vários pontos dentro do mesmo gráfico da calibração.
	Avançar/retroceder entre os vários gráficos de calibração que tenham sido realizados.
	Remover/reinserir pontos dos gráficos de calibração.

10. Para remover um ponto que seja considerado ***outlier***, clicar sobre ele ou chegar até ele pelas primeiras setas da **Tabela 1**. Clicar no terceiro ícone da **Tabela 1** para o remover. O ponto mantém-se no gráfico, mas a vermelho (**Figura 4**), e automaticamente os valores dos coeficientes da reta de calibração, assim como o coeficiente de correlação, são alterados. Caso se queira voltar a adicionar o

ponto, basta selecioná-lo e voltar a clicar no mesmo ícone. Os coeficientes são também restabelecidos.

11. Após a finalização da edição do gráfico relativo ao parâmetro NPOC, passar para o gráfico seguinte nas segundas setas da **Tabela 1**. Na área do gráfico do software passa então a estar disponível o da calibração de TNb. Realizar o mesmo processo de remoção dos ***outliers*** (caso se verifiquem) e no final avançar no processo de calibração novamente no mesmo ícone.

12. Com a ação anterior, abre-se a janela ***Calibration coefficients*** como apresentado na **Figura 5**. Nesta janela são visualizados os coeficientes de cada uma das retas de calibração dos vários parâmetros e ainda as áreas mínima e máxima, já com o desconto do branco, que lhes deram origem. Nos separadores relativos aos parâmetros NPOC e TNb, os coeficientes visualizados dizem respeito à nova calibração. Clicar em ***OK*** para aceitar os novos coeficientes, ou em ***Cancel***, caso não se queira aplicar os novos coeficientes e manter os anteriores à calibração.

    Importante: como a calibração de TNb é realizada sempre em conjunto com outro parâmetro (neste caso NPOC), na janela ***Calibration coefficients*** é possível inserir manualmente os coeficientes de NPOC anteriores, caso só se queira alterar a calibração de TNb.

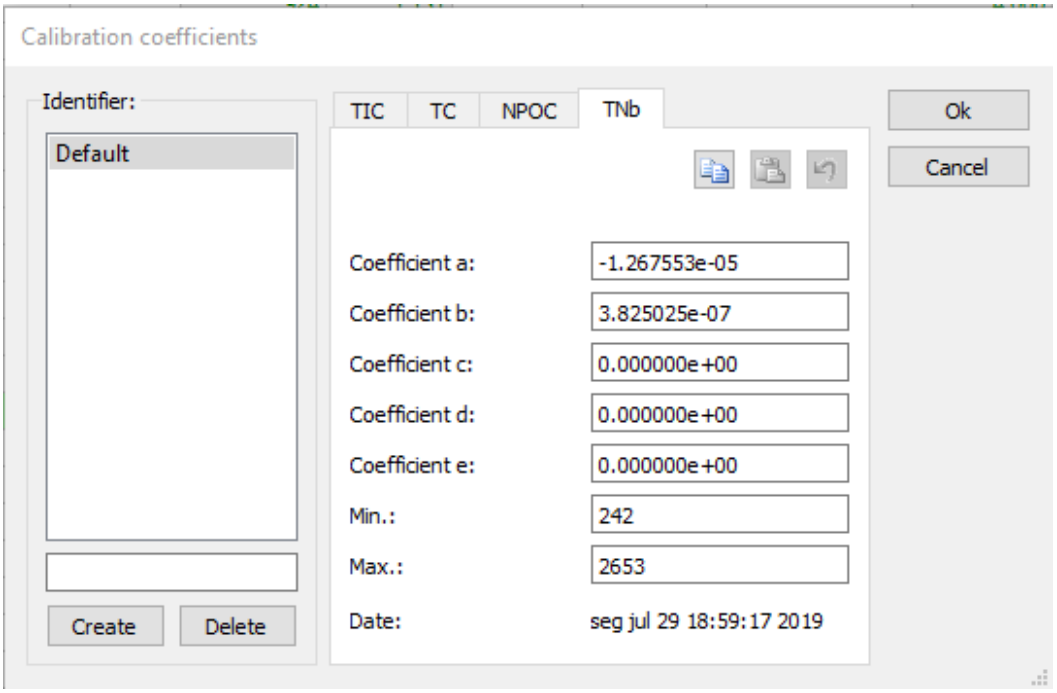


Figura 22 – Vista dos coeficientes de TNb na janela ***Calibration coefficients*** do software.

13. Clicando em ***Ok*** a calibração está finalizada e os novos coeficientes serão aplicados no cálculo da massa do respetivo parâmetro, sempre que este seja analisado.

14. Para ter acesso a ficheiros mais específicos gerados pela calibração, ir a ***File*** > ***Print*** > ***Configure report***. Nesta janela representada na **Figura 6** é possível escolher a informação a sair no relatório de calibração. Em ***File*** > ***Export/Import*** > ***Export sheet data (for Excel)*** é possível ainda extrair o ficheiro da calibração em formato Excel.

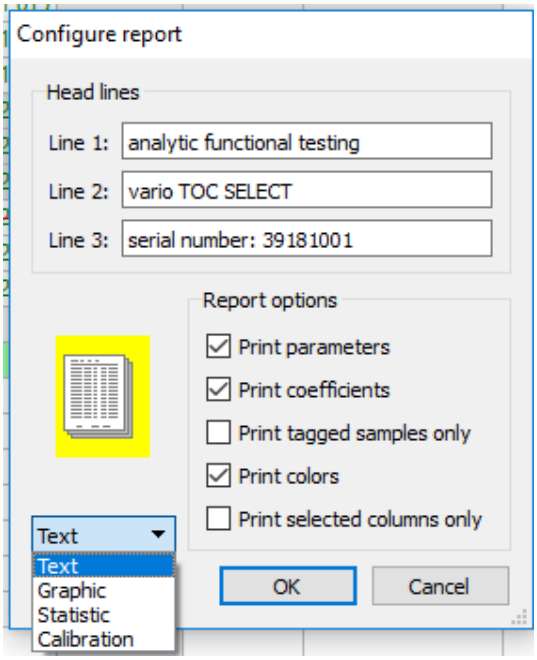


Figura 23 – Vista da janela ***Configure report*** do software.

2.3. Análise de amostras pelo método TNb

Após a realização da calibração, é então possível analisar amostras da matriz correspondente à calibração realizada, neste caso da matriz águas superficiais. Para isso deve ter-se um conhecimento prévio da concentração de azoto total nas amostras. A análise pode ser realizada na mesma folha de cálculo onde foi efetuada a calibração, a seguir aos padrões; ou pode optar-se por abrir um novo documento no software. É apenas necessário que o procedimento da calibração esteja concluído até à tarefa **13.** do ponto **2.2.2. Procedimento da calibração**, de forma a que os novos coeficientes sejam aplicados no cálculo das amostras a analisar.

No caso de se optar por realizar a análise no mesmo ficheiro da calibração, colocar a ***Stop tag*** numa amostra de limpeza, depois da análise dos padrões de calibração, de forma a que a análise não prossiga para as amostras, como apresentado na **Figura 7**.

Proceder da seguinte forma para a análise de amostras:

- Inserir na coluna respetiva o nome das amostras;
- Preencher a coluna ***Method*** com o método **NPOC/TNb – precise**. **NOTA**: Se as amostras necessitarem de agitação para análise, usar o método **NPOC/TNb – particle** e colocar um magneto no vial da amostra. No entanto, este método exige que o volume de injeção seja 0.200 ml, o que por vezes pode não ser útil, dependendo da concentração das amostras.

Por isso, no caso das amostras da **Figura 7**, foi usada a ferramenta de agitação do analisador, que pode ser ativada de forma manual em ***System*** > ***Stirrer***, promovendo a agitação da mesma forma, quando um magneto se encontra dentro do vial das amostras. A função é desativada novamente em ***System*** > ***Stirrer***.

Hole Pos.	Name	Method	TIC vol. [ml]	TC vol. [ml]	NPOC vol. [ml]	TIC Area	TC Area	NPOC Area	TNI
1	Blank	NPOC/TNb - ...			0.600			361	
2	Blank	NPOC/TNb - ...			0.600			207	
3	Blank	NPOC/TNb - ...			0.600			198	
4	std-npoc/tnb 1ppm	NPOC/TNb - ...			0.100			292	
4	std-npoc/tnb 1ppm	NPOC/TNb - ...			0.100			360	
4	std-npoc/tnb 1ppm	NPOC/TNb - ...			0.100			271	
5	std-npoc/tnb 2ppm	NPOC/TNb - ...			0.100			506	
5	std-npoc/tnb 2ppm	NPOC/TNb - ...			0.100			475	
5	std-npoc/tnb 2ppm	NPOC/TNb - ...			0.100			496	
6	std-npoc/tnb 4ppm	NPOC/TNb - ...			0.100			859	
6	std-npoc/tnb 4ppm	NPOC/TNb - ...			0.100			924	
6	std-npoc/tnb 4ppm	NPOC/TNb - ...			0.100			865	
7	std-npoc/tnb 6ppm	NPOC/TNb - ...			0.100			1 242	
7	std-npoc/tnb 6ppm	NPOC/TNb - ...			0.100			1 227	
7	std-npoc/tnb 6ppm	NPOC/TNb - ...			0.100			1 265	
8	std-npoc/tnb 8ppm	NPOC/TNb - ...			0.100			1 621	
8	std-npoc/tnb 8ppm	NPOC/TNb - ...			0.100			1 658	
8	std-npoc/tnb 8ppm	NPOC/TNb - ...			0.100			1 643	
9	std-npoc/tnb 10ppm	NPOC/TNb - ...			0.100			1 982	
9	std-npoc/tnb 10ppm	NPOC/TNb - ...			0.100			2 046	
9	std-npoc/tnb 10ppm	NPOC/TNb - ...			0.100			2 023	
10	Limpeza	NPOC - fast			0.600			869	
11	Caniceira	NPOC/TNb - ...			0.500			0	
11	Caniceira	NPOC/TNb - ...			0.500			0	
11	Caniceira	NPOC/TNb - ...			0.500			0	
12	Queiriga	NPOC/TNb - ...			0.500			0	
12	Queiriga	NPOC/TNb - ...			0.500			0	
12	Queiriga	NPOC/TNb - ...			0.500			0	
13									

Figura 24 – Vista do software do ficheiro tipo calibração de TNb, com a análise das amostras de águas superficiais.

- Caso as amostras tenham sido diluídas, colocar o fator de diluição na coluna respetiva do software para que a concentração das mesmas venha já com o desconto do branco. Caso não tenham sido, não fazer qualquer alteração na coluna do fator de diluição. Para uma melhor compreensão do modo de desconto do valor do branco, consultar o **Procedimento geral de Operação do analisador.**
- Preencher a coluna do volume de injeção com o volume pretendido;
- Dar início à análise. Dar a indicação para a realização a acidificação prevista no método NPOC, caso as amostras não estejam a pH inferior a 2. Para isso colocar um vial com 10 % HCl na posição 32 do carrossel, para que a acidificação seja realizada antes da análise. Ver o funcionamento do método NPOC no **Procedimento geral de Operação do analisador**.
- Deixar correr a análise;

- No final da análise, para obter os resultados de forma correta, há que ter em conta as seguintes considerações:
    - Padrões de calibração: a concentração dos padrões que é apresentada na respetiva coluna do software (**TNb mg/l**) necessita de correção com o valor da taxa do branco, uma vez que, como se tratam de padrões de calibração, não foi introduzido o factor de diluição na respetiva coluna (**Dilut. Factor**). Caso se queira utilizar as áreas dos padrões para algum tipo de cálculo, há que ter em consideração que as áreas na coluna **TNb Area** dizem respeito às áreas brutas. Para obter o valor das áreas compensadas com o branco é necessário subtrair, aos valores da coluna **TNb Area**, a respetiva taxa do valor do branco, que se encontra calculada para cada valor de área individual na coluna **TNb blank**. NOTA: Relembrar que este valor é calculado sem ser necessário introduzir o factor de diluição na respetiva coluna, pois trata-se do processo de calibração do equipamento, funcionando, portanto, de forma diferente da análise de uma amostra.
    - Amostras: caso estas tenham sido diluídas antes da análise e na coluna **Dilut. Factor** do software este tenha sido introduzido, o valor da concentração já vem corrigido com o valor da taxa do branco (a concentração foi calculada a partir da área compensada). No caso das áreas, as apresentadas na respetiva coluna dizem respeito à área bruta, logo necessitam de ser corrigidas como os padrões de calibração.
- No final retirar o ficheiro correspondente à análise em ***File*** > ***Export/Import*** > ***Export sheet data (for Excel)***.