Istraživanje kalibracijskih metoda za izravnu desorpciju koja prethodi plinskoj kromatografiji

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Investigation of calibration methods for direct desorption prior to gas chromatography

DIPLOMA THESIS

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This thesis has been submitted to the University of Zagreb, Faculty of Pharmacy and Biochemistry under the supervision of Prof. Dr. Biljana Nigović.
The experimental work was conducted at the laboratory of Pharmaceutical Analysis of the Department of Pharmaceutical and Pharmacological Sciences at KU Leuven, under the expert guidance of Prof. Dr. Erwin Adams and under the supervision of Senior Technician Kris Wolfs and Pharm. Wenping Huang.

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1. INTRODUCTION

1.1. Residual solvents

The European Pharmacopoeia (Ph. Eur.) defines residual solvents (RS) as volatile organic chemicals (VOCs) which are used or produced during the manufacturing process of active pharmaceutical ingredients (APIs) and excipients, or during preparation of medicinal products. They are termed 'residual' as they cannot be completely removed from the medicinal product by typical manufacturing techniques. RS play a significant role in production processes such as synthesis, separation and purification, and in product formulation procedures like granulation or coating. (*Tankiewicz et al., 2016*) Appropriate solvent selection is important as it can impact the characteristics of the active substance, such as its crystal form, solubility or purity. Even though solvents may be a critical parameter in the drug production process, they themselves have no therapeutic benefit, and some are even known to cause health and environmental hazards. *(Ph. Eur. 11.0, 50400)*

The International Council on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use classified RS in 3 classes by risk assessment:

1) **Class 1 solvents: solvents to be avoided.**
   This category consists of chemicals which are known, or strongly suspected, human carcinogens and environmental hazards. As they cause unacceptable toxicities, the Ph. Eur. states that their use should be avoided in the manufacturing process of APIs, excipients or medicinal products unless their necessity can be strongly justified.

2) **Class 2 solvents: solvents to be limited.**
   Class 2 solvents are non-genotoxic animal carcinogens, potential causative agents of some irreversible toxicities (teratogenicity or neurotoxicity) or other significant, but reversible, toxicities. Their practical use should be limited.

3) **Class 3 solvents: solvents with low toxic potential.**
   Solvents categorized in this class have low toxic potential for humans and therefore, no exposure limits are needed. The Ph. Eur. specifies this class of solvents should be used where practically possible.

Considering their lack of therapeutic benefit and their potentially hazardous nature, RS should be removed as good as possible from the final medicinal product in order to meet product specifications and ensure patient safety. As it isn't possible to achieve complete elimination, the Ph. Eur. defined a "permitted daily exposure" (PDE). PDE is a pharmaceutically acceptable amount of RS for intake by a patient, in a day. A PDE value along with a limit concentration for each solvent is stated in the Ph. Eur. Concentration limits for highly toxic Class 1 solvents are in the 2 – 8 ppm range, with the exception of 1,1,1-trichloroethane, an environmentally hazardous solvent, whose concentration limit is 1500 ppm. In case Class 1 solvents are expected to be present, they must be identified and quantified. For Class 2 solvents, the limit concentrations range from 50 – 4500 ppm. Class 3 solvents, which are deemed less toxic, are considered safe if their daily intake is below 5000 ppm. If Class 2 and 3 chemicals are present in a quantity higher than 0.5% (w/w), they must be identified and quantified, as well. *(Ph. Eur. 11.0, 50400)*

In order to verify if a pharmaceutical product contains higher amounts of RS than their respective limit, or to identify the solvent(s) present, testing for RS should be performed. Testing is done only on actives, excipients and pharmaceutical products, when a manufacturing or purification process is known to utilize a RS or it results in formation of one. Analytical procedure of choice for identification and control of RS in pharmaceuticals, as stated in the Ph. Eur., is headspace – gas chromatography (HS-GC). *(Ph. Eur. 11.0, 50400)*

1.2. Headspace – Gas Chromatography for RS determination

Gas chromatography (GC) is an analytical technique used for investigation (separation, detection, quantification) of volatile analytes in various sample matrices. In GC, the sample is introduced into a flow of an inert gas (nitrogen, helium or hydrogen) – the mobile phase, which transfers the sample to the analytical column containing a stationary phase. The mobile phase, also referred to as the "carrier gas", further transports the sample through the column and during this process separation of sample components takes place. Mixture components are separated on the basis of selective partitioning between the mobile and stationary phase and therefore, different components reach the end of the column at different times. This allows for each sample component to be detected individually at the end of the column. Routinely applied detectors are flame ionization detectors (FID) and mass spectrometers (MS). (*Kolb and Ettre, 2006*)

As a result, a graphical representation of the detected signals intensity as a function of time – *a chromatogram*, is produced. In a chromatogram, sample components appear as Gaussian shaped peaks. The size of each peak, height or area under the peak, is proportional to its analyte's concentration in the original sample matrix. The time indicated under each peak (retention time) is, under given conditions, a characteristic of each analyte and can be further used to help identify the analyte. (*Kolb and Ettre, 2006*)

When performing GC analysis, sample preparation is of utmost importance. While GC serves as a powerful technique for separating and identifying compounds within sample mixtures, the efficacy of the process hinges on the thoroughness of sample preparation. There are two main reasons why this is. Firstly, at times it is necessary to concentrate target analytes prior to GC analysis in order to reach concentration levels that permit reliable identification and quantification. Secondly, removing sample matrix interferences (e.g. biological or environmental, solids or strongly adsorbing materials) is occasionally needed to make sure such compounds don't adversely affect the GC set-up or that they don't hinder the isolation of volatile analytes from the sample matrix. (*De Koning et al., 2009*)

Headspace (HS) is the Ph. Eur. sampling technique of choice for determining RS in pharmaceuticals. HS has an inherit advantage when coupled with GC - it provides a sample extract limited to volatile components, which is perfectly suited to be analyzed by GC. Furthermore, unlike other traditional extraction and enrichment techniques which use extracting solvents, it doesn't suffer from co-extraction of matrix components and contamination from extraction solvents. With HS, an extracting solvent is not needed. Additionally, HS requires minimal sample preparation, it is applicable to a wide range of sample types (solids, liquids) and all modes of HS can be easily automated, which significantly lowers processing time, increases sample throughput and ensures consistency and reproducibility (*Dettmer-Wilde and Engewald, 2014; Robards and Ryan, 2022*).

HS sampling, in its simplest form, consists of placing and sealing a solid or liquid sample in a gas-tight vial. The vial is then heated at a set temperature, causing the volatile sample components to evaporate into the gas phase above the sample, the so called "headspace". When the volatile sample components reach a thermodynamic equilibrium between the sample and the HS, an aliquot of the headspace is transferred to the GC. Sample transfer can be done manually, using a gas-tight syringe, or automatically, by pressurizing the sample vial and

performing a time- or volume-controlled withdrawal of an aliquot of the HS by an autosampler. The concentration of the volatile analytes in the HS aliquot should be an accurate representation of the volatile analytes' concentration in the sample. Because an aliquot of the sample is transferred to the GC after the two phases have reached equilibrium, this type of HS analysis is called **static headspace (sHS)**. (*Kolb and Ettre, 2006*)

In a sealed HS vial containing a thermally equilibrated solid or liquid sample, **Dalton's law** defines the total pressure of the vapor phase above the sample (i.e. the headspace) - p_{total}, as a sum of partial pressures (p_i) of the gases present in the gas mixture:

$$p_{total} = \sum p_i \tag{1}$$

From Dalton's law it further follows that the partial pressure of one component of the gas mixture is also proportional to the fraction of its molecules in relation to the total molecules (n_{total}) present:

$$\frac{p_i}{p_{total}} = \frac{n_i}{n_{total}} = x_{G(i)} \tag{2}$$

$$p_i = p_{total} \cdot x_{G(i)} \tag{3}$$

where $X_{G(i)}$ represents the mole fraction of a component in the gas mixture.

For an ideal sample solution, the equilibrium between the gaseous and liquid phase is described by **Raoult's law**:

$$p_i = p_i^0 \cdot x_{S(i)} \tag{4}$$

It states that the partial pressure of a dissolved solute over its solution is directly proportional to its mole fraction in the solution, $x_{S(i)}$. The proportionality constant $p^0{}_i$, is the vapor pressure of the pure analyte.

For non-ideal solutions, meaning sample solutions with intermolecular interactions between the analyte and other sample components, especially the sample matrix (solvent), a correction factor to the concentration (γ_i) has to be introduced into eq. 4:

$$p_i = p_i^0 \cdot \gamma_i \cdot x_{S(i)} \tag{5}$$

This correction factor is called the activity coefficient of compound *i*. It depends on the nature of component *i* and it reflects the aforementioned intermolecular interactions of component *i* and other sample components, the so-called **matrix effect**. The activity coefficient is unknown for most analytes and is accounted for by calibrating the HS sampling system. Because of this, the calibration must be performed with such a mixture, which composition corresponds to that of the analyzed sample.

The partition of the analyte of interest between the sample and the gaseous phase can be expressed by the **partition coefficient (K)**:

$$K = \frac{c_s}{c_g} \tag{6}$$

C_s being the concentration of the analyte in the sample phase and C_g the concentration of the same analyte in the headspace (gas phase).

The ratio of the gaseous phase volume (V_g) to the sample phase volume (V_s) in the HS vial is described by the **phase ratio (β):**

$$\beta = \frac{V_g}{V_s} \tag{7}$$

If a sample with a volume of V_0 and a concentration of c_o is brought in a HS vial, the situation after equilibrium can be formulated as:

$$c_0 \cdot V_0 = c_s \cdot V_s + c_g \cdot V_g \tag{8}$$

$$c_0 \cdot V_0 = K \cdot c_g \cdot V_s + c_g \cdot V_g \tag{9}$$

For normal HS temperatures and volumes, the starting sample volume (V_0) doesn't change significantly during equilibration, meaning V_s stays equal to V_o:

$$c_0 \cdot V_s = K \cdot c_g \cdot V_s + c_g \cdot V_g \tag{10}$$

Introducing β into eq. 10 and expressing C_g as a function of C_o gives:

$$C_g = C_0 \cdot \frac{1}{K+\beta} \tag{11}$$

Eq. 11 is the basis of sHS analysis. It states that the concentration of an analyte in the original sample (and therefore chromatographic peak area) is directly proportional to its concentration in the headspace phase, considering K and β are both constants in a given system and under a given temperature. *(Kolb and Ettre, 2006; Kialengila et al., 2013)*

Besides statically, HS can also be performed dynamically. In **dynamic headspace (dHS),** equilibrium is never reached, unlike in its static equivalent. Here, the heated sample vial is continuously purged with an inert gas, as volatile analytes try, and fail, to reach equilibrium between the sample and the gas phase above. By doing so, the majority of volatile analytes, (besides the ones with a high affinity for the sample matrix) will be entirely removed from the sample by the inert gas flow. A variant technique of dHS is "**Purge and Trap**", where instead of directing the inert gas flow above the sample, the gas flow is flushed *through* the sample. Volatiles collected by either of these methods end up in a greatly diluted gas extract and are therefore, subsequently concentrated using a cold trap or a sorbent trap. Finally, the trap is heated and the volatile analytes are released by thermal desorption and transferred by a carrier gas to the GC column for analysis. The dynamic HS approach offers improved sensitivity compared to the static technique. However, the automation of dHS remains complicated to execute in practice. *(Kolb and Ettre, 2006)*

A prerequisite for a successful HS-GC analysis, performed as described above, is a homogenous sample (ideally a solution), as a homogenous sample ensures that the vapor phase above it, and therefore the sampled HS aliquot, accurately represents the true composition of the sample.

1.3. Sample

As stated earlier, regulatory agencies recommend using static HS-GC in identification and quantification of RS in medicinal products. The procedures described in pharmacopoeias start from diluting the sample in an appropriate solvent (water, dimethylformamide (DMF) or 1,3-dimethyl-2-imidazolidinone), depending on the solubility of the sample, and analyzing the resulting solution. *(Ph. Eur. 11.0, 20424)* While this agrees with a lot of potential analytes, not everything is easy to dissolve.

The Drug Delivery and Disposition laboratory of the Department of Pharmaceutical and Pharmacological Sciences at KU Leuven developed multiple types of modified cellulose beads to potentially be used as a drug carrier. (*Xie et al., 2023, 2022, 2021*) During preparation and

processing of the beads, organic solvents were used and, as is the requirement for all medicinal products, the residual content of such chemicals in the final product had to be determined. Cellulose is however insoluble in the majority of common solvents, including water and DMF, owning to both its strong intermolecular hydrogen bonding and hydrophobic interactions *(Medronho et al., 2012; Norgren et al., 2023*). Therefore, cellulose beads cannot be dissolved to obtain a homogenous mixture and analyzed using the suggested Ph. Eur. method.
In theory, it is also possible to analyze solid samples as solids, rather than solutions, using HS-GC. However, in this case, such a thing wouldn't be possible because we are dealing with an experimental sample, no standard reference material is available to us and without reference, complete release of RS can't be proven. (*Kolb and Ettre, 2006*)

Recently, a thermal desorption - gas chromatography (TD-GC) method has been successfully developed and used as an alternative way to determine RS in complex matrices (albumin, gelatin) such as ours. (*Asfaw et al., 2020, 2018*)

1.4. Thermal Desorption - Gas Chromatography

Thermal desorption (TD) is an all-encompassing sample preparation, concentration and introduction technique to GC. At its essence, it is an extraction technique, based on physical separation - heating of a sample or sorbent material in order to volatilize the retained analytes and 'desorb' them into a gas stream. The technique was developed as an alternative to the traditional solvent-based extraction methods, in an effort to eliminate the use of toxic organic solvents, enhance sensitivity and reduce the duration and complexity of sample preparation, while also enabling automation of the entire procedure.

TD-GC was originally intended for monitoring of airborne chemicals. Hence, in its earliest form, TD analysis was carried out by filling a GC injector liner with a sorbent material, sampling a fixed volume of air or a gas by a sampling pump, or sampling air by passive diffusion, and then quickly transferring the liner back to the GC inlet to be thermally desorbed and analyzed. These early **single-stage desorption** methods showed to be quite flawed. Issues such as air ingress, variability, and volatile losses hindered the accuracy of the sampling part of the analysis and huge volumes of carrier gas needed to extract the analytes off of the sorbent material proved to be incompatible with GC, as they compromised the analytical resolution and sensitivity. (*Woolfenden, 2021*)

Addressing these issues lead to development of the present-day TD itineration – a **two stage desorption** system. Here, a sample tube packed with one or more sorbent materials is used to sample a gaseous phase, either by passive diffusion or by forced transport, after which the tube is placed in the TD-GC apparatus. The TD process starts with a leak test of the sample tube and, if the test is passed successfully, a tube purging step, to reduce the risk of sample or adsorbent oxidation during desorption. Then, the first desorption stage takes place. The sample tube gets heated by an oven, which causes volatiles to vaporize, and is thermally desorbed. The extracted volatiles are transferred, under a flow of inert gas, to a cold trap, composed of one or more adsorbents, where they are collected and concentrated. Next, the second desorption stage takes place. The cold trap is rapidly heated and the retained volatiles are released from the trap into a gas flow, which carries them to the GC column in a small and concentrated volume of carrier gas. This allows for better analytical resolution, enhanced sensitivity and improved quantitative accuracy, compared to the single-stage TD setup. *(Woolfenden, 2021*)

TD efficiency can be further improved for- and before- each specific analysis by **optimizing different TD parameters** such as desorption time and temperature, carrier gas flow and sorbent type. The desorption temperature should be set with consideration to the volatility and thermal stability of the sample and the compounds of interest, and the temperature limits of trap sorbent materials. Usually, in order to achieve complete separation of analytes from the sorbent material, the sample tube temperature should be set as high as possible. As well as temperature, optimizing the desorption time is equally as important in ensuring the complete extraction of all analytes from the sorbent material, but also minimizing the analysis time and maximizing sample throughput. As an alternative to increasing desorption temperature, for instance when dealing with reactive compounds, or simply as an addition to the high temperature, the thermal desorption process can further be enhanced by increasing the carrier gas flow. A well-known general rule states, that by doubling the desorption flow, the desorption time is roughly halved. It's important to note that gas flow should be lowered through the trap, in order to avoid sample breakthrough. (*Woolfenden, 2021*) Lastly, when choosing a sorbent material, either for the sample tube or the trap, the analyst should pick one strong enough to retain target analytes during sampling/concentration on the trap, but not so strong that it inhibits the release of those same analytes during the thermal desorption step. In a situation where there are multiple compounds with widely different volatilities being determined, the sample tube/trap can be packed with more than one sorbent material, arranged in order of increasing retentive strength from the sampling end. (*Woolfenden, 2002*)

1.4.1. Modifications of the TD-GC setup

The TD-GC system employed for determination of RS in albumin and gelatin (as previously mentioned), was however a modified one, with all of the modification done in-house by the Pharmaceutical Analysis laboratory of the Department of Pharmaceutical and Pharmacological Sciences, at KU Leuven. The system was modified in 2 parts.

Firstly, a direct injection GC injector was connected to the TD apparatus by a heated transfer line. The entire injector setup was inserted into the primary desorption flow of the TD machine after the pressure control module and before the sample tube, so that potential leaks or septum failures get detected by the TD firmware as a tube seal leak (*Figure 1*). This modification has been used for inline liquid calibration as explained in 1.4.2.

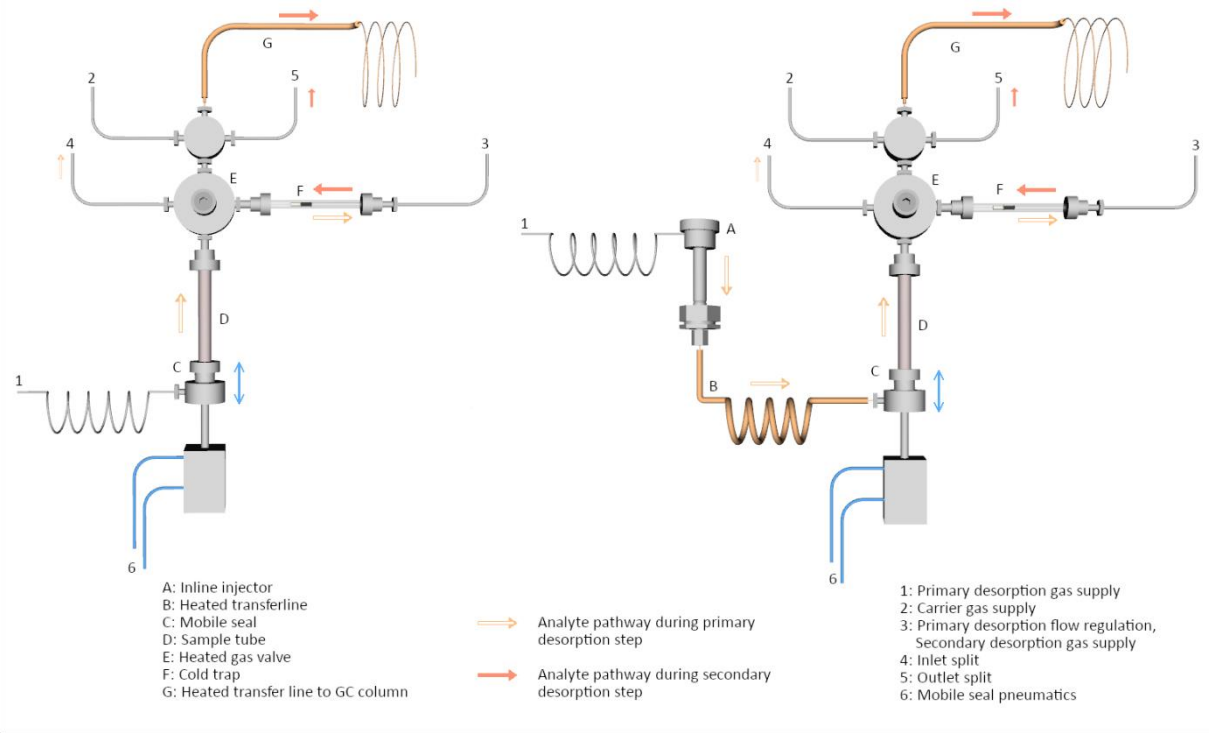

Figure 1. Thermal desorber without (left) and with (right) the inline injector installed.

Secondly, the typically sorbent-filled sample tube, made for sampling a gaseous sample, was replaced by an empty TD sample tube intended to be packed with a solid sample to be analyzed. The immobilized solid sample would then be directly subjected to the classic two-stage desorption, without being preconcentrated on a sorbent material beforehand. Meaning, this modified TD-GC was technically used as a high temperature dynamic HS-GC, rather than a traditional TD-GC. (*Asfaw et al., 2019*)

1.4.2. Calibration

When the TD technique is used for its original purpose (monitoring of volatile air-born chemicals) the calibration of the method should, ideally, be performed using gas-phase standards. The calibration procedure then consists of loading known amounts of gaseous standards onto a TD tube, filled with the same sorbent used for sampling. This way, sample and reference compounds are treated identically throughout the analysis, all systematic deviations are compensated for and an accurate calibration is guaranteed. Gas-phase standards can, for some analytes, be quite expensive and difficult to obtain, and even when they are acquired, it is often difficult to generate low concentrations necessary for calibration. Consequently, using liquid references to calibrate a TD-GC has become standard practice. *(Demeestere et al., 2008; Woolfenden, 2021*)

In **offline liquid calibration (OLC)** liquid-phase standards are diluted in a suitable solvent to create serial dilutions. Microliter volumes of those dilutions are then transferred onto sorbent-filled TD tubes using a micro syringe, ideally through an unheated GC injector connected to the sampling end of the TD tube. The syringe should be inserted through the septum of the injector port, so that it touches the sorbent-retaining material within the tube (*Figure 2*) before injecting the solution volume onto the sorbent. (*Woolfenden, 2021*) The transferring process can be mediated by a flow of an inert gas, which vaporizes the liquid standards and enables both the analytes and the solvent to reach the sorbent material in the vapor phase, just like they do during sample collection. The inert carrier gas can also be used to selectively remove the solvent from the sorbent material, if the appropriate solvent-sorbent combination and gas flow conditions are selected. *(https://markes.com; Martin et al., 2007*) Considering the concentration and volume of the used liquid references are known and under assumption that the adsorption and desorption processes of VOCs are complete, it is possible to calculate the amount of reference compounds reaching the GC detector. The entire process is significantly easier to execute compared to gas-phase calibration. However, it does have its drawbacks, the main ones being the possibility of an incomplete transfer of reference solution onto the sorbent-filled sample tubes and the potential loss of reference volatiles due to an inadequate adsorption system or during tube manipulation. (*Asfaw et al., 2019*) This is why TD-GC procedures using OLC as a calibration method must always be validated against another analytical method or gas-phase standards.

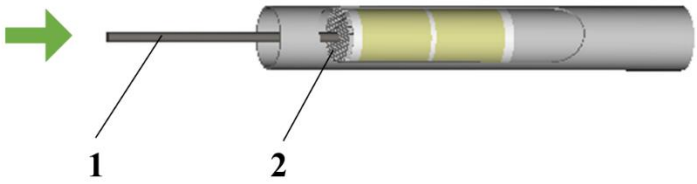

Figure 2. TD-OLC sample tube loading.
The syringe needle (1) touching the sorbent retaining stainless-steel mesh (2). The green arrow represents the direction of the sample loading gas flow.

Modifications done to the TD apparatus, specifically the added heated injection port, allow for another type of liquid calibration – **inline liquid calibration (ILC)**. In ILC, reference is injected into the heated injector port where it vaporizes entering the primary desorption gas stream. It then travels through the transfer line and an empty sample tube, all the way to the cold trap, where it is retained and concentrated, after which it follows the typical secondary desorption and GC analysis. This type of calibration completely avoids the primary adsorption of reference onto the sorbent material in the sample tube and ensures 100% analyte introduction, therefore eliminating the issues present in OLC. For the same reason, ILC can also be used to optimize OLC. (*Asfaw et al., 2019)*

2. AIM OF THE STUDY

RS determination is mandatory for all active substances, excipients and medicinal products upon release testing and recommended during the entire manufacturing process. Official methods, recommended by the ICH and implemented by the USP and Ph. Eur., are however only applicable to soluble samples and consequently, some formulations are either difficult or impossible to analyze using those procedures. One such formulation are modified cellulose beads developed by the Drug Delivery and Disposition laboratory of the Department of Pharmaceutical and Pharmacological Sciences at KU Leuven. A recently presented TD-GC method, developed by the Pharmaceutical Analysis laboratory of the Department of Pharmaceutical and Pharmacological Sciences, at KU Leuven, has been successfully used as an alternative way to determine RS in complex, insoluble matrices, such as cellulose. This new method will be adapted and applied to all types of modified cellulose beads in order to try and determine the RS content and it will finally be compared against a more traditional approach, enzymatic degradation of the cellulose matrix followed by HS-trap GC analysis.

The specific focus of this work will be the search for an optimal calibration method for the TD-GC analysis of RS in modified cellulose beads. Ideally, TD should be calibrated using gas-phase standards in order to ensure there is no difference in the adsorption and desorption pathways between the sample and the reference. In practice however, gas-phase standards are expensive and/or difficult to obtain, so calibration is routinely done using liquid standards. Calibration becomes a critical step in an analysis like this, where TD is used for a direct thermal extraction of RS from a solid sample and where the calibration is performed using a standard liquid mixture, because in this case, the adsorption and desorption pathways of RS in the sample and the reference differ significantly. Offline (OLC) and inline (ILC) liquid calibration will be explored and compared, as they have already been successfully used for calibration of a TD-GC method for determination of RS in insoluble solid matrices, such as ours.

3. MATERIALS AND METHODS

3.1. Chemical reagents

- methanol (MeOH), 99.8% (Fisher Scientific, Loughborough, UK)
- ethanol (EtOH), 99.8+% (Fisher Scientific, Loughborough, UK)
- tert-Butanol (2-methyl-2-propanol) (tBA), 99.7% (Honeywell, Seelze, Germany)
- toluene, 99,8% (Acros Organics, Geel, Belgium)
- acetonitrile (ACN), 99.9% (Fisher Scientific, Loughborough, UK)
- lithium chloride (LiCl), 99% (for analysis, anhydrous) (Acros Organics, Geel, Belgium)
- mesoporous silica (MPSi) (Syloid AL-1FP (pore size 2-3 nm) and Syloid XDP3050 (pore size 25 nm)), provided by Grace Davison (Worms, Germany)

3.2. Samples

Samples of modified cellulose beads were provided by Prof. Dr. Van den Mooter of the Drug Delivery and Disposition laboratory at the Department of Pharmaceutical and Pharmacological Sciences at KU Leuven (Leuven, Belgium). They manufactured and provided us with four different types of beads:

1. **unmodified cellulose beads (CB)**, prepared by following the protocol from Trygg. et al. (*Trygg et al., 2013*)
2. **dialdehyde cellulose beads (DAC)**, prepared by periodate oxidation of CBs (*Xie et al., 2022*)
3. **ethylenediamine dialdehyde cellulose beads (DAC-EDA)**, developed by introducing ethylenediamine (EDA) on dialdehyde cellulose (DAC) (*Xie et al., 2023*)
4. **TEMPO-oxidized cellulose beads (OCB)**, prepared by introducing carboxyl groups on the cellulose beads by TEMPO-mediated cellulose oxidation (*Xie et al., 2021*)

3.3. Instrumentation: TD-GC-FID/MS

Sample introduction was performed using a modified Turbomatrix ATD 350 thermal desorber. Modifications were made in-house (*Asfaw et al., 2019*) to allow for direct introduction of standard solution into the desorption flow, therefore enabling ILC. This was done by connecting a GC injector (150 °C) to the sampling end of an empty TD sample tube by a heated transfer line. Empty stainless steel TD tubes (89 mm x 6.35 mm o.d.) were used to carry samples as well as drying and sorbent materials used for calibration. They were capped with polytetrafluoroethylene (PTFE) caps containing an O-ring as a sealing element. An Air monitoring trap from Perkin Elmer (Waltham, MA, USA) was used as TD trap material. GC analyses were executed on a Clarus 680 GC with an FID detector and a SQ8T mass spectrometer. Tubes, caps, traps and equipment were all purchased from Perkin Elmer (Waltham, MA, USA), except the tubes prepacked with Carbosieve SIII 60/80, which were acquired from Camsco (Houston, TX, USA). GC separations were carried out on ZB-624plus column (30 m x 0.53 mm, df = 3 µm) from Phenomenex (Torrance, CA, USA). A 10-microliter syringe with an 80 mm needle (Hamilton, Reno, NV, USA) was used in application of reference solutions. Quartz filter paper (MN QF-10, Ø = 50 mm) from Macherey-Nagel (Düren, Germany) was used to immobilize the sample and the sorbent and drying materials inside the tubes.

3.4. Standard and sample preparation

For optimization of TD parameters (whether ILC or OLC), a reference mixture was prepared by adding 100 mg of MeOH, EtOH and tBA each, to a 50 mL volumetric flask, already containing a small volume of water to minimize analyte loss by evaporation. The process of adding the reference to a partially filled volumetric flask was used for all further described solutions prepared in this work. The flask was then brought to volume with water. Finally, 5 µL of the reference mixture was injected directly into the TD desorption flow with a microliter syringe through the installed GC injector (in ILC) or transferred onto adsorbent-filled TD sample tube under a constant flow of nitrogen, again using a microliter syringe (in OLC). The suitability of toluene and ACN as solvents was also investigated, and reference mixtures using them as solvents, instead of water, were prepared and analyzed in the same manner.

To test the effect of water on the cold trap, a toluene solution was made by weighing of 50 mg of each analyzed alcohol (MeOH, EtOH and tBA) into a 25 mL volumetric flask and bringing the flask to volume. The solution was then dried with sodium carbonate. 5 µL of the dried toluene solution was injected directly into the TD desorption flow through the installed GC injector, with a microliter syringe, either alone, preceded by or followed by 5 µL of demineralized water, depending on the test run.

For calibration, two 25 mg/mL standard stock solutions were prepared: MeOH in water and EtOH in water in order to avoid potential weighing mistakes and reduce MeOH evaporation. Five ILC calibration solutions, ranging from 0.1 mg/mL to 5 mg/mL for MeOH and EtOH and from 5 mg/mL to 100 mg/mL for tBA, were prepared by directly weighing the tBA into a 25 mL volumetric flask, pipetting the same volume of each stock solution and finally bringing the flask to volume with water.

3.5. Tube filling

For the OLC approach, empty TD sample tubes were manually filled with of 2 types of MPSi, 100 mg of each, sandwiched between two triple layers of QF and separated by 1 layer of QF, to avoid mixing (*Figure 3*). The two kinds of MPSi, Syloid XDP 3050 and Syloid AL-1 PF silica, were inserted so that the one with larger pores sat first in the direction of the loading flow. A 5 µL calibration solution volume was then loaded onto the MPSi tube, by a microliter syringe, under a flow of nitrogen gas. The syringe was positioned in such a way, that the tip of the needle gently rests on the stainless-steel mesh of the tube so that the solution is directly taken up by the adsorbent material.

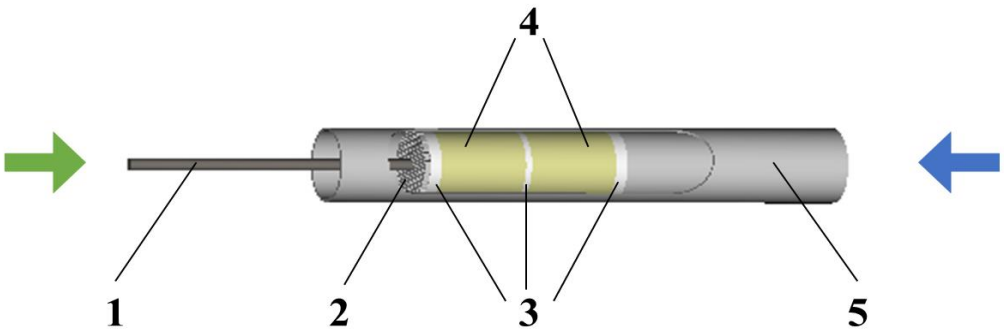

Figure 3. OLC tube filling.
The syringe needle (1) touching the sorbent retaining mesh (2). A TD sample tube (5) filled with two kinds of MPSi (4), sandwiched between two triple layers of QF (3) and separated by a single layer of QF (3). The blue arrow represents the direction of the primary desorption flow and the green arrow the direction of the sample loading gas flow.

The TD sample tube which connects the heated GC injector to the cold trap in ILC was manually filled with 500 mg of anhydrous LiCl. LiCl was sandwiched in the sample tube between 3 layers of QF and immobilized by a sorbent-retaining mesh on either side (*Figure 4*).

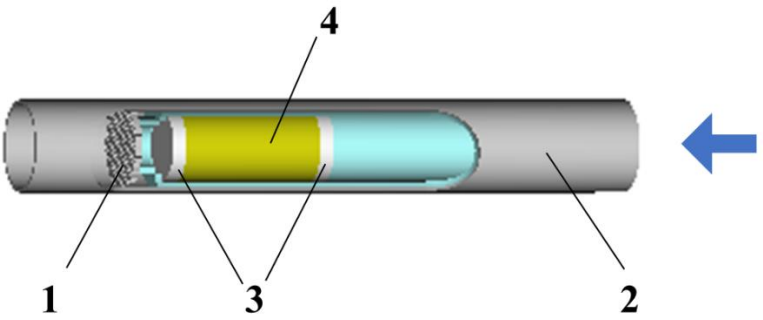

Figure 4. ILC tube filling.
A TD sample tube (2) filled with 500 mg of LiCl (4), sandwiched between two triple layers of QF (3) and immobilized by a stainless-steel mesh (1). The blue arrow represents the direction of the primary desorption flow.

In regards to sample analysis, 10 mg of one kind of cellulose beads (CBs) was placed in an empty TD sample tube, sandwiched between two triple layers of QF (*Figure 5*), and analyzed by TD-GC.

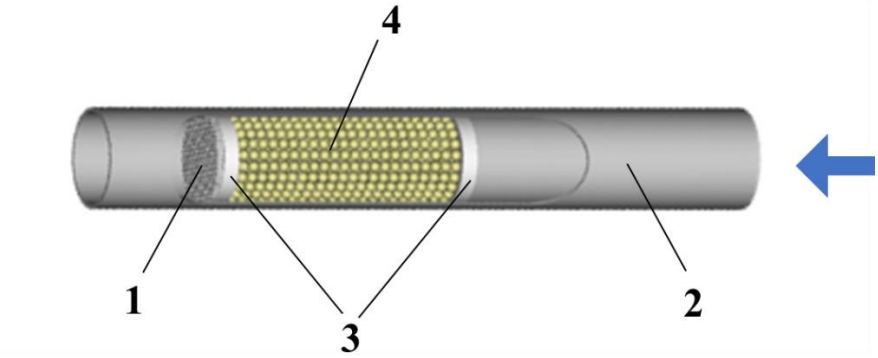

Figure 5. Sample analysis tube filling.
A TD sample tube (2) filled with 10 mg of CBs (4), sandwiched between two triple layers of QF (3) and immobilized by a stainless-steel mesh (1). The blue arrow represents the direction of the primary desorption flow.

3.6. Conditioning and drying

MPSi tubes were conditioned in the thermal desorber at 400 °C for 20 minutes under a constant nitrogen flow >130 mL/min.

LiCl filled tubes were dried at 250 °C for 25 minutes under a constant nitrogen flow > 400 mL/min.

4. RESULTS AND DISCUSSION

4.1. Preliminary analyses

Preliminary TD-GC analyses of all 4 types of CB samples showed the presence of MeOH, EtOH and tBA. This discovery was the basis of choosing a Perkin Elmer Air monitoring trap as our cold trap. Furthermore, given the physio-chemical properties of the encountered alcohols, water, toluene and acetonitrile were chosen as reference solution solvents to be explored for method optimization. Water would be the healthier, more eco-friendly option, but it is not a particularly "GC-friendly" solvent *(Kuhn, 2002*). So, toluene and acetonitrile were investigated as solvents as well.

4.2. Optimization of TD parameters for ILC

As stated earlier in this work, ILC can be used to validate OLC or it can be used as a calibration method by itself. OLC is however, the preferred method because of its simplicity, ease of operation and yearlong experience of using the method. Our original aim was to use ILC to optimize and validate OLC, but in order to do that we had to optimize ILC first.

Throughout the entire process of ILC optimization the same GC parameters (*Table 1*) were used:

Parameter	**Settings**
Column flow	4.50 mL/min
GC oven program	40 °C hold 5 min, 45 °C/min to 230 °C, 230°C hold 5 min
Auxiliary pressure	20 kPa

Table 1. GC parameters, which were kept constant.

All direct (inline) injections were performed by injecting 5 µL of a reference mixture volume (either water, toluene or acetonitrile solution) through the installed GC injector using a microliter syringe.

The starting TD conditions (*Table 2*) were set as follows:

Parameter	Settings
Desorption temperature	350 °C
Desorption time	20 min
Desorption flow	20 mL/min
Trap low temperature	30 °C
Trap high temperature	350 °C
Valve temperature	290°C
Trap hold time	10 min
Inlet split	2 mL/min
Outlet split	4 mL/min

Table 2. Staring TD parameters.

To examine the influence of different TD parameters, a set of experiments was performed in which the detector response for each RS was supervised in function of a single parameter. Once a seemingly optimal value for the investigated parameter was chosen, that one became the new constant parameter and a different parameter was explored.

4.2.1. The isobutene issue

The first few completed test-runs of both the water and toluene reference solution, showed the presence of some unexpected peaks in both chromatograms (*Figures 6 and 7*). MS was used to help identify them as isobutene and acetaldehyde, and some further research was done to investigate where they could be coming from (contamination from alcohol standards, contamination from sample tubes, degradation products…).

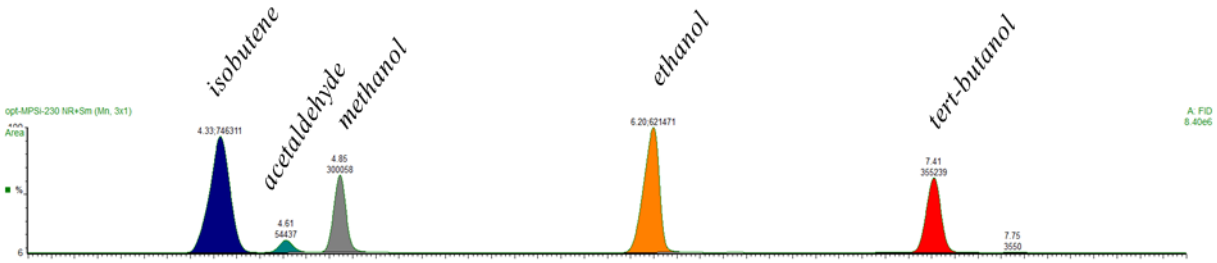

Figure 6. Toluene reference solution. Peaks from left to right: isobutene, acetaldehyde, methanol, ethanol, tert-butanol.

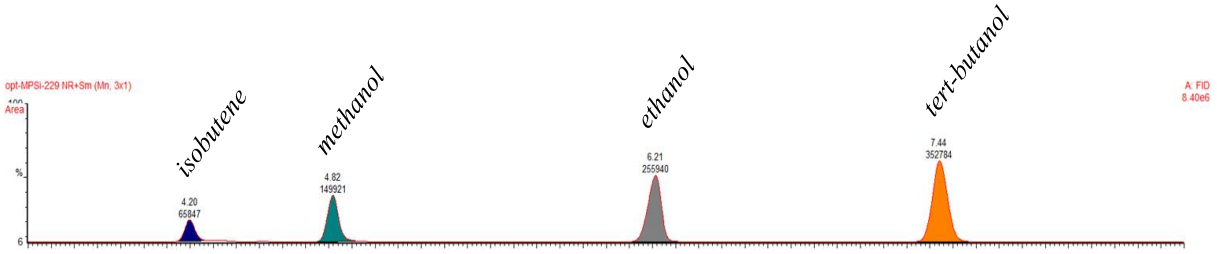

Figure 7. Water reference solution. Peaks from left to right: isobutene, methanol, ethanol, tert-butanol.

Literature research (*Brzeski and Skurski, 2019; Knifton et al., 2001; Sheu et al., 1994*) showed that isobutene can be used as a starting material to synthesize tBA, so we assumed that the reverse process could also be possible. A quick stability analysis of a tBA in water solution indicated a small quantity of isobutene immediately present in the first sample, which was a freshly made solution, meaning that the isobutene is probably a contaminant in the tBA standard to begin with. Furthermore, during the first 2 days of the stability analysis, the isobutene to tert-butanol ratio remained stable. However, after 4 days, the tBA peak area decreased, the isobutene peak area increased, and consequently, the isobutene to tBA ratio increased. This, proved that isobutene is also formed spontaneously in an aqueous tBA solution and that it could be a tBA degradation product. This is however, not the focus of this particular research. So, even though it is certainly an interesting issue worth exploring in more detail, the topic wasn't pursed any further on this occasion.

4.2.2. Trap Low Temperature

The first investigated parameter was the trap low temperature. The temperature was lowered from 30 °C to 4 °C in order to capture the volatile gases more effectively on the focusing cold

trap, to avoid breakthrough and therefore achieve a higher signal GC signal. Temperatures lower than 4 °C weren't tested (at first) for the fear of the cold trap freezing over.

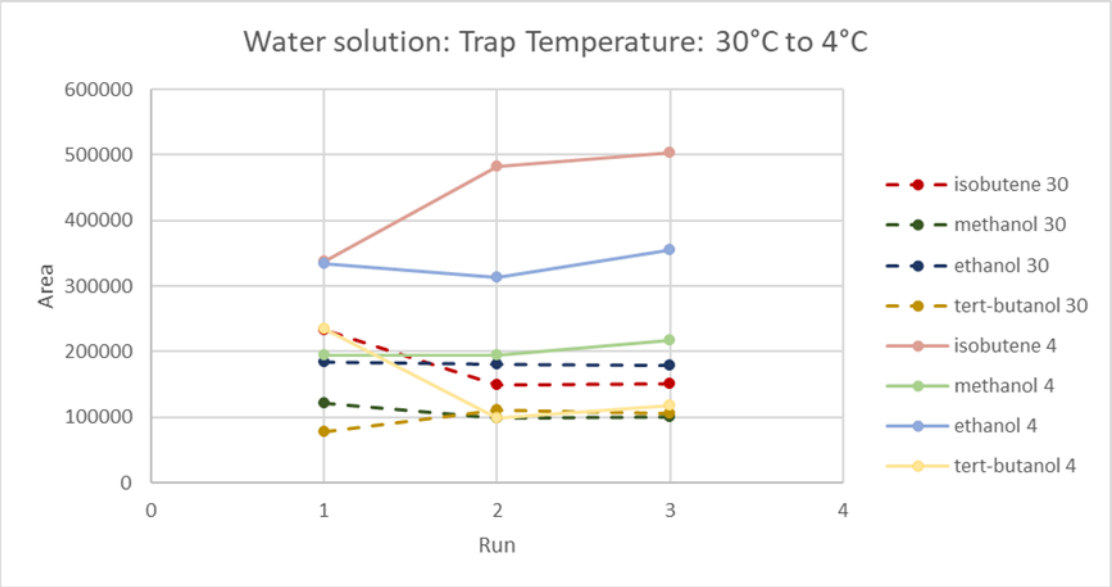

Figure 8. Illustration of the influence of the lower trap temperature (30 and 4 °C) on the detector response, for the water solution.

The water reference solution showed bigger peak areas for MeOH, EtOH and tBA at a trap temperature of 4 °C than 30 °C (*Figure 8.*), which was what we wanted, whereas the toluene reference solution showed smaller peak areas for MeOH and EtOH at 4 °C and a bigger tBA peak area than at 30 °C.

However, there was an additional problem with the toluene solution. The isobutene peak showed peak splitting at 4 °C (*Figure 9.*), which could potentially signify further isobutene (tBA) degradation.

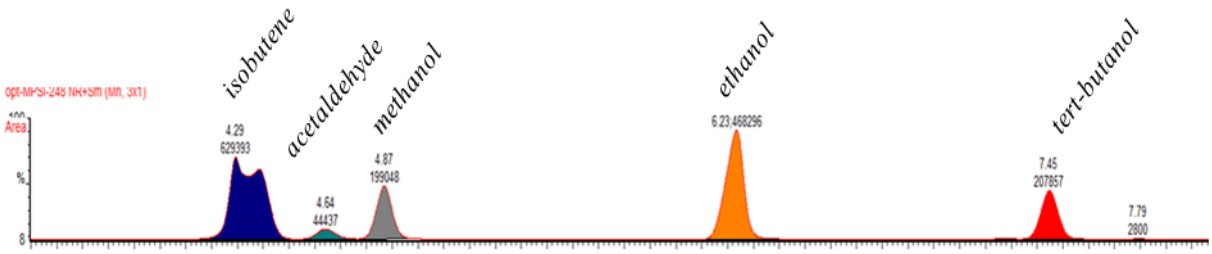

Figure 9. TD-GC-FID chromatogram of the toluene solution at the lower trap temperature (4 °C).

This behavior of the toluene solution combined with the fact that toluene wasn't showing any relevant advantages compared to the water solution, while also being the ecologically less favorable solvent, made us decide to abandon the toluene reference solution. The decision was made to continue TD optimization with only the aqueous reference solution and the newly adopted trap low temperature of 4 °C.

4.2.3. Valve and Tube Temperatures

Next two parameters that were explored were valve and tube temperature. Previous tests pointed us in the direction of lowering system temperatures in order to gain larger alcohol recoveries. However, it was important to keep in mind that these temperatures could not go lower than the boiling temperature of the solvent. Otherwise, the solution might condense inside the apparatus. Therefore, the tube temperature was lowered from 350 °C to 150 °C and valve temperature was decreased from 290 °C to 150 °C.

With the newly set conditions, peak areas for MeOH and EtOH remained similar to the values obtained in the previous test, but the tBA, isobutene and acetaldehyde peak areas changed drastically (*Figure 10*). The tBA peak area was significantly larger than before while the isobutene peak area was smaller, demonstrating a seemingly inversely proportional relationship to each other. There was no acetaldehyde peak detected (*Figure 11*).

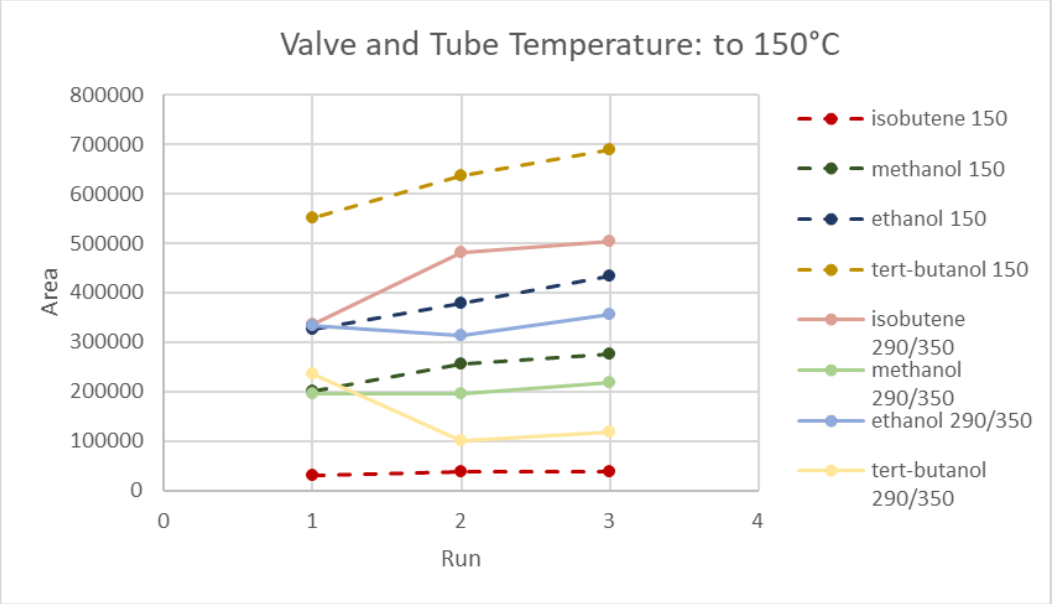

Figure 10. Shows the influence of lowering valve and tube temperatures on the detector response.

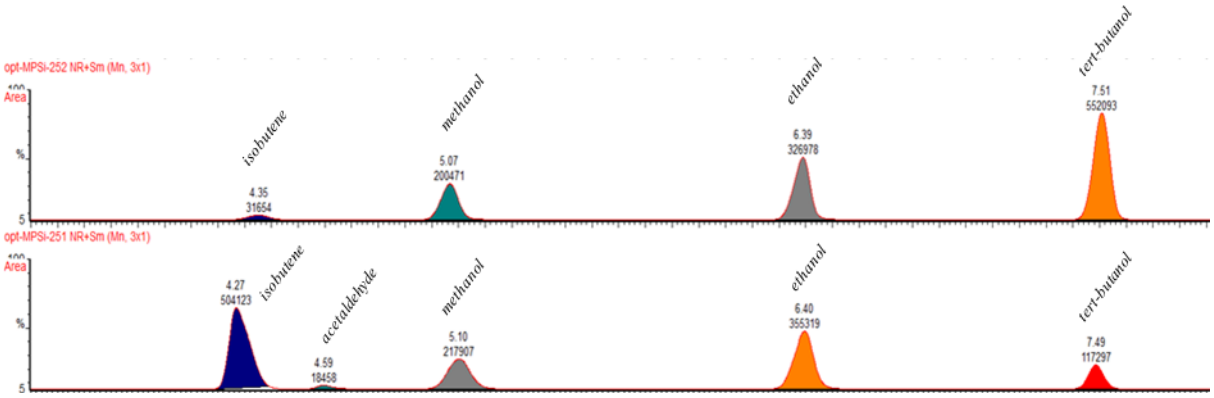

Figure 11. Effect of the lower valve and tube temperatures on the detector response. The upper chromatogram shows the chromatogram recorded under new TD conditions (Valve and Tube temperature at 150 °C) and the chromatogram below under the old conditions (Valve temperature at 290 °C and Tube temperature at 350 °C).

One conclusion that could be derived from these results, and one that fits well in our "tBA degradation to isobutene" theory, is that there was significant tBA heat-triggered degradation happening inside the system, and that the degradation was avoided by reducing the temperature inside the apparatus. Therefore, for all further experiments both the valve and tube temperature were set to 150 °C.

4.2.4. Acetonitrile as solvent

In order to go lower with the trap temperature to try and recover even more alcohols, it was necessary to change the solvent. Because water freezes at 0 °C, lowering the trap low temperature below 0 °C would make water freeze on the trap, which could potentially cause mechanical issues or damage to the instrument. Acetonitrile was chosen as the alternative solvent because it is compatible with the investigated alcohols and has a freezing point at -35 °C.

To start, it was necessary to check if we could get good peak separation between all our analytes and the solvent. The newly adapted TD parameters were kept the same for each run, with multiple GC temperature and pressure setups tried out. Relevant chromatograms are presented below (*Figure 12*).

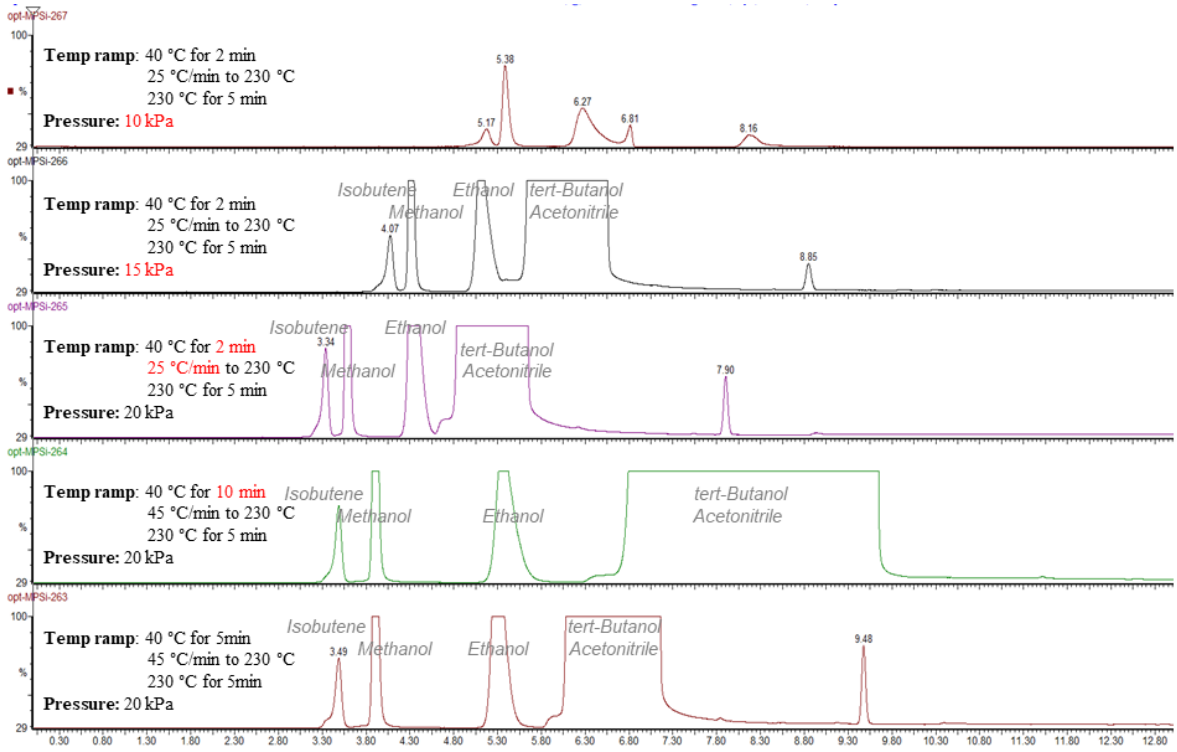

Figure 12. Influence of different GC setups on analyte elution.

As depicted in the chromatograms, the tBA peak couldn't be separated from the acetonitrile peak, so acetonitrile was dropped as a potential solvent.

4.2.5. Effect of water on the cold trap

Considering water proved to be the best performing solvent so far, the issue of increasing recovery needed to be approached from a different angle. The initial idea was that water molecules might be taking up binding spaces on the TD cold trap and therefore preventing the alcohols from being adsorbed to and retained on the cold trap. Instead of being trapped, the majority of the analyzed alcohols were then flushed away from the system. To explore the hypothesis, a small experiment was carried out.

Three direct injections were made following the same ILC procedure that was used so far (*see 4.2.*):

1) For the first run, a minute after the primary desorption time had started, 5 µL of the dried toluene solution was directly injected into the TD. 10 minutes later, 5 µL of demineralized water was injected in the same manner.

2) For the second run, a minute after the primary desorption started, 5 µL of demineralized water was injected and after 2 minutes 5 µL of the toluene solution.

3) Lastly, only the toluene solution was injected, a minute after the primary desorption had started.

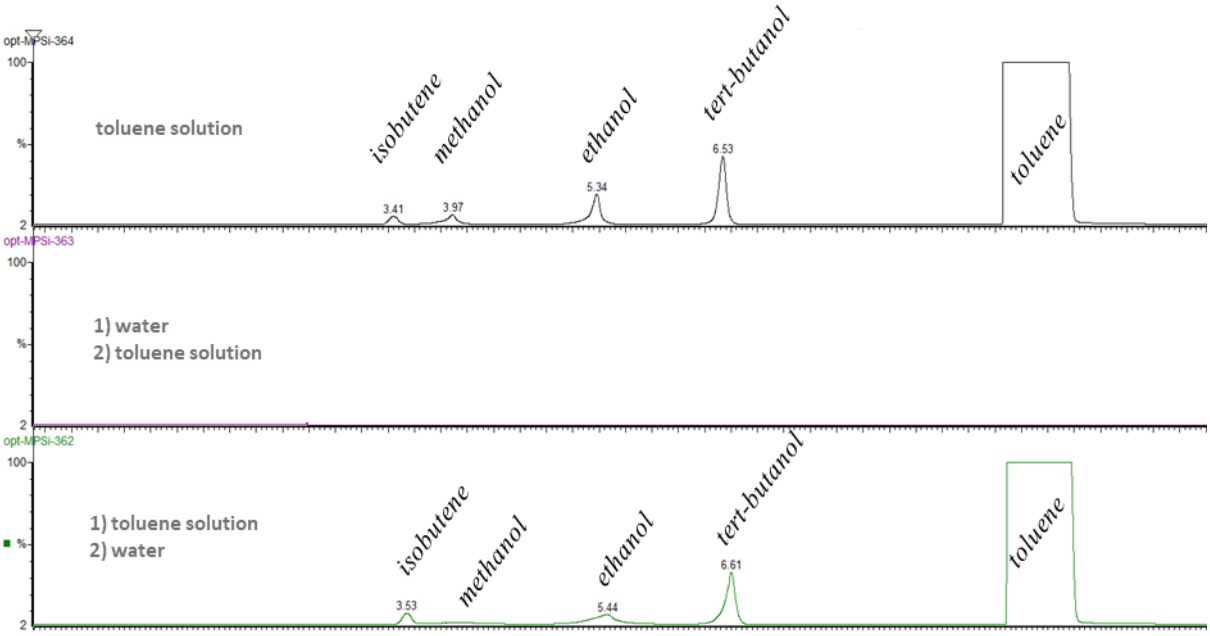

Figure 13. Effect of water on the cold trap. The bottom chromatogram shows the first run (toluene solution, water), the middle one shows the second run (water, toluene solution) and the top chromatogram the third run (just the toluene solution).

The main takeaway from this experiment was that the FID trace of the second direct injection (where water was injected first) showed no alcohol or toluene peaks, compared to the first direct injection (where toluene solution was injected first) whose FID trace contained peaks of all the analyzed alcohols and the solvent. Additionally, in the FID trace of the last run, where no water was injected, the peak shape of all analyzed alcohols was better than that of the first run (toluene solution injection, followed by a water injection) and the alcohol recovery was larger (*Figure 13*). This tentatively confirmed the hypothesis that there was a significant water interference on the VOCs adsorption on the cold trap.

4.2.6. LiCl as water absorbent

The best way to combat the water interference issue, would be to prevent water from reaching the cold trap in the first place. The idea was to place an adsorbent material in the, thus far, empty sample tube connecting the direct injector port to the TD, so that when the injected solution passed through to the trap, water molecules got captured on the adsorbent material, while the VOCs passed through to the TD cold trap. The adsorbent material chosen was anhydrous LiCl.

Literature (*Masset, 2009*) suggested that the dehydration of $LiCl \cdot H_2O$ is a two-step process, happening between 99-110 °C and 160-180 °C. Thus, the tube temperature needed to be lowered so that the water could be trapped by the LiCl, but not released from it. Two tube temperatures were tested: a higher one, at 98 °C, because of the fear of water condensation, and a lower one, at 50 °C. Other TD parameters were kept the way they have been optimized: trap low temperature at 4 °C and valve temperature at 150 °C.

To start, we checked if LiCl retained any water and at which temperature, by analyzing the water MS trace of 3 different direct injection TD-GC runs:

1) a blank run, to check for pre-existing water in the system
2) a direct injection of the reference solution, at a tube temperature of 98 °C
3) a direct injection of the reference solution, at a tube temperature of 50 °C

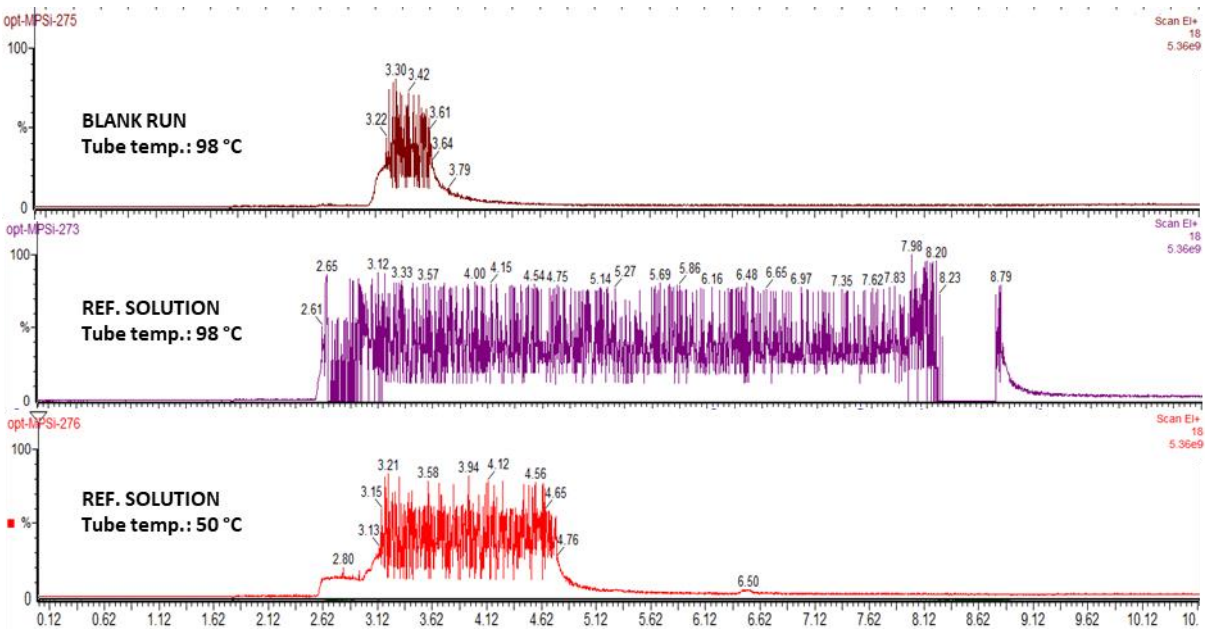

Figure 14. Adsorbent properties of anhydrous LiCl.

Comparing the blank run to the reference solution runs (*Figure 14*), it was clearly visible that a significant amount of water was brought onto the GC column by injecting the reference solution. Moreover, a tube temperature of 98 °C seemed to be too high, with the dehydration of LiCl seemingly already happening, which was why more water was detected at 98 °C, compared to 50 °C.

From the FID traces of the reference solutions (*Figure 15*), it was apparent that there was more alcohol recovery at 50 °C than at 98 °C, almost 45% more for methanol and ethanol and around 30% more for tBA. This made sense considering the information obtained from the MS traces above: at 50 °C there is significantly less water on the cold trap than at 98 °C, and therefore more alcohol molecules are retained and later injected onto the GC column.

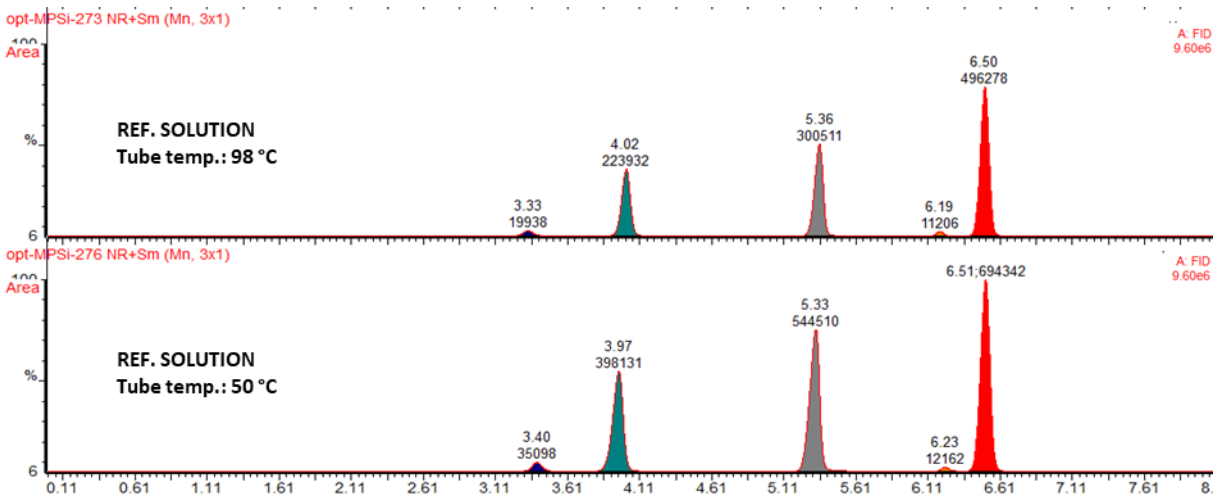

Figure 15. Influence of LiCl and lower tube temperature on alcohol recovery.

All in all, anhydrous LiCl as a water adsorbent proved to be a good solution, simultaneously eliminating the water interference issue and delivering a larger alcohol recovery. Another benefit of the lack of water on the cold trap was that the issue of water potentially freezing on the cold trap was eliminated. The trap low temperature could now be decreased even further, which was the original request. Further ILC optimization was therefore continued using LiCl as water adsorbent.

Trap low temperatures of -10 °C and -20 °C were investigated and compared to the current trap low temperature of 4 °C.

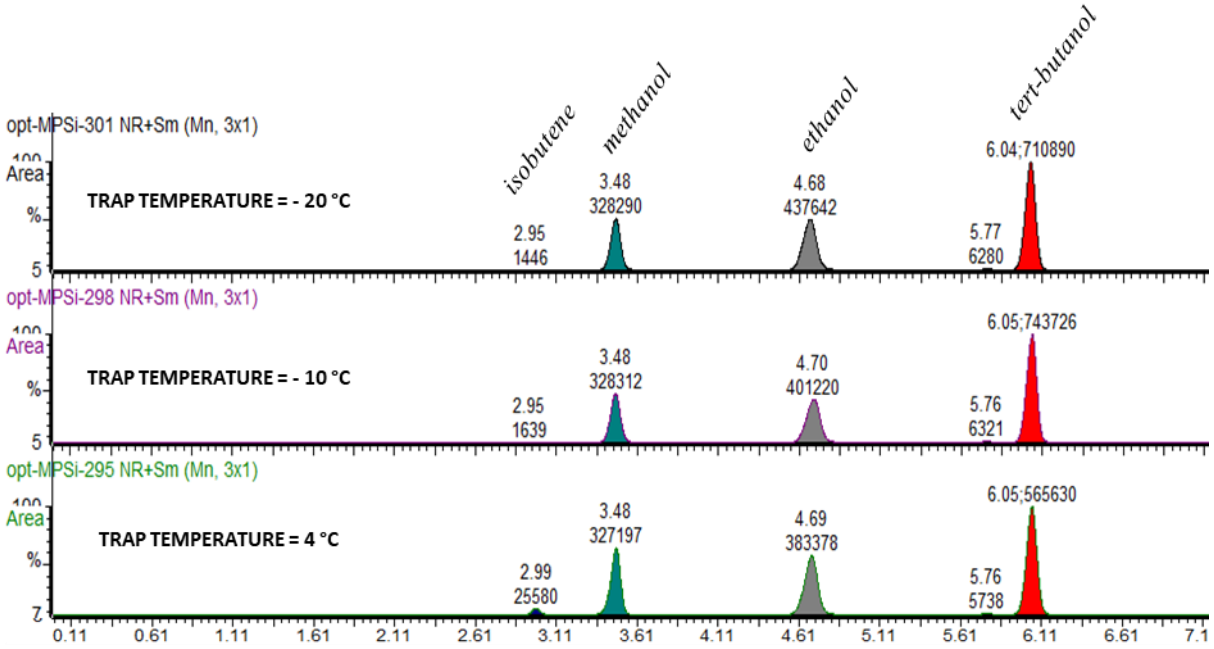

Figure 16. Influence of trap low temperature on alcohol recovery.

The decrease in trap temperature to -10 °C had the largest effect on the tBA recovery. However, slightly more ethanol was detected as well. Additionally, as we saw before by lowering the tube and valve temperature, the increase in tBA recovery came at the expense of isobutene (*Figure 16*). By lowering the trap temperature, the isobutene peak further decreased in size, which could be taken as additional evidence to support the theory that there is degradation of tBA to isobutene happening inside the apparatus, at higher system temperatures.

Further decreasing the trap low temperature to -20 °C showed no significant difference in the amount of alcohols recovered (*Figure 16*). So, it was decided that a trap low temperature of -10 °C was the optimum condition.

Parameters	Settings	
	Initial	**Optimized**
Desorption temperature	350 °C	50 °C
Desorption time	20 min	20 min
Desorption flow	20 mL/min	20 mL/min
Trap low temperature	30 °C	-10 °C
Trap high temperature	350 °C	350 °C
Valve temperature	290 °C	150 °C
Trap hold time	10 min	10 min
Inlet split	2 mL/min	2 mL/min
Outlet split	4 mL/min	4 mL/min

Table 3. Overview of TD settings after optimization. Parameters which have been optimized are marked in black, and parameters which were not are marked in grey.

4.3. Optimization of TD parameters for OLC

Once the TD-GC system has been optimized to detect 100% of the alcohols injected directly onto the system (*Table 3*), the inline approach could be used to help optimize the standard offline approach. For offline calibration optimization, 2 adsorbent materials were tested: mesoporous silica (MPSi) and Carbosieve S-iii (CS). CS was chosen as it was the only commercially available adsorbent that successfully trapped methanol. MPSi was selected as an alternative adsorbent material, with which the lab had a lot of positive experience.

In order to optimize OLC, it was necessary to optimize two separate processes: sample tube loading and primary desorption. Within the tube loading process, the parameter to be investigated was the total volume of nitrogen necessary to successfully load the tube with reference solution (sweep volume). The amount of reference solution injected onto the tube was kept constant at 5 µL. To optimize the primary desorption, the ideal length and temperature of the process needed to be evaluated. Other TD-GC parameters were kept constant and were set as follows (*Table 4*):

Parameter	Value
Desorption flow	20 mL/min
Trap low temperature	-10 °C
Trap high temperature	350 °C
Valve temperature	200 °C
Trap hold time	10 min
Inlet split	2 mL/min
Outlet split	4 mL/min
Desorption temperature	350 °C
Desorption time	20 min

Table 4. TD parameters used for OLC optimization.

The desorption time and temperature given in Table 4. were only used to optimize OLC tube loading, but were later optimized during primary desorption optimization.

GC parameters were kept the same as for ILC optimization (*Table 1*).

4.3.1. Tube loading: sweep volume

Using an inert gas to facilitate the transport of analytes from the injection point in the tube to the sorbent bed is advisable when performing OLC *(https://markes.com; Woolfenden, 1997*). However, because a too high gas flow might cause analyte breakthrough, it was necessary to find an optimal loading procedure that would ensure that no analyte was lost during the loading process. Furthermore, considering OLC tubes were filled with an adsorbent material, rather than anhydrous LiCl, previous issues encountered with water on the TD trap might resurface. Using a sweep gas to load the sample could potentially help with this problem as well. It might be possible to find a combination of nitrogen flow and loading time, which would flush out (some of the) solvent from the tube, but still retain our analytes on the sorbent bed.

Even though the parameters varied in this experiment were the nitrogen gas flow and loading time, the combined effect of them on the reference loading was better represented by expressing them as a total sweep gas volume used to load the tube. The graphs below (*Figures 17 and 18*) demonstrate the influence of different sweep gas volumes on both the analyte loading efficiency and the elimination of water.

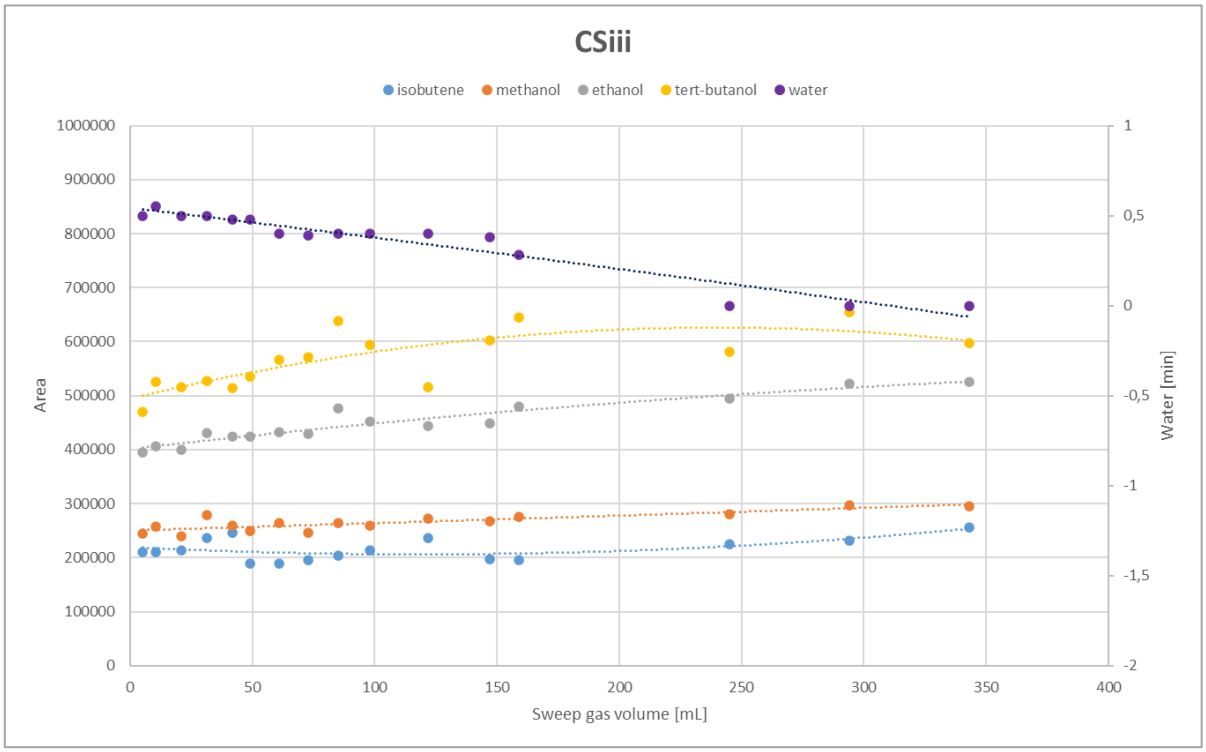

Figure 17. Influence of increasing the sweep gas volume on the efficiency of reference loading and water elimination, with CS as the adsorbent material.

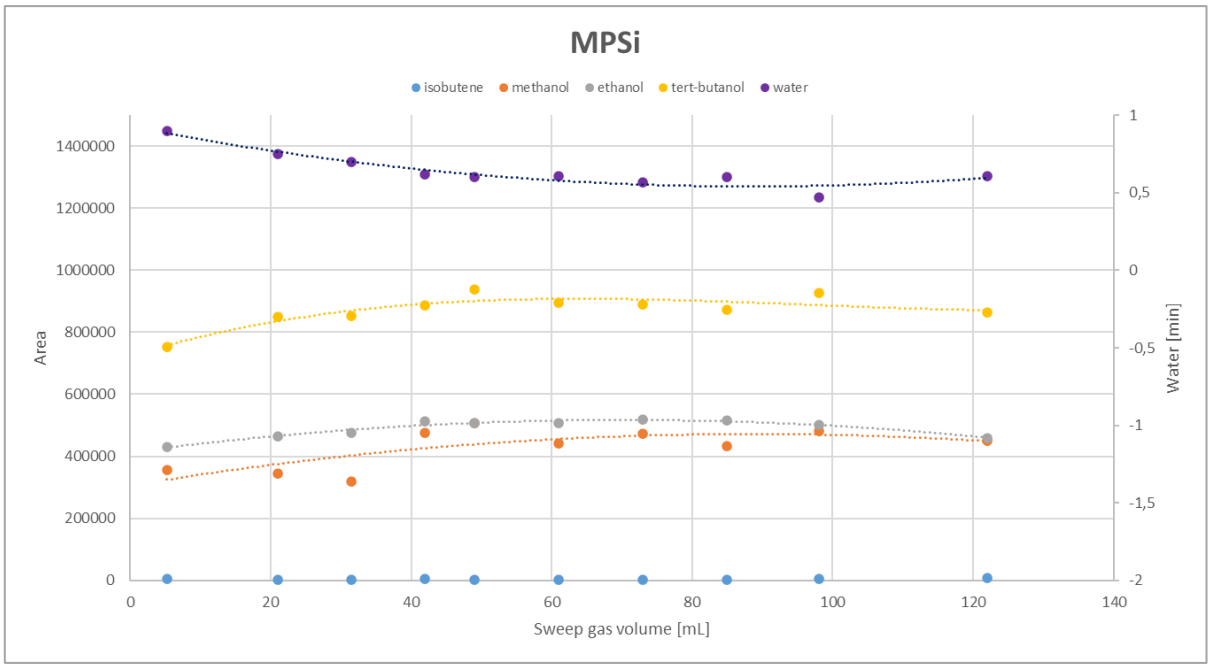

Figure 18. Influence of increasing the sweep gas volume on the efficiency of reference loading and water elimination, with MPSi as the adsorbent material

'Water [min]' (right Y-axis in *Figures 17 and 18*) was used to describe the amount of water reaching the GC detector in a single TD-GC run. This originated from an attempt to quantify the amount of water in the system for each TD-GC run so that water elimination could be tracked and compared between different runs. The minutes in 'water [min]' represent the length of time on an MS water trace during which the detector was so overloaded with water that it couldn't produce a typical MS trace and it flat-lined (*as shown in Figure 19.*).

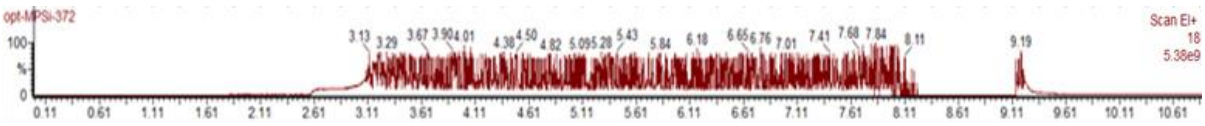

Figure 19. An MS trace showing a water overload of the detector.

It is evident from Figures 17 and 18 that tubes filled with MPSi yielded higher analyte recoveries than the CS packed tubes, even though the water content did not drop as much as it did for CS tubes. Additionally, significantly less isobutene was detected on the MPSi tubes compared to the CS ones.

The optimal total sweep gas volume for the MPSi tubes was 73.5 mL, achieved by injecting the reference solution on the tube under a constant flow of nitrogen of 24.5 mL/min, and leaving the tube to be flushed with nitrogen, under the same flow, for 3 minutes. For CS-filled tubes the optimal total sweep gas volume chosen was 245 mL, acquired by setting the gas flow to 24.5 mL/min as well, but leaving the tubes to be flushed with nitrogen for 10 minutes.

4.3.2. Primary desorption: time and temperature

Considering the desorption parameters used in the loading optimization investigation weren't yet optimized, the absolute recoveries found in that experiment weren't reliable values. This is why in order to decide the optimal adsorbent material for OLC, TD tubes filled with both adsorbents (CS and MPSi) were subjected to desorption optimization. The range of desorption time investigated was the same for both adsorbents: 10 to 40 minutes. The range of potential ideal desorption temperatures is, however, restricted by the thermal stability of the adsorbent material inside the sample tube. In this case, the declared maximum temperature for CS is 400 °C, while MPSi is thermally stable up to almost 600 °C (*Mitran et al., 2020*). This was why the investigated temperature ranges for CS were 300 to 400 °C and for MPSi 300 to 450 °C.
Because changes in desorption temperature might impact the necessary desorption time, and vice versa, optimizing these two parameters wasn't done in the same way as for ILC. To optimize ILC, the detector response for each analyte was supervised in function of a single parameter (with all other parameters kept constant), whereas here it was investigated as a function of two codependent parameters. A set of multiple GC runs was performed (*Figures 20 – 23*), testing out every desorption temperature with every desorption time, for both adsorbent materials.

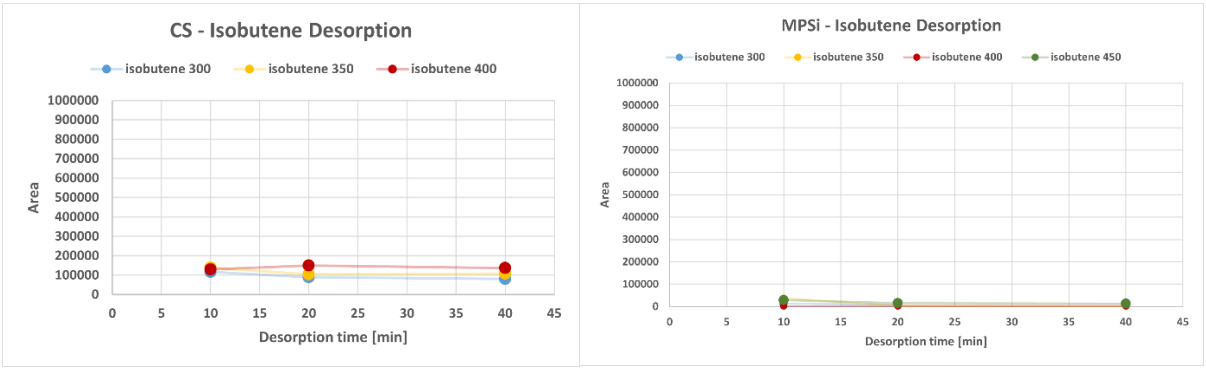

Figure 20. Effect of the primary TD desorption time and temperature on the detector response (illustrated as peak area) for isobutene, for CS (on the left) and MPSi (on the right) tubes, respectively.

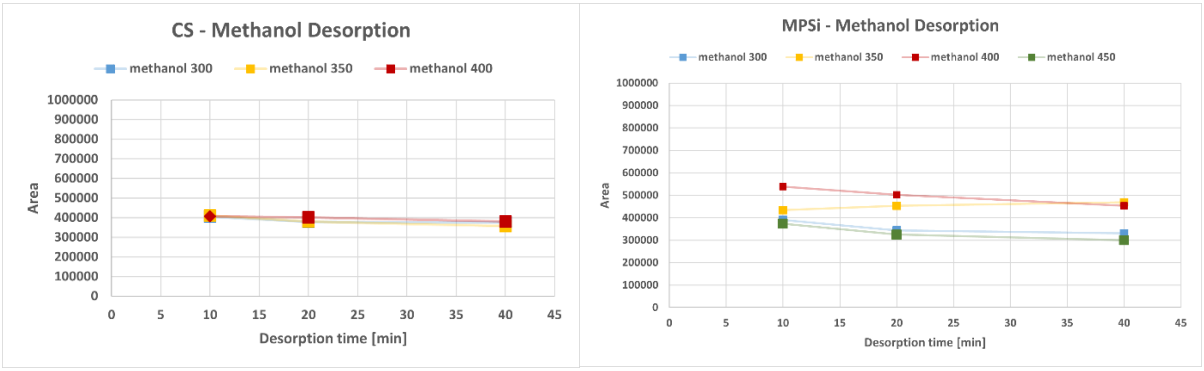

Figure 21. Effect of the primary TD desorption time and temperature on the detector response (illustrated as peak area) for methanol, for CS (on the left) and MPSi (on the right) tubes, respectively.

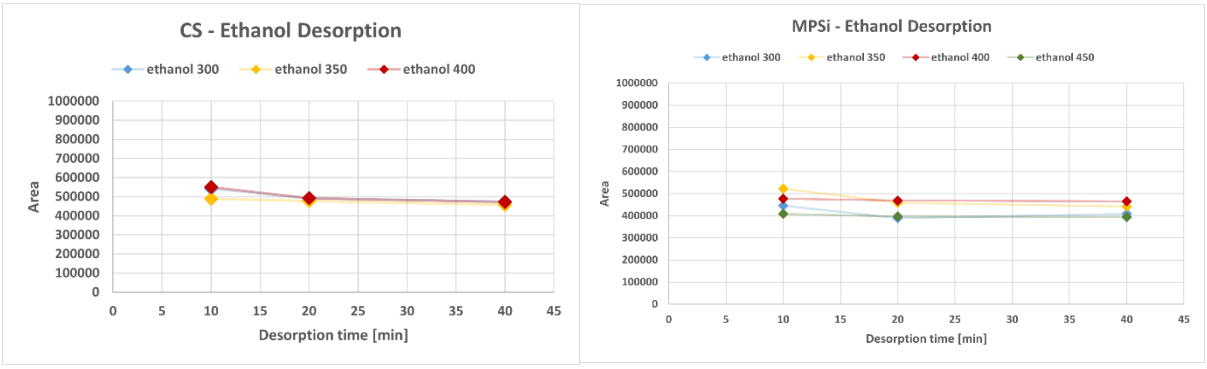

Figure 22. Effect of the primary TD desorption time and temperature on the detector response (illustrated as peak area) for ethanol, for CS (on the left) and MPSi (on the right) tubes, respectively.

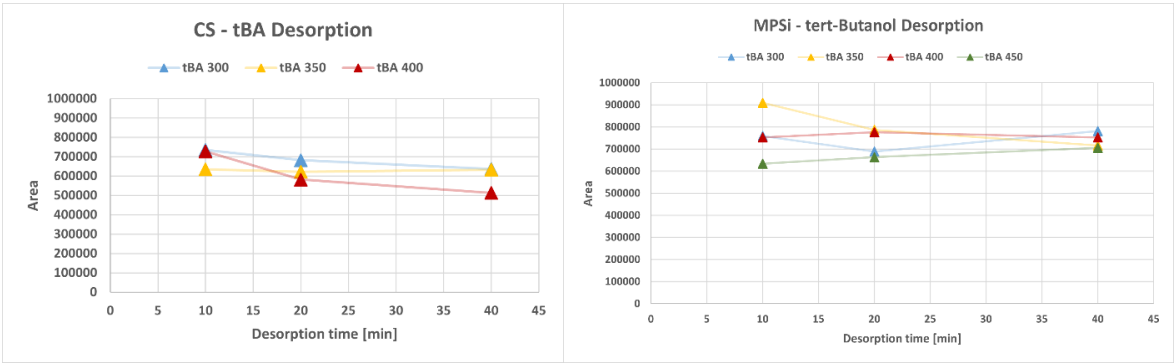

Figure 23. Effect of the primary TD desorption time and temperature on the detector response (illustrated as peak area) for tert-butanol, for CS (on the left) and MPSi (on the right) tubes, respectively.

To achieve maximum primary desorption of all analytes from the sorbent material, the sample tube temperature needed to be set as high as possible. High system temperatures have previously (during ILC optimization) led to formation of isobutene in the apparatus and OLC unfortunately seems to be not different. Both the tube loading chromatograms and desorption optimization chromatograms showed isobutene peaks and in both cases more isobutene seemed to be formed on the CS tubes, compared to the MPSi tubes (*Figures 17, 18 and 20*). Potential explanations could be that there is tBA degradation happening on or is catalyzed by CS or that CS simply retains isobutene better than MPSi. However, these theories weren't explored further since MeOH, EtOH and tBA were the primary analytes of interest.

The greater formation of isobutene on CS compared to the MPSi was the reason that MPSi was chosen as the preferred adsorbent material for this analysis and the optimal desorption time and temperature were set at 25 min and 400 °C, respectively. A desorption time of 25 min was chosen as optimal as there seemed to be too much variation in detector response before the 20-minute mark and desorption temperatures higher than 400 °C seemed to be causing some breakthrough.

4.3.3. Calibration curve

With all parameters optimized and MPSi chosen as the sample tube adsorbent material, the next step was the construction of a calibration curve using OLC. A five-point calibration curve (10, 20, 40, 80 and 100 µg of MeOH and EtOH each, and 10, 100, 250, 400 and 500 µg of tBA on tube) was constructed with each concentration being injected five times. Prior to each OLC concentration quintuplicate, two ILC injections of the same concentration were made. This allowed us to track analyte recovery of OLC as compared to ILC, which we assumed to be 100%.

The resulting OLC calibration curve showed significantly poorer recovery compared to ILC, ranging from 55-65% for MeOH and EtOH and 75-85% for tBA. The variation in recovery presented an additional issue for this type of calibration as it prevented the determination of a correction factor. Secondly, the amount of alcohols recovered throughout the same five concentration injections exhibited an unacceptable RSD: ranging from 3-12% for different MeOH and EtOH concentrations and between 7-10% for tBA. Finally, injecting the two highest concentrations also resulted in overloading of the system with tBA.

Taking all of this into consideration, it was decided that OLC would be dropped as the calibration method of choice and that the calibration would be done by ILC.

4.4. ILC calibration curve

4.4.1. tBA overloading and poor RSD

Simply transferring to ILC didn't resolve all the issues that arose during OLC, mainly the tBA overloading and the poor RSD for MeOH and EtOH. So, it was necessary to address that before attempting a new calibration curve.

In order to avoid overloading of high concentrations of tBA, a switch in detector attenuation (ATT) was introduced. The starting ATT was set at -4 for MeOH and EtOH detection. Then at 5.9 min, the ATT was switched to 0, effectively lowering the detector sensitivity for tBA. This allowed us to continue working simultaneously (one TD-GC injection for all of the three RS) on two calibration curves at lower concentrations for MeOH and EtOH, and another one at higher concentrations for tBA, which was present in samples at a significantly higher amount then the other two RS.

In addition to switching ATT, different split flows were tested in order to reduce the amount of reference, and therefore tBA, reaching the GC column and causing the overload. The outlet split was increased from 4 mL/min to 10 mL/min and the auxiliary pressure from 20 kPa to 23 kPa, to keep the same flow through the GC column as it was earlier. These two combined changes successfully resolved the problem of tBA overloading.

To improve the RSD values of MeOH and EtOH by focusing them on the trap as much as possible, the trap low temperature was further lowered to -30 °C. This resulted in lower RSD values and even better alcohol recovery, with all three alcohol peaks increasing in area threefold, which then consequently, once again led to tBA overloading. However, with MeOH and EtOH peaks now significantly larger, halving the amount of sample that reached the cold trap (and subsequently the GC column and detector) would still give quantifiable peaks at the lowest calibration concentration of MeOH and EtOH and it resolved the tBA overloading at the highest tBA concentrations. To achieve this, the desorption flow was decreased to 10

mL/min and the inlet split was increased to 10 mL/min. Finally, to get the most reliable peak integration the Analog – Digital Conversion had to be tuned. So, the FID detector time constant was lowered from 200 to 50.

TD parameter	ILC Initially optimized	Final ILC settings
Desorption temperature	50 °C	50 °C
Desorption time	20 min	20 min
Desorption flow	20 mL/min	10 mL/min
Trap low temperature	-10 °C	-30 °C
Trap high temperature	350 °C	350 °C
Valve temperature	150 °C	150 °C
Trap hold time	10 min	10 min
Inlet split	2 mL/min	10 mL/min
Outlet split	4 mL/min	10 mL/min

Table 5. TD parameters used for ILC. Parameters which have been optimized are marked in black, and parameters which were not are marked in grey.

GC parameter	Original	Optimized
Column flow	4.50 mL/min	4.50 mL/min
GC oven program	40 °C hold 5 min, 45 °C/min to 230 °C, 230°C hold 5 min	40 °C hold 5 min, 45 °C/min to 230 °C, 230°C hold 5 min
Auxiliary pressure	20 kPa	23 kPa
ATT	0	-4 / 0
ADC	200	50

Table 6. GC parameters used for ILC. Parameters which have been optimized are marked in black, and parameters which were not are marked in grey.

4.4.2. Calibration curve

Three five-point calibration curves (*Figures 24 and 25*) were constructed using the newly optimized ILC parameters (*Tables 5 and 6*). Calibration points chosen were 0.5, 2.5, 5, 10 and 25 µg for MeOH and EtOH, and 25, 50, 100, 200 and 500 µg of tBA injected onto the tube, with each concentration being injected five times.

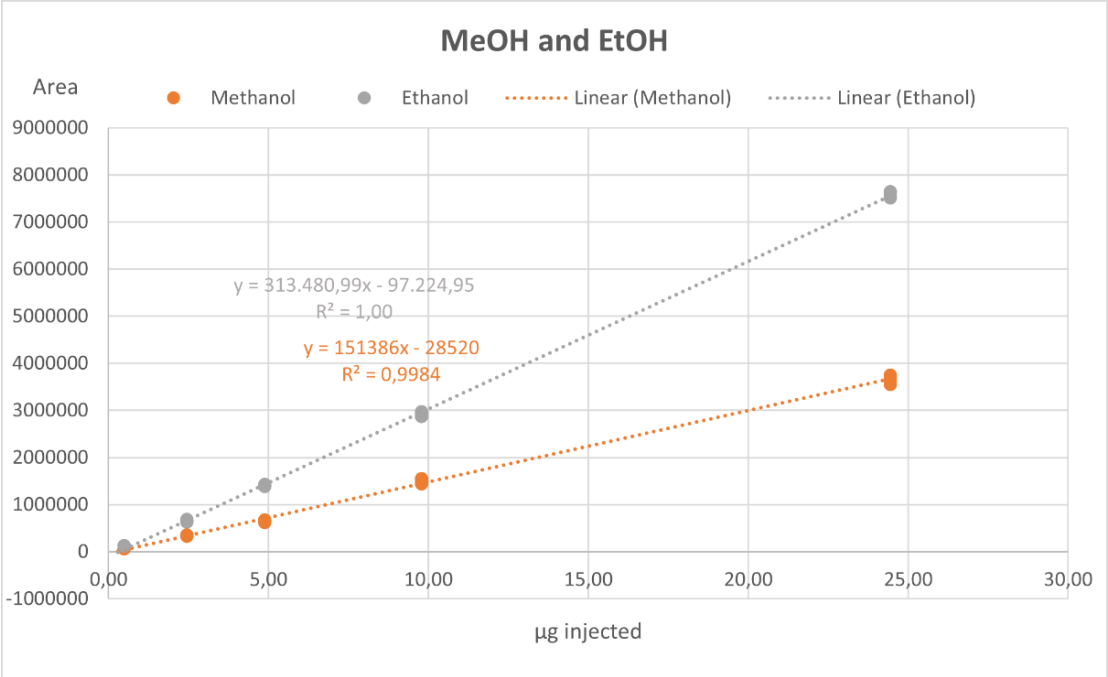

Figure 24. MeOH and EtOH calibration curves.

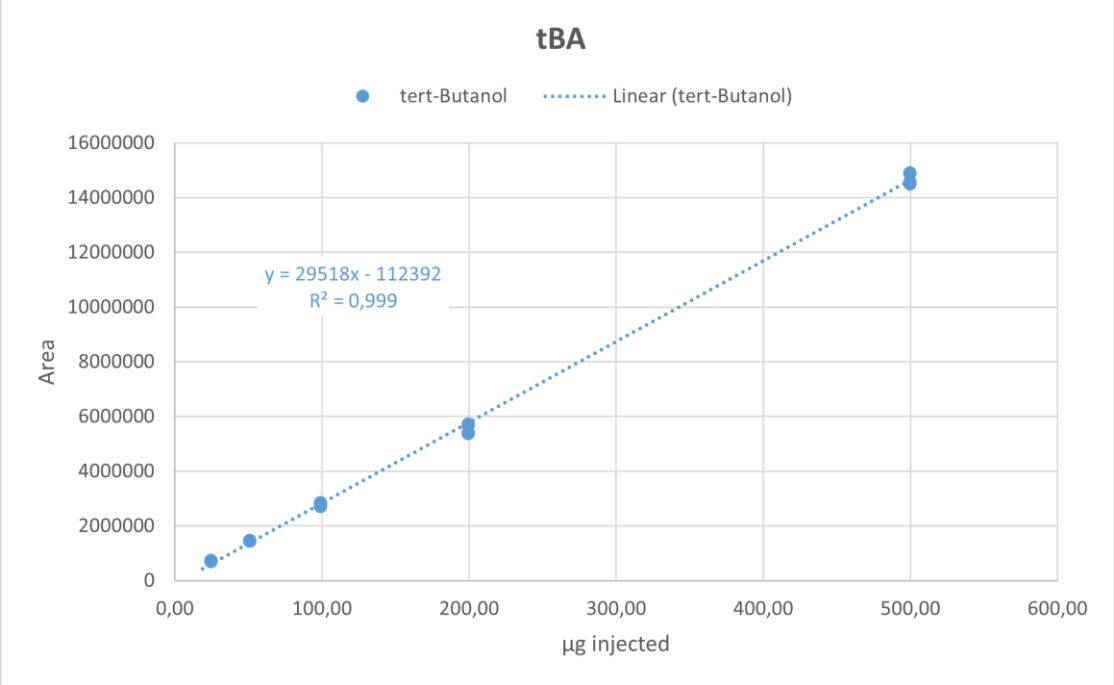

Figure 25. tBA calibration curve.

The R^2-values for the three RS varied between 0.998 and 1.0, with RSD-values < 4 %, indicating that good linearity and precision were obtained.

4.5. Cellulose samples

The newly developed method was then finally tested for determination of RS in samples that were provided by the Drug Delivery and Disposition laboratory of the Faculty of Pharmaceutical Sciences, KU Leuven. TD-GC parameters used for sample analysis were the optimized parameters stated in Tables 5 and 6, with the exception of the desorption temperature which was set to 280 °C, not 50 °C. For sample analysis the sample tube doesn't need to be packed with LiCl, as it has to be for calibration. This is because water, which was causing issues by covering the cold trap, came from the standard solutions which were used to optimize the system and generate calibration curves. Because there is no excess of water in the sample material, there is no need for LiCl traps and therefore the desorption temperature can be set to a higher temperature. The new desorption temperature needed to be lower than 300 °C, at which temperature cellulose starts to degrade, but high enough to guarantee complete desorption of all RS from the sample material, so 280 °C was chosen. MS was used to identify the peaks.

First, a blank tube run was performed on 6 QFs to make sure no contaminants from the QF affected the results of the sample analysis. The FID chromatogram of the blank run showed no significant peaks, which meant that it was safe to proceed with sample analysis. Five sample tubes were packed each with 10 mg of a single cellulose bead type or microcrystalline cellulose, immobilized between 3 QF on either side, and subjected to the TD-GC-MS analysis.

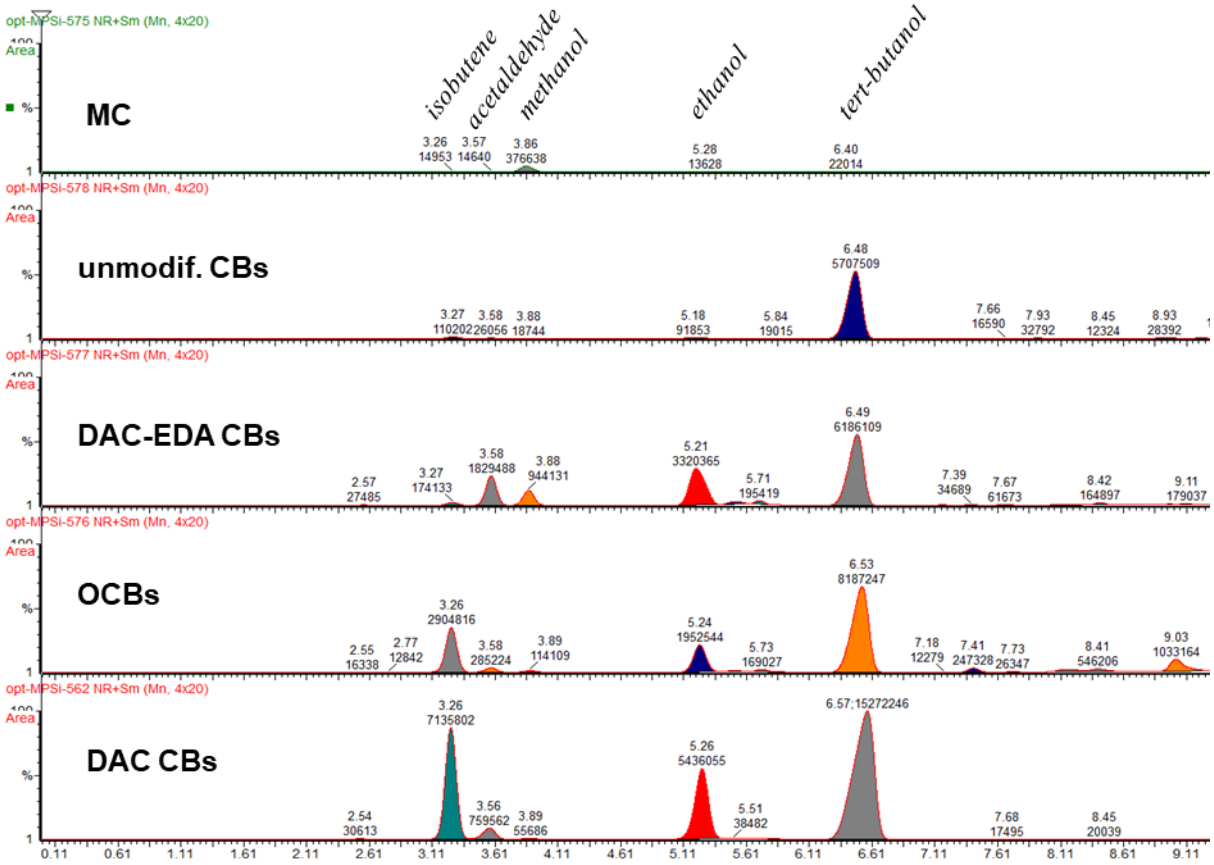

Figure 26. TD-GC-FID chromatograms of (top to bottom): microcrystalline cellulose (MC), unmodified cellulose beads (CB), ethylenediamine dialdehyde cellulose beads (DAC-EDA), TEMPO-oxidized cellulose beads (OCB) and dialdehyde cellulose beads (DAC).

TD-GC-FID chromatograms (*Figure 26*) of microcrystalline cellulose (MC) and the unmodified CBs were looking quite clean. The only significant RS peak in the MC chromatogram was MeOH, and in the CB chromatogram, tBA, together with some small quantities of EtOH and isobutene. However, the chromatograms of the CBs that underwent modifications (OCB, EDA, DAC) contained a lot more peaks, including all the expected RS (MeOH, EtOH and tBA), as well as tBA degradation products (isobutene, acetaldehyde) and others.

Regarding the hypothesis that isobutene, acetaldehyde, etc. are tBA degradation products, formed inter alia under high temperature conditions (inside the TD apparatus) and in contact with carbon structures (cellulose, carbosieve, TD cold trap), which is exactly the way TD works (i.e. high desorption temperatures and carbon-based trap materials), using TD to determine tBA didn't seem to be the best option. Such an analysis resulted in (as shown in Figure 26 for OCB,

EDA and DAC) chromatograms containing peaks of compounds that did not come directly from the sample, but were formed inside the TD-GC system during analysis. This could then subsequently lead to wrong interpretation of results.

With regard to the determination of MeOH and EtOH in simple cellulose matrices, this TD-GC method seemed adequate.

4.6. Comparison with HS

This thesis was a part of a bigger, more encompassing research project, investigating the applicability of TD-GC for determination of RS in complex matrices. A colleague from the same laboratory tried out a more traditional approach for RS determination in the same CB samples that were investigated in this work. He enzymatically degraded the CB matrix prior to analysis to circumvent the issue of cellulose solubility and achieve a homogenous sample. The destroyed matrix was then analyzed with HS-trap GC.

CB type		**CB**	**DAC-EDA**	**OCB**	**DAC**
% (MeOH)	TD - ILC	not detected	0.07	0.01	0.01
	HS trap	not detected	not detected	not detected	not detected
% (EtOH)	TD - ILC	0.01	0.11	0.07	0.18
	HS trap	0.06	0.08	0.10	0.15
% (tBA)	TD - ILC	2.01	2.20	2.86	5.38
	HS trap	1.42	1.49	1.99	4.24

Table 7. Comparison of MeOH, EtOH and tBA content (%) determined by the new TD-GC method and HS-trap GC

In comparison to HS-trap, the TD-GC method exhibits a higher sensitivity for MeOH, the detected ethanol content was similar for both methods and finally, regarding tBA, even though the TD-GC method shows higher sensitivity than HS-trap, the results should be considered inconclusive because the TD-GC analysis of tBA did not prove to be reliable (*Table 7*).

All things considered, the advantages of the TD-GC method are a higher sensitivity for MeOH, and therefore the ability to successfully quantify MeOH at lower concentrations and a significantly easier and shorter sample preparation process, which results in shorter analysis times. On the other hand, the inability to straightforwardly determine RS in more complex cellulose matrices is its major drawback.

5. CONCLUSION

The determination of RS in active substances, excipients, and medicines is required during release testing and advisable throughout the entire drug manufacturing process when a manufacturing or purification process is known to utilize a RS or it results in formation of one. Official regulatory bodies (ICH, USP, Ph. Eur.) recommend HS-GC as the analytical procedure of choice for identification and control of RS in pharmaceuticals. The official HS-GC methods assume that the sample can be made into a homogenous solution which would then be analyzed. However, not all compounds can be easily dissolved. One such complex sample is cellulose beads, developed by the Drug Delivery and Disposition laboratory of the Department of Pharmaceutical and Pharmacological Sciences at KU Leuven. Cellulose is a notoriously insoluble polysaccharide and as such, the sample preparation required for a typical HS-GC analysis is intricate and time-consuming. A recently presented TD-GC method, developed by the Pharmaceutical Analysis laboratory (Department of Pharmaceutical and Pharmacological Sciences - KU Leuven) and successfully used as an alternative way to determine RS in complex, insoluble matrices, has been adapted and applied to the modified cellulose beads to determine the RS content.

The focus of this work was the search for an optimal calibration method for the TD-GC analysis. Ideally, TD should be calibrated using gas-phase standards, but these are expensive and difficult to obtain. So, calibration is routinely done using liquid standards. However, calibration becomes critical when TD is used for direct thermal extraction of RS from a solid sample because then the adsorption and desorption pathways of RS in the sample and the reference differ significantly. Offline (OLC) and inline (ILC) liquid calibration were both optimized, tested and compared as calibration methods and finally, the TD-GC method was compared against a more traditional approach of RS determination: the enzymatic degradation of the cellulose matrix followed by HS-trap GC analysis.

Preliminary TD-GC analyses of all 4 types of cellulose beads samples showed the presence of MeOH, EtOH and tBA. So, the focus of our research was on those RS. The initial plan was to optimize ILC first, and then use it to optimize and validate OLC, which was the originally preferred calibration method as it is more common in general practice and easier to perform.

The original idea to use OLC as the calibration method fell through when the generated OLC calibration curve showed significantly poorer recovery compared to ILC (55-65% for MeOH and EtOH and 75-85% for tBA). The variation in recovery presented an additional issue: it prevented the determination of a correction factor. Secondly, the amounts of alcohols recovered exhibited an unacceptable RSD (3-12% for different MeOH and EtOH concentrations and between 7-10% for tBA). Consequently, OLC was dropped and the calibration curve was constructed by ILC.

Finally, the ILC method was used to analyze the samples. TD-GC-FID analysis of the unmodified cellulose matrices (microcrystalline cellulose and unmodified cellulose beads) was successful. It resulted in clean-looking chromatograms, which contained the expected RS peaks. However, the chromatograms of the cellulose matrices that underwent modifications (modified cellulose beads: OCB, EDA, DAC) contained a lot more peaks in addition to the expected RS, including isobutene and other compounds, which were presumably formed inside the TD-GC system during analysis. To summarize, using TD-GC to determine tBA in complex matrices doesn't seem to be a good option, but the method works well for determination of MeOH and EtOH, especially in simple matrices.

When compared to the traditional HS trap analysis, the advantages of the TD-GC method are a higher sensitivity for MeOH and a simpler and quicker sample preparation process, but the major disadvantage is the inability to straightforwardly determine RS in more complex cellulose matrices.

There were two additional, unexpected issues that were encountered during the course of this research: the isobutene formation and water interference on the TD cold trap. The underlying mechanisms of both phenomena were not explored in this study and were simply worked around of for the time being, but they remain intriguing topics that merit exploration in some future studies.

6. LIST OF ABBREVIATIONS AND SYMBOLS

ACN	acetonitrile
APIs	active pharmaceutical ingredients
ATT	detector attenuation
β	phase ratio
C_0	analyte concentration in original sample
CB	cellulose beads
C_g	concentration in the headspace (gas phase)
CS	Carbosieve III
C_s	concentration in the sample phase
DAC	dialdehyde cellulose beads
DAC-EDA	ethylenediamine dialdehyde cellulose beads
dHS	dynamic headspace
DMF	dimethylformamide
EtOH	ethanol
FID	flame ionization detector
GC	gas chromatography
HS	headspace
ICH	International Council on Harmonization of Technical Requirement for Registration of Pharmaceuticals for Human Use
ILC	inline liquid calibration
K	partition coefficient
LiCl	lithium chloride
MeOH	methanol
MPSi	mesoporous silica
MS	mass spectrometer
n_i	amount of sample component i, in moles
n_{total}	total amount of moles in the sample
OCB	TEMPO-oxidized cellulose beads
OLC	offline liquid calibration
p^0_i	vapor pressure of a pure analyte
PDE	permitted daily exposure
Ph. Eur.	European Pharmacopoeia

p_i	partial pressure of sample component i
p_{total}	total pressure of the vapor phase (headspace) above the sample
QF	quartz filter paper
RS	residual solvents
sHS	static headspace
tBA	tert-Butanol
TD	thermal desorption
USP	The United States Pharmacopeia
V_0	original sample volume
V_g	gas phase volume
VOCs	volatile organic chemicals
V_s	sample phase volume
$X_{G(i)}$	mole fraction of component i in the gas mixture
γ_i	activity coefficient of compound i

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8. SUMMARY

The determination of residual solvents in active substances, excipients, and medicines is mandatory during release testing and advisable throughout the entire drug manufacturing process. Official regulatory bodies recommend headspace-gas chromatography (HS-GC) as the analytical procedure of choice for identification and control of residual solvents in pharmaceuticals. The official headspace-gas chromatography methods assume that the sample can be made into a homogenous solution, which is then analyzed. However, not all compounds can be easily dissolved. A recently presented thermal desorber-gas chromatography (TD-GC) method, developed by the Pharmaceutical Analysis laboratory of KU Leuven and successfully used as an alternative way to determine residual solvents in complex matrices, was adapted and applied to determine residual solvents in different types of modified cellulose beads. Ideally, thermal desorption should be calibrated using gas-phase standards. However, in practice, gas-phase standards are expensive and difficult to obtain, so calibration is routinely done using liquid standards. Calibration becomes critical when thermal desorption is used for direct thermal extraction of residual solvents from solid samples because then the adsorption and desorption pathways of residual solvents in the sample and the reference differ significantly. Inline (ILC) and offline (OLC) liquid calibration were explored as potential calibration methods. After optimization of both methods, offline liquid calibration showed poorer recovery and precision compared to inline liquid calibration. So, inline liquid calibration was chosen as the preferred calibration method for this kind of analysis.

Određivanje ostatnih otapala u aktivnim farmaceutskim supstancama, ekscipijensima i gotovim lijekovima je obavezno tijekom završnog ispitivanja, a preporučuje se tijekom cijelog procesa proizvodnje lijeka. Regulatorna tijela definiraju *headspace*–plinsku kromatografiju (HS-GC) kao preferiranu metodu za identifikaciju i određivanje ostatnih otapala u farmaceutskim oblicima. Službena metoda polazi od pretpostavke da se uzorak može otopiti te da se zatim analizira nastala otopina, no ne mogu se sve tvari lako homogenizirati. Nedavno opisana metoda analize plinskom kromatografijom s termalnom desorpcijom (TD-GC), uspješno razvijena u Laboratoriju za farmaceutsku analizu KU Leuvena-a kao alternativna metoda za analizu ostatnih otapala u složenom matriksu, prilagođena je i primjenjena za određivanje ostatnih otapala u više vrsta celuloznih zrnaca. Idealno, termalnu desorpciju bi trebalo kalibrirati standardima u plinskoj fazi, međutim plinski standardi su kompliciranizanabaviti, pa se kalibracija rutinski vrši tekućim standardima. Kalibracija metode je kritični korak u analizi u kojoj se termalni desorber koristi za izravnu desorpciju uzorka u čvrstom stanju, zbog toga što se tada značajno razlikuju adsorpcijski i desorpcijski putevi ostatnih otapala u uzorku i tekućem standardu. *Inline* (ILC) i *offline* (OLC) tekućinska kalibracija su istražene kao potencijalne kalibracijske metode. Po završetku optimizacije obiju metoda, *offline* tekućinska kalibracija je pokazala manji prinos i lošiju preciznost u usporedbi sa *inline* kalibracijom. Zbog toga je *inline* tekućinska kalibracija odabrana kao preferirana metoda kalibracije.

Temeljna dokumentacijska kartica

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Diplomski rad

ISTRAŽIVANJE KALIBRACIJSKIH METODA ZA IZRAVNU DESORPCIJU KOJA PRETHODI PLINSKOJ KROMATOGRAFIJI

Ana Modrić

SAŽETAK

Određivanje ostatnih otapala u aktivnim farmaceutskim supstancama, ekscipijensima i gotovim lijekovima je obavezno tijekom završnog ispitivanja, a preporučuje se tijekom cijelog procesa proizvodnje lijeka. Regulatorna tijela definiraju *headspace*–plinsku kromatografiju (HS-GC) kao preferiranu metodu za identifikaciju i određivanje ostatnih otapala u farmaceutskim oblicima. Službena metoda polazi od pretpostavke da se uzorak može otopiti te da se zatim analizira nastala otopina, no ne mogu se sve tvari lako homogenizirati. Nedavno opisana metoda analize plinskom kromatografijom s termalnom desorpcijom (TD-GC), uspješno razvijena u Laboratoriju za farmaceutsku analizu KU Leuvena-a kao alternativna metoda za analizu ostatnih otapala u složenom matriksu, prilagođena je i primjenjena za određivanje ostatnih otapala u više vrsta celuloznih zrnaca. Idealno, termalnu desorpciju bi trebalo kalibrirati standardima u plinskoj fazi, međutim plinski standardi su komplicirani za nabaviti, pa se kalibracija rutinski vrši tekućim standardima. Kalibracija metode je kritični korak u analizi u kojoj se termalni desorber koristi za izravnu desorpciju uzorka u čvrstom stanju, zbog toga što se tada značajno razlikuju adsorpcijski i desorpcijski putevi ostatnih otapala u uzorku i tekućem standardu. *Inline* (ILC) i *offline* (OLC) tekućinska kalibracija su istražene kao potencijalne kalibracijske metode. Po završetku optimizacije obiju metoda, *offline* tekućinska kalibracija je pokazala manji prinos i lošiju preciznost u usporedbi sa *inline* kalibracijom. Zbog toga je *inline* tekućinska kalibracija odabrana kao preferirana metoda kalibracije.

Rad sadrži: 50 stranica, 26 grafičkih prikaza, 7 tablica i 28 literaturnih navoda. Izvornik je na engleskom jeziku.

Ključne riječi: kalibracija, termalna desorpcija, plinska kromatografija, ostatna otapala

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Diploma thesis

INVESTIGATION OF CALIBRATION METHODS FOR DIRECT DESORPTION PRIOR TO GAS CHROMATOGRAPHY

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SUMMARY

The determination of residual solvents in active substances, excipients, and medicines is mandatory during release testing and advisable throughout the entire drug manufacturing process. Official regulatory bodies recommend headspace-gas chromatography (HS-GC) as the analytical procedure of choice for identification and control of residual solvents in pharmaceuticals. The official headspace-gas chromatography methods assume that the sample can be made into a homogenous solution, which is then analyzed. However, not all compounds can be easily dissolved. A recently presented thermal desorber-gas chromatography (TD-GC) method, developed by the Pharmaceutical Analysis laboratory of KU Leuven and successfully used as an alternative way to determine residual solvents in complex matrices, was adapted and applied to determine residual solvents in different types of modified cellulose beads. Ideally, thermal desorption should be calibrated using gas-phase standards. However, in practice, gas-phase standards are expensive and difficult to obtain, so calibration is routinely done using liquid standards. Calibration becomes critical when thermal desorption is used for direct thermal extraction of residual solvents from solid samples because then the adsorption and desorption pathways of residual solvents in the sample and the reference differ significantly. Inline (ILC) and offline (OLC) liquid calibration were explored as potential calibration methods. After optimization of both methods, offline liquid calibration showed poorer recovery and precision compared to inline liquid calibration. So, inline liquid calibration was chosen as the preferred calibration method for this kind of analysis.

The thesis is deposited in the Central Library of the University of Zagreb Faculty of Pharmacy and Biochemistry.

Thesis includes: 50 pages, 26 figures, 7 tables and 28 references. Original is in English language.

Keywords: calibration, thermal desorption, gas chromatography, residual solvents

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