Tutor/s
Pilar Rodríguez Martínez
Laboratori Agroalimentari de Cabrils
Generalitat de Catalunya

Dra. Encarnación Moyano Morcillo
Departament d'Enginyeria Química i Química Analítica

Grau de Química

Treball Final de Grau

DART-HRMS analysis of gossypol and theobromine in feed samples

Anàlisi de gossipol i teobromina en mostres de pinso amb DART-HRMS

Daniel Rozúa Álvarez

June 2022

Universitat de Barcelona

B:KC Barcelona Knowledge Campus
Campus d'Excel·lència Internacional

Things sometimes don't work in the lab simply because God hates chemists.

Paul Lloyd-Williams

Dono les gràcies al Laboratori Agroalimentari de Cabrils (LAC), en especial a Pilar Rodríguez, Meritxell González i Mireia Medina per oferir-me aquest projecte i per permetre'm veure com es treballa a un laboratori d'anàlisi.

Vull agrair a la doctora Encarnación Moyano la seva tutela durant la realització d'aquest projecte, on he après molt sobre la química analítica en tots els àmbits. Vull agrair-li també la oportunitat que em va donar de poder donar visibilitat al meu treball, portant-me a un congrés nacional.

Dono també les gràcies a tot el grup ChroMS EnviFood, per ajudar-me quan la meva tutora no podia fer-ho, i per ser allà per vigilar-me que no fiqués la pota.

Finalment, vull agrair la companyia a totes les persones que m'han estat amb mi al llarg d'aquests sis mesos. La meva parella, els meus companys de classe, els meus amics, tant els que ja coneixia com els que he fet de nous... totes les persones que m'han animat quan m'han vist abatut pel caprici de la ciència, de sempre resistir-se a ser entesa.

REPORT

IDENTIFICATION AND REFLECTION ON THE SUSTAINABLE DEVELOPMENT GOALS (SDG)

Of the 5 P's that define the United Nations' Sustainable Development Goals (Peace, People, Prosperity, Planet and Partnership), the one that would better suit this project is "Prosperity". Optimizing the working procedures in analytical laboratories increases their productivity, leading to higher quality service. In addition, the method proposed in this project uses fewer consumable materials, especially organic solvents, which are gradually being displaced by other alternatives with less impact on the environment. With the method proposed in this project, the overall productivity of food control laboratories should increase significantly.

The results supporting this hypothesis are that the developed DART-based method works and is one order of magnitude faster than the standard HPLC-MS method.

The SDG that better defines the subject matter of this project is SDG number 9, "to build resilient infrastructure, promote sustainable industrialization and foster innovation". This is because the project proposed is based on a relatively new invention. Among the goals included in the SDG number 9, the one that better suits this project is 9.3, "to enhance research and upgrade industrial technologies", and the indicator that would be able to monitor the progress of this project is "the money spent on research and development in proportion to the PIB". However, if another more suitable indicator was proposed, it would be one that monitors the productivity of the industrial sector compared to previous years.

Laboratori Agroalimentari de Cabrils has no 2030 agenda of their own.

CONTENTS

1. SUMMARY

DART (Direct Analysis in Real Time) is a relatively new ionization technique that belongs to the so-called *Ambient techniques*, which ionize analytes from sample surface and in their own environment without the need of any sample treatment or chromatographic separation. The combination of DART with high-resolution mass spectrometry and tandem (quadrupole-Orbitrap) provides the sensitivity and selectivity needed for a fast and direct analysis of analytes in complex samples. The presence of gossypol and theobromine in feed is regulated to 20 mg/kg and 50 mg/kg, respectively. Generally, the analysis of these compounds in feed requires a long and tedious sample treatment before the LC-MS/MS analysis, which usually becomes the bottleneck of food control laboratories.

In this project, a new DART-HRMS method has been developed for the direct analysis of gossypol and theobromine in feed samples. The most critical DART parameters (ionization gas, temperature, analysis mode, etc.) were optimized to effectively ionize these analytes. Nitrogen gave better results than helium as ionization gas, and temperatures of or above 400 °C were needed for the effective thermal desorption of both compounds, having been previously deposited in a QuickStrip™ rack with their extraction solvent and totally or partially dried. Scanning mode gave better results than pulsing mode. Fragmentation pathways were also studied for both compounds using MS/MS in order to obtain structural information and to identify the most characteristic product ions. The DART-HRMS/MS method developed was fast, selective and sensitive enough to detect both regulated compounds 50 and 8 times, theobromine and gossypol respectively, below the legislated level using a simple extraction of 0.5-1 g of feed samples before the DART-HRMS/MS analysis. Positive samples previously analysed by UHPLC-MS were easily detected applying the new DART-HRMS/MS. Results obtained demonstrate the good performance of the proposed method.

Keywords: DART, Ambient MS, theobromine, gossypol, screening analysis, high-resolution mass spectrometry

2. RESUM

El DART (*Direct Analysis in Real Time*) és una font d'ionització relativament nova que pertany a les anomenades *tècniques Ambient*, les quals consisteixen en ionitzar mostres fent servir els elements del seu entorn, sense necessitat de tractament de mostra ni de separació cromatogràfica. La combinació del DART amb HRMS/MS (espectrometria de masses d'alta resolució i tàndem, amb un quadrupol-Orbitrap) dona la sensibilitat i selectivitat necessàries per un anàlisi ràpid i directe d'analits en matrius complexes. La presència de gossipol i teobromina en pinsos es troba regulada i no deu sobrepassar 20 mg/kg pel primer i 50 mg/kg pel segon. Generalment, l'anàlisi d'aquests compostos requereix un llarg i tediós tractament de mostra previ a l'anàlisi amb LC-MS/MS, i és un coll d'ampolla pels laboratoris de control d'aliments.

En aquest projecte, s'ha treballat per desenvolupar d'un nou mètode DART-HRMS/MS per l'anàlisi directe de gossipol i teobromina en mostres de pinso. S'estudiaren les rutes de fragmentació d'ambdós compostos amb MS/MS per obtenir informació estructural i identificar els fragments més característics. Els paràmetres més crítics del DART (gas d'ionització, temperatura, mode d'anàlisi, etc.) van ser optimitzats. El nitrogen va ser el gas d'ionització més efectiu, i temperatures iguals o superiors a 400 °C van ser necessàries per la desorció tèrmica efectiva d'ambdós compostos, havent sigut prèviament dipositats a una reixeta QuickStrip™ en el seu dissolvent d'extracció i assecats totalment o parcial. El mode d'anàlisi per escombrat va donar millors resultats que el mode en pols. El mètode DART-HRMS desenvolupat permet l'anàlisi ràpid i selectiu d'ambdós compostos a concentracions inferiors a 500 µg/kg en el pinso, rere una simple extracció de 0.5-1.0 g de mostra previ a l'anàlisi. Mostres positives per UHPLC-MS/MS van ser estudiades per DART-HRMS/MS per confirmar el funcionament del mètode. Els resultats demostren el bon rendiment del mètode proposat.

Paraules clau: DART, Ambient MS, pinso, teobromina, gossipol, anàlisi de cribratge, espectrometria de masses d'alta resolució.

3. INTRODUCTION

The use of mass spectrometry (MS) has grown from its discovery to become one of the most used analytical techniques in all fields of chemistry, not only for its unique ability to provide information on the molecular weight and the chemical structure of analytes, but also because of its selectivity and sensitivity. To analyse complex samples, chromatography is usually coupled to mass spectrometry. However, in this TFG project we want to explore the feasibility of a relatively new ionization technique (DART, "Direct Analysis in Real Time") for the direct analysis of complex samples such as feed, without applying any sample treatment and without performing any chromatographic separation before the analytical detection/determination.

3.1. DART IONIZATION

Some decades ago, it would have been unthinkable to hold an object in front of a mass spectrometer in open air and record mass spectra in real time. Nowadays, almost any sample can be analysed instantaneously (liquids, solids, and gases) by using Ambient Ionization-Mass Spectrometry (AIMS). DART (Direct Analysis in Real Time) is one of the ionization sources belonging to the family of AIMS [1]. These techniques ionize samples in their own environment without chromatographic separations and without (or with a very simple) previous sample treatment, which allows for faster analyses than the chromatographic-mass spectrometry-based methods [2].

DART is an ionization source based on atmospheric-pressure chemical ionization (APCI) mechanism, and it consists in a tubular chamber with a needle electrode inside, as is shown in Figure 1 (scheme of the DART source) [3]. The ionization gas, generally nitrogen or helium, flows inside this chamber and is exposed to a high electric field (on the tip of the needle electrode), which initiates a *glow discharge*. The plasma generated in the atmospheric-pressure glow discharge contains a variety of highly energetic species, including ions, electrons, and excited-state neutral species (*metastable species*). Some of these metastable species last long enough to survive transport by the DART stream gas to exit the DART source. A gas heater gets the DART gas to the desired temperature to facilitate the thermal desorption of analytes from the

sample surface. The neutral molecules (analytes, solvents, permanent gases, etc.) in the gas-phase interact with metastable species to produce positive radical ions $[R]^{+\bullet}$ and electrons. These new ionic species interact with neutral molecules (analytes) through proton transfer and charge exchange mechanisms to ionize analytes thermally desorbed from the sample surface. DART is a form of APCI, and it generally produces protonated and/or ammoniated molecules in positive-ion mode and deprotonated molecules in negative-ion mode. Ions formed by DART have relatively low internal energies, so they are stable ions that do not fragment during the ionization process, and for this reason this ionization source can be considered a "soft" ionization technique. This fact is an advantage for the analysis of complex samples since each compound generates one ion (more specifically, an isotope cluster) and each signal in the mass spectrum can be assigned to one compound, which simplify the mass spectrum interpretation process [4].

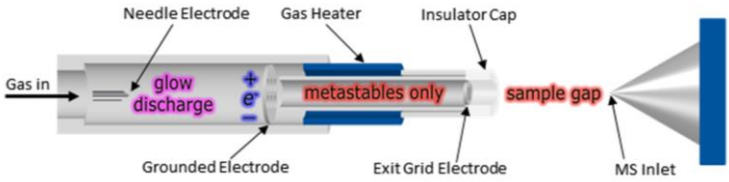


Figure 1. Representation of the insides of a DART source.[5]

For the sample analysis, only few microliters of sample solution have to be deposited onto a QuickStrip® rack, which is positioned within the stream gas way from DART to the mass spectrometer inlet. The rack moves at a constant speed (defined by the user), so the signal is recorded (mass spectrum) *vs* time across the sample area in the QuickStrip® window, obtaining the corresponding *chronograms* (time domain) for each sample analyzed.

3.2. HIGH RESOLUTION MASS SPECTROMETRY: USING A Q-ORBITRAP

Although the ionization in DART is soft, all compounds on the surface that can be thermally desorbed and ionized in the gas-phase will contribute with a signal in the mass spectrum. For this reason, DART-MS spectra are inherently complex, with a high probability of the presence of isobaric interferences (ions with the same nominal mass but different exact mass). Therefore, low-resolution mass spectrometry has some limitations in its application with DART-MS, the number of false positives being quite significant. The great selectivity of high-resolution mass spectrometry (HRMS) offers a good alternative to reduce both false positives and false negatives in DART-MS analysis, as it overcomes isobaric interferences and reduces background noise, thus improving signal-to-noise ratio (S/N) and limits of detection (LODs). Additionally, HRMS

instruments allow accurate mass measurement of the molecular weight of analytes detected, as well as the detection of isotope clusters with almost no interference, which allows the identification/calculation of the molecular formula and gives valuable chemical information for confirmatory purposes.

The mass spectrometer used in this TFG is a hybrid instrument equipped with a quadrupole-Orbitrap® mass analyzer, which can be seen in Figure 2. Orbitrap allows for the selective analysis of ions operating at a mass resolution ranging from 17,000 to 140,000 FWHM (full width of half-maximum) and a mass accuracy of 5 ppm [6]. Orbitrap is a kind of ion trap-based mass analyzer that traps ions in a constant radial electrostatic field. The centrifugal force that arises from the initial tangential velocity of ions compensates the electrostatic attraction towards the central electrode, like a satellite in orbit. These ions start to move in an axial oscillation around and along the central electrode with a defined frequency that is a function of their *m/z* value. A differential amplifier connected to the outer electrodes halves monitors the frequency of the harmonic axial oscillation providing a sine function that can be transformed into the accurate monitoring of the *m/z* signals by using a Fourier transform.

Among the high-resolution mass analyzers, Orbitrap offers the highest sensibility, mass resolution and mass accuracy while being quite robust. For instance, TOF mass analyzers offer lower mass resolution than Orbitrap and they have to be calibrated each time they are used [7]. The only significant drawback of Orbitrap is its rather long scan time when the mass resolution is increased, which can make it difficult when coupling with very fast separation techniques and it is simultaneously required ultra-high mass resolution. However, this fact is not problem to a DART-based method, since the signal is acquired during a relatively long period of time [8].

As commented above, DART is a soft ionization technique and Orbitrap provides the accurate mass and isotopic pattern, thus the elemental composition of ions detected can be easily obtained. However, this is not enough for those compounds with the same elemental composition, or with very similar ones in mass, so information about the chemical structure is necessary as an additional tool for confirmatory purposes and for the characterization of suspect and unknown compounds.

For these reasons, Orbitrap is frequently combined with other mass analyzers in a hybrid instrument configuration. In this project, we have used a quadrupole-Orbitrap to perform experiments of “tandem mass spectrometry-in-space”.

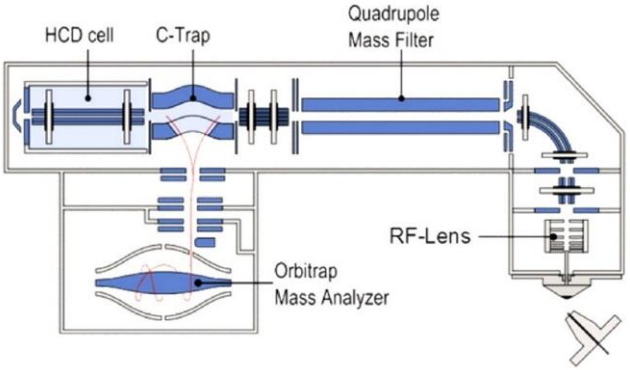


Figure 2. Scheme of a Q-Orbitrap instrument. Image extracted from Thermo Fisher Scientific®'s Q-Exactive Orbitrap™ manual.

Ions generated in the DART source are isolated (precursor ions) by the quadrupole and transported to the high-collision dissociation cell (HCD) for their fragmentation into product ions, which are later analyzed by the Orbitrap to provide a high-resolution product ion mass spectrum [9].

3.3. ANALYSIS OF UNDESIRABLE SUBSTANCES IN FEED SAMPLES

One of the most important bottlenecks in an analytical laboratory is the long and tedious analytical methodologies and chromatographic separations required for the analysis of complex samples, especially when the result of most samples is expected to be negative. This is the case of the analytical control of theobromine and gossypol in feed samples. For theobromine, very few samples are identified as positive annually. For gossypol, no positive samples have been found in agrifood control laboratories in the last decade. Legislation, nonetheless, requires the mandatory control of this compounds. Under this frame is when screening analysis plays an important role, and methods such as a DART-based screening method would help to save time improving the productivity of agrifood laboratories.

3.3.1. Theobromine and gossypol

The chemical structure of theobromine is shown in Figure 3. It is an alkaloid present in food products like chocolate and tea [10]. It belongs to the same family of compounds as caffeine. Although it is not considered toxic to humans, animals like dogs metabolize it slower, and thus can suffer from theobromine poisoning at low concentrations [11]. Theobromine presence in feed is regulated in Europe, with a maximum concentration limit value of 50 mg/kg [12]. Theobromine can be present in feed if one of the ingredients for feed production is, for example, cocoa sheaths.

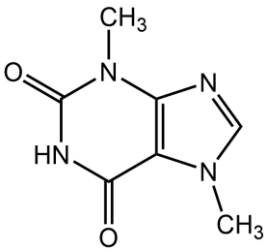


Figure 3. Theobromine.

The chemical structure of gossypol is shown in Figure 4. It is a polyphenolic aldehyde present in cotton, classified as a terpenoid [13]. It acts as a dehydrogenase inhibitor and can be toxic to both humans and animals. This has led to regulate its presence in feed in Europe, with a maximum concentration limit value of 20 mg/kg [12]. Gossypol can be present in feed if one of the ingredients for its production is the cotton plant.

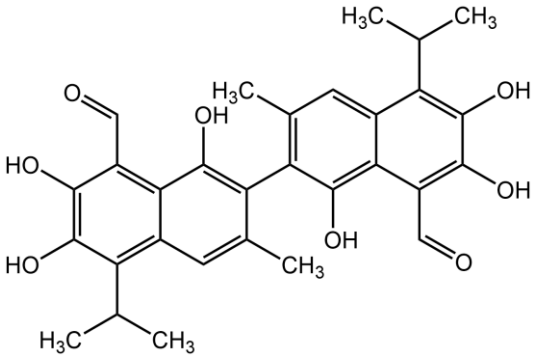


Figure 4. Gossypol

The legislated levels of these two compounds can be considered “high”, therefore they are ideal to be analysed with an Ambient technique, that lacks the detectability of other methods.

Table 1 summarizes some physicochemical properties of theobromine and gossypol that are relevant for the development of the DART-HRMS method. Gossypol can be easily deprotonated, meaning that the easiest way to detect it is as its negative ion $[M-H]^-$. Theobromine, on the other hand, will be protonated up to high pH values, meaning that the easiest way to detect it is as its positive ion $[M+H]^+$. Theobromine is much more polar than gossypol, and it can be extracted with water. Gossypol will need an organic solvent for performing the extraction. Theobromine sublimes at a relatively low temperature, meaning that it will be easy to desorb. Gossypol, however, should

not be possible to desorb alone, since it sublimes at a higher temperature that the maximum DART gas temperature, which is 500 °C.

Table 1. Physicochemical properties of theobromine and gossypol.

	Theobromine	**Gossypol**
Monoisopotic mass	$[M+H]^+$ *m/z* 181.0717	$[M-H]^-$ *m/z* 517.1867
CAS Number	83-67-0	303-45-7
pKa	9.9 [14]	6.7 7.4 11.8 12.7 13.9 (est) [15]
Log P	- 0.78 [14]	8.2 [15]
Boiling point (°C)	290-295 °C (sublimes) [14]	707 °C (est) [15]

Both extraction processes are summarized in Table 2. The current analytical method applied by *Laboratori Agroalimentari de Cabrils* (LAC) *(Generalitat de Catalunya)* to determine gossypol is accredited and based on ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) using electrospray ionization (ESI) as source and a single-quadrupole as mass analyser. First, an UHPLC-MS screening method is applied, analysing 3 blank samples spiked at three different concentration levels every 10 feed samples. In case of detecting a potential positive feed sample, its concentration level can be predicted based on the quantitation using the 3 spiked blank samples (basic calibration curve). If the positive sample is above the maximum concentration limit legislated, it is determined more accurately using a 6-points standard addition method. This method is, comprehensively, quite slow.

In the case of theobromine, the official method is based on an ultra-high performance liquid chromatography with UV-vis spectrophotometry detection, accreditation. Both instrumental methods are summarized in Table 3. LAC is developing an LC-MS-based method that could potentially be used in the near future.

A literature search has been performed, and there are currently no publications on the analysis of theobromine and gossypol in feed using AIMS techniques, making it an interesting field of study.

Table 2: Current extraction methods used in LAC for the analysis of theobromine and gossypol.

Theobromine	Gossypol
1. Weigh 5.0 g homogenized feed sample	1. Weigh 1.0 g homogenized feed sample
2. Add 30 mL petroleum ether at 40-60 °C	2. Add 40 mL AcCN:H_2O 8:2 (v/v)
3. 2 min vortex, 10 min centrifuge (4000 rpm)	
4. Decant and discard organic phase. Repeat steps 1-4 **two times**	3. 15 s vortex, 5 min ultrasound, 1 h horizontal agitator, 5 min centrifuge (3000 rpm)
5. Dry in a water bath at 90 °C	
6. Add 30 mL H_2O at 80 °C	4. Filter liquid phase with PFTE filter
7. 2 min vortex, 15 min ultrasound, 10 min centrifuge (10000 rpm)	
8. Decant liquid phase. Repeat steps 6-8 **three times**	5. Dilute 5 times with additional solvent
9. Filter with 0.45 µm filter	
10. Analyse with HPLC (10 min per sample)	6. Analyse with HPLC (8 min per sample)

Table 3: Current chromatographic conditions for the analysis of theobromine and gossypol.

	Theobromine	Gossypol
Column	C18 150x3.9 mm 4 µm	Phenyl 2.1x100 mm 1.7 µm
Mobile phase	A: H_2O Acetic acid 1% B: MeOH	A: AcCN B: H_2O Formic acid 0.05%
Gradient	From A:B 100:0 to A:B 70:30	From A:B 70:30 to A:B 95:5
Flow [mL/min**]**	1.0	0.40
Injection volume [µL**]**	25	2.0
Detector or coupled technique	UV-vis (monitor λ=272 nm)	MS/MS (monitor m/z=517.2, 471.2, 259.2, 231.2, positive ion)

4. OBJECTIVES

The general objective of this TFG project is to develop a fast and sensitive method based on DART-MS to detect theobromine and gossypol in feed samples. To achieve this goal, the following specific objectives should be addressed:

- To study the ionization of theobromine and gossypol under DART conditions.
- To optimize the DART most critical parameters to obtain the best signal intensity.
- To evaluate the limitations and advantages of both low- and high-resolution mass spectrometry for the study of these analytes.
- To study the fragmentation of these analytes by tandem mass spectrometry.
- To select the best DART-MS conditions to achieve the lowest LODs with standards.
- To study the matrix effect on DART ionization.
- To design the strategy with minimal manipulation to determine these analytes in feed samples below the maximum concentration limits stablished by the EU.

5. EXPERIMENTAL SECTION

5.1. MATERIALS AND EQUIPMENT

- Stock standard solutions:
  - 1,000 mg/L solution of theobromine in water
  - 1,000 mg/L solution of gossypol in acetonitrile:water (8:2, v/v)
- Working solutions were prepared by dilution in the appropriate solvent depending on the experimental work
- Micropipettes of 10, 200 and 1000 µL, GMBH and Co. (Wertheim, Germany)
- Micro syringe of 100 µL, Hamilton Company (Reno, NV, USA)
- Eppendorf Tube™ plastic vials, 2 mL, Nirco (Barcelona, Spain)
- Topaz glass screw vials, 8 mL, Thermo Fisher Scientific (San Jose, CA, USA)
- 0.22 µm pore size Nylon syringe filters, Whatman (Clifton, NJ, USA)
- Vortex Stuart™ (Staffordshire, United Kingdom)
- Falcon Tube™ plastic vials, 50 mL, Serviquimia (Tarragona, Spain)
- Ultrasound bath Bransonic™ B-5510 (Soest, Germany)
- Centrifuge Selecta Centronic™ (Barcelona, Spain)
- Water, LC-MS grade, Sigma Aldrich (St Louis, MO, USA)
- Acetonitrile, LC-MS grade, Sigma Aldrich (St Louis, MO, USA)
- Methanol, LC-MS grade, Sigma Aldrich (St Louis, MO, USA)
- Formic acid 88%, J.T. Baker Chemical Co. (Philipsburg, MO, USA)

The instruments and ionization sources used are cited in the following list:

- Finnigan LTQ™ Ion Trap Mass Spectrometer, Thermo Fisher Scientific (San Jose, CA, USA)
- Q-Exactive Orbitrap™ Thermo Fisher Scientific (San Jose, CA, USA)
- DART JumpShot™ Ion Sense (Saugus, MA, USA)
- ESI Source Thermo Fisher Scientific (San Jose, CA, USA)
- APCI Source Thermo Fisher Scientific (San Jose, CA, USA)
- Analytical balance Mettler Toledo AG425 (Colombus, OH, USA)

5.2. SAMPLE MANIPULATION AND FINAL CONDITIONS

Tables 4 and 5 summarize the final conditions of the DART-HRMS screening method. In the "Results and discussion" section is included the information on how these parameters were determined and optimized.

Table 4. Final conditions of the extraction process.

Theobromine	Gossypol
Weigh 0.5 g pulverized and homogenized feed sample	Weigh 1.0 g pulverized and homogenized feed sample
Add 40 mL water as extractant	Add 5 mL AcCN:H_2O 8:2 as extractant
15 s vortex	
15 min ultrasound bath	
15 min centrifuge 3000 rpm	
Decantation and filtration with 0.2 µm syringe filter	
Store in glass or plastic	Store in plastic only
Add 10 µL of the filtered solution to a QuickStrip™ rack (explained in text)	

Table 5: Final conditions of the DART analysis.

	Theobromine	Gossypol
Ion mode	Positive	Negative
Analysis mode	Scanning mode, 0.8 mm/s	
Source-to-inlet distance [cm]	2.6	
DART gas, pressure	N_2, 10 bar	
DART temperature [°C]	500	400

There were five different type feed blank samples available (pig, bird, rabbit, cow and veal feed), which have been spiked and used for the method development and matrix effect studies. Additionally, 10 theobromine positive feed samples were available, five of which had been analysed and quantified by the official LC-MS method. There were no positive gossypol feed samples available.

DART uses a certain fungible material called QuickStrip™ to work. QuickStrip's are small, disposable metal racks covered with an inert polymer. On these racks, a small quantity of liquid is deposited (10 µL were used throughout all the project) and left to dry. As it dries, the substances dissolved stick to the rack. The interaction with the excited air particles that the DART source produces desorbs and ionizes them. The most common ions produced are protonated and deprotonated species.

QuickStrip's have 12 positions. In the even positions, standards, or samples in the form of a solution are deposited, and in the odd positions, the solvent used in the preparation or extraction is deposited, making it work as a blank. The QuickStrip is inserted in a slot that moves following the desired scanning mode and its speed. This allows the user to regulate the amount of time the sample deposited is exposed to the metastable air species. Other parameters, like gas temperature, type of gas and ionizing mode can all be set through the DART's control program.

The reproducibility of DART methods is rather low, since the place and shape of the liquid drop in the rack has a big effect on the signal but cannot be controlled by a human user.

6. RESULTS AND DISCUSSION

6.1. DEVELOPMENT OF THE DART-MS METHOD

The first part of the experiments was to optimize the analytical conditions that were to be used later to detect the analytes in feed samples. Firstly, fragmentation studies were made to be able to monitor correctly the analytes using MS/MS. Then, DART parameters were optimized to maximize the signal, along with other parameters.

6.1.1. Ionization and fragmentation studies

DART ionization mechanism is quite similar to the atmospheric pressure chemical ionization (APCI). The ionization of both techniques takes place in the gas-phase by interaction of the neutral molecules with ionic/neutral species and both techniques is expected to generate similar ions for both gossypol and theobromine. In this TFG project, we explored the ionization of these analytes by electrospray (ESI) and APCI using both ionization modes, positive and negative. Theobromine provided better results in positive mode, generating the ion corresponding to the protonated molecule $[M+H]^+$, while gossypol produced a more intense signal in negative mode yielding the ion corresponding to the deprotonated molecule $[M-H]^-$. These ions were expected to be also produced under DART conditions.

The infusion mode, unlike DART's Quickstrips, allows a continuous flow of analytes into the ionization source, which is much more convenient to study the fragmentation of these analytes.

As commented before, tandem mass spectrometry (MS/MS) allows a more selective signal, reducing the background noise and improving the signal-to-noise ratio. For this reason, to obtain low limits of detection is recommended to use MS/MS whenever possible. To perform MS/MS in the Q-Orbitrap instrument, precursor ions $[M+H]^+$ or $[M-H]^-$ are isolated by the quadrupole at low resolution (1 $\Delta m/z$ window), and these ions are sent to the high-energy collision cell (HCD), where they are fragmented into product ions by applying enough collision energy. Afterward, product ions generated in the HCD are injected into the Orbitrap mass analyser for their separation and measurement at high-resolution (HRMS). If compounds with the same *m/z* value yield different

characteristic product ions, they can be perfectly identified and differentiated without worrying about isobaric interferences.

Although HRMS can provide the accurate mass and the isotope distribution to identify the compound via elemental composition, when working with very complex matrices, MS/MS can still improve the selectivity and the signal-to-noise ratio. For instance, some structural isomeric ions can provide different product ions allowing their differentiation and helping to overcome some significant interferences.

The collision energy curves for theobromine and gossypol were obtained using tandem in-time. Figures A1 and A2 (see Appendix section) show how the normalized collision energy (NCE) applied affect the abundance of the most important product ions. However, when working with tandem in-time, fragmentations by multiple collisions can occurred, generating product ions of different generation, which makes difficult to assign the genealogical origin of each product ion. For this reason, an ion trap mass analyser was used to work in multiple stage mass spectrometry (MS^n), which allowed to establish the fragmentation pathway of each compound and the correct relationship between product ions. Figures A3 and A4 show the fragmentation pathways of these compounds with the most important product ions for each compound. These product ions were detected in the tandem in-time in the Q-Orbitrap and their accurate mass were measured. Table 6 shows the accurate mass and the ion assignment of the most relevant ions.

Table 6. Accurate mass and ion assignment of the most characteristic ions observed (precursor and product ions) for each compound.

Theobromine (NCE: 30%)		**Gossypol (NCE: 35%)**	
m/z	**Ion assignment**	***m/z***	**Ion assignment**
181.0720	$[M+H]^+$	517.1868	$[M-H]^-$
110.0718	$[M+H-C_2HNO_2]^+$	259.0976	$[M-H-C_{15}H_{14}O_4]^-$
108.0556	$[M+H-H_2O-C_2HNO]^+$	231.1027	$[M-H-C_{16}H_{14}O_5]^-$

In the previous table, the $[M+H]^+$ and $[M-H]^-$ are the precursor ions. They should always be present in the product ion scan at least at 5 to 10% of the base peak of the mass spectrum. The other ions are product ions used for the quantification and confirmation purposes; they are the most characteristic product ions of each compound. Although no quantitative studies were made

in this project, the intensity of the quantification fragment was used as comparison between analyses.

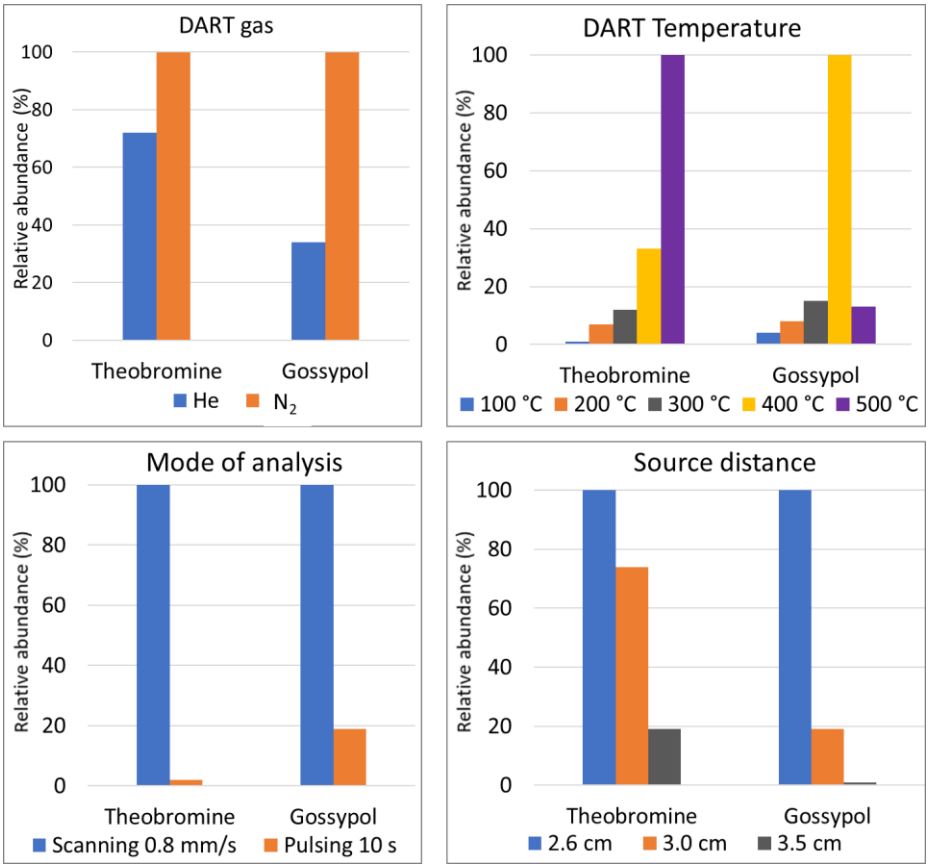
6.1.2. DART conditions

In the development of the DART method, the most critical parameters have been optimized: DART gas type, source temperature, source-to-MS inlet distance, and analysis mode (pulsing mode 10 s or scanning mode 0.8 mm/s). As a starting point, the standard conditions used in the ChroMS EnviFood research group for the DART source were used. Table 7 summarizes these initial parameters.

Table 7. DART conditions used as starting parameters [16]

	Theobromine	**Gossypol**
Analyte concentration [mg/L]	2	2
Ionization	Positive $[M+H]^+$ (181.07 *m/z*)	Negative $[M-H]^-$ (517.18 *m/z*)
Ionizing gas	Helium	Helium
Temperature [°C]	500	500
Source-to-inlet distance [cm]	2.6 (minimum possible)	2.6 (minimum possible)
Mode of analysis	Scanning, 0.8 mm/s	Scanning, 0.8 mm/s

Figures 8 to 11 show the results of the optimization. As can be seen, nitrogen provided better signal intensity than He. The closest possible source-to-inlet distance gave the highest signal, and the scanning mode produced gave better results than the pulsing mode. Regarding DART temperature, it is at first shocking the fact that gossypol gave better results at a lower temperature than theobromine, despite having a higher boiling point. The possible explanation must be related to the fact that gossypol was scanned while partially humid, unlike theobromine, which was completely dry. This phenomenon is explained in the following section “other parameters”.



Figures 8 to 11. Optimization of DART parameters. Figure 8 shows the optimization of the DART gas, Figure 9 the temperature, Figure 10 the mode of analysis and Figure 11 the source-to-inlet distance.

6.1.3. Other parameters

Gossypol was expected to have problems of desorption due to its high boiling point (707 °C), so different strategies were tested to help the thermal desorption of this compound and improve its response in the DART analysis. The way the sample is deposited and its state on the sample support (QuickStrip™) is of great importance. We evaluated two strategies:

1) Analysing the gossypol standard after letting it dry on the QuickStrip,
2) Analysing the same gossypol standard deposited on the QuickStrip while it is still humid in order to allow the DART gas to evaporate gossypol from the microdroplets that could be generated by the stream gas.

Results show an increase in the signal intensity for gossypol when using a wet rack. With a 0.5 mg/L gossypol standard, the signal of the analyte could only be seen when a thin liquid film was left in the QuickStrip. The same test was done on theobromine, but no differences were observed. In both cases, dry or humid rack, the signal was similar, but the variability was higher, probably due to the fact that the amount of solvent deposited onto the rack was more difficult to control and it spread more. Parameters like air humidity, temperature and salinity of the sample could affect the evaporation of sample solvent, even when left to dry the same amount of time.

The waiting time between deposition and analysis is critical, since it was noticed that a thin film of liquid was necessary, but too much liquid (thick film) proved to be negative for the signal intensity and the reproducibility. Too much liquid on the rack produces large solvent droplets and spreads the liquid in the areas near the deposition in such a way that the signal widens on the chronogram (persists for a longer time) decreasing the intensity (height) of the signal. That way, the DART peaks in the chronogram (signal of different racks) get closer to each other, which is not an ideal situation as they can partially overlap, and it is desirable to have well separated peaks between two sample positions on the QuickStrip™ to avoid cross-contaminations.

Additionally, during a DART analysis session using the wetting strategy, an abnormal increase in theobromine signal was observed and the signal persisted in all analysis, blanks, or standards. This fact was probably due to an excess of liquid on the rack. If we do not dry the sample until a minimum film thickness, the excess of solvent is introduced in the mass by being blown by the DART gas. The large droplets cannot release analytes into the gas-phase, and they can stick on the walls of the transfer line. It was possible to make the signal disappear after cleaning the transfer line.

Another parameter studied was the solvent of the sample. We evaluate methanol and acetonitrile. Acetonitrile provided improved signal for gossypol, and it was chosen as the optimal organic solvent for samples.

The addition of formic acid is frequently recommended to avoid the adsorption of gossypol on the glass surface of vials. However, this procedure would hinder the ionization of gossypol in DART negative ion mode to generate the $[M-H]^-$. This was significant at low concentration levels (0.05, 0.01 mg/L), making gossypol signal not detectable in the mass spectrometer. For this reason, the use of Eppendorf Tube™ plastic vials is recommended to prepare/store samples and standards of gossypol.

6.1.4. Reproducibility and instrumental limits of detection (iLOD)

Reproducibility was evaluated using standard solutions of 2.0 mg/L for theobromine and 20 mg/L for gossypol and analysing 5 replicates by depositing 10 µL in 5 racks of the QuickStrip. Table 8 summarizes the results of the 5 replicate measurements. The RSD% values obtained are relatively high, but these results are quite normal and expectable when working with Ambient-based methods. Frequently, if the label standard is available, like deuterated isotopologues, it is used as an internal standard correcting the signal variability. In our case, there were not labelled standards available.

Table 8. Results of the reproducibility tests on theobromine and gossypol.

	Theobromine (2 mg/L) **Signal Intensity [**abundance**]**	**Gossypol (20 mg/L)** **Signal Intensity [**abundance**]**
Replicate 1	9.2E7	1.2E8
Replicate 2	4.8E7	8.1E7
Replicate 3	6.2E7	9.3E7
Replicate 4	6.7E7	6.9E7
Replicate 5	8.8E7	3.7E7
RSD%	**26%**	**38%**

Gossypol analysis was less reproducible than theobromine. As commented before, this effect can be due to gossypol standards having to be analysed partially humid.

After optimizing all parameters, the instrumental limits of detection were estimated. For theobromine, the iLOD calculated was **0.0050 mg/L**, while for gossypol the iLOD value observed was **0.50 mg/L** probably due to the difficulties with the thermal desorption of gossypol. In MS/MS, there is a problem to estimate the iLODs, since normally there is no background noise. For this reason, iLODs have to be estimated experimentally by analysing standard solutions diluted at low concentration level instead of estimating them by the traditional method of extrapolating from the calibration curve and taking into account the signal-to-noise ratio (S/N) of 3.

6.2. ANALYSIS OF SAMPLES

For the analysis of samples, the matrix effect in the DART ionization was studied. These experiments were done by using spiked blank samples. Blank samples were submitted to a simple extraction with the extracting solvent and a defined amount of analyte was added to an aliquot of the extract. After the homogenization, 10 µL of this spiked extract were analysed by DART-MS.

6.2.1. Matrix effect and isobaric interferences of the DART-HRMS method

The main reason to use DART with HRMS is to avoid isobaric interferences and to reduce background noise. Isobaric interferences are caused by ions that have the same nominal mass as the analyte, but different exact mass. As can be seen in Figures 12 and 13, in the m/z zone were the analytes (theobromine and gossypol) should be detected appear an important number of isobaric ions. If the analysis is done at LRMS, these signals will contribute to the same peak of theobromine and gossypol. In Ambient Ionization methods, all analytes that can be desorb/ionized providing a signal in the mass spectrum, so isobaric interferences are quite common.

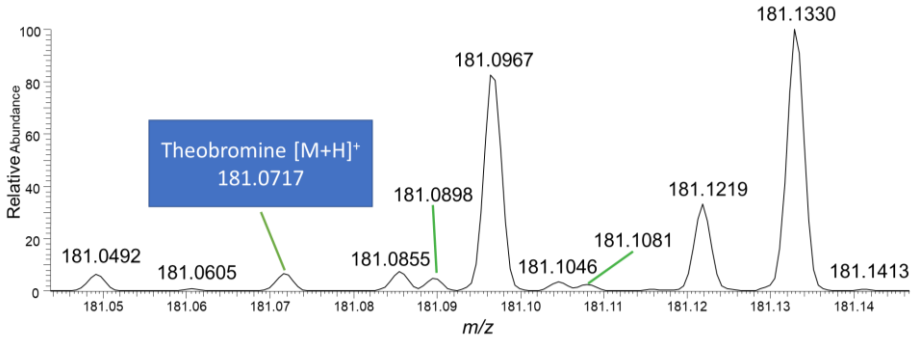


Figure 12. DART-HRMS full scan mass spectrum of an extract of matrix sample spiked with theobromine. A 1 *m/z* window in the zone where theobromine should appear.

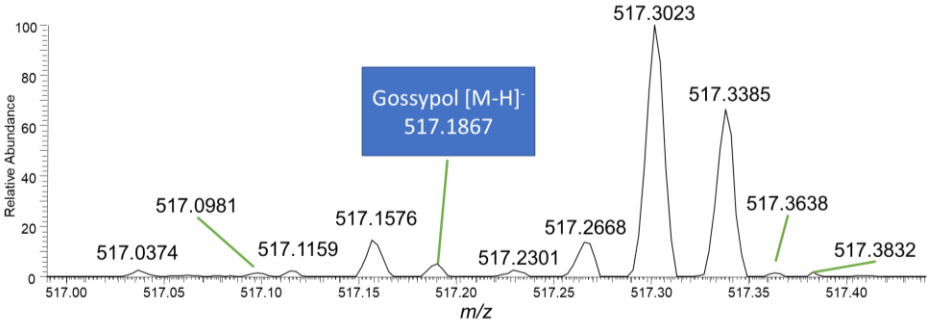


Figure 13. DART-HRMS full scan mass spectrum of an extract of matrix sample spiked with gossypol. A 1 *m/z* window in the zone where gossypol should be appeared.

Regarding the matrix effect on the signal intensity, blank samples of feed were extracted, then spiked with analytes' aliquots of this extract at the same concentration level than a reference standard solution prepared in the same extraction solvent, H_2O for theobromine and AcCN:H_2O 8:2 v/v for gossypol. By comparing the DART-HRMS signal obtained from both solutions (spiked extract and standard solution), we evaluated the matrix effect on the DART-HRMS signal. Figures 14 and 15 show the chronograms obtained for the feed sample extract diluted from 1:80, 1:40 and 1:20 and spiked at the same concentration level with theobromine (0.50 mg/L, Figure 14) and diluted 1:20, 1:10 and 1:5 for gossypol (5 mg/L, Figure 15). As can be observed, when diluting the matrix, the signal becomes higher. Additionally, we observed a decrease in background noise. Figures 16 and 17 show bar diagrams to compare the reference standard solution and the feed extracts diluted and spiked at the same concentration level. As can be observed, the matrix effect is significant.

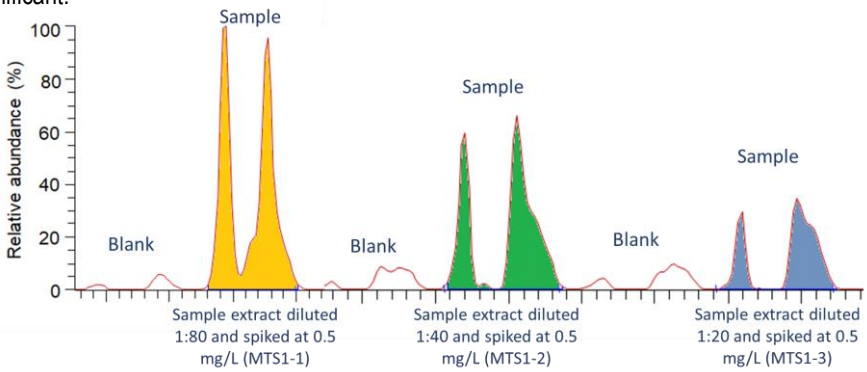


Figure 14. Chronogram of an extract feed sample diluted at three different levels and spiked at 0.5 mg/L of theobromine.

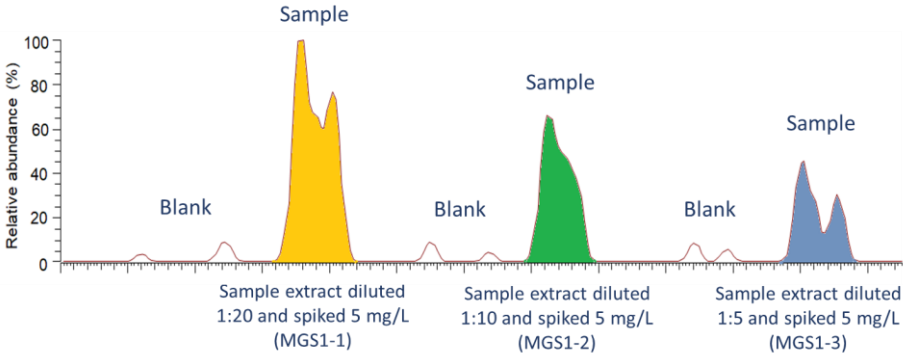
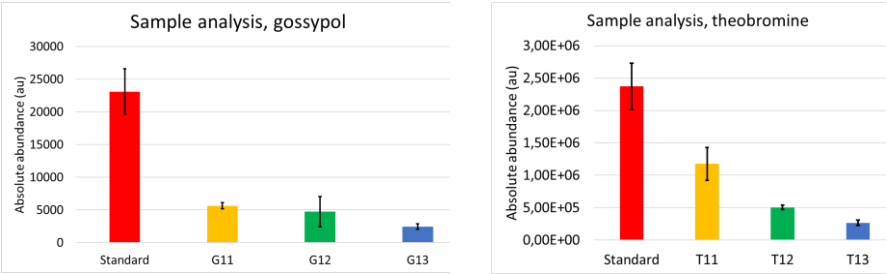
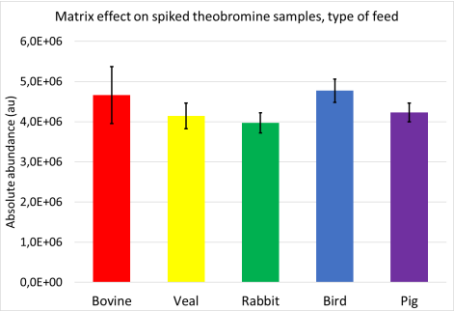


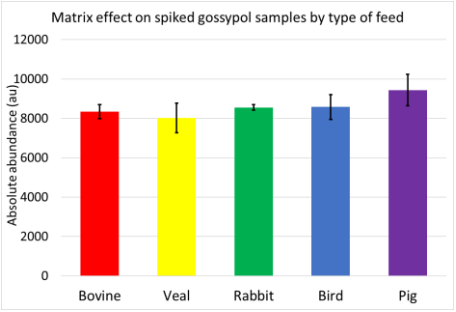
Figure 15. Chronogram of and extract feed sample diluted at three different levels and spiked at 5 mg/L gossypol.



Figures 16 and 17. Bar diagrams corresponding to the signal intensity of gossypol and theobromine in the reference standard and in the matrix, extract diluted at three different levels and spiked at the same concentration than the reference standard (0.5 mg/L of theobromine and 5 mg/L of gossypol).

Additionally, we also extracted different types of feed samples (bovine, veal, rabbit, bird, pig), all of them blank feed samples. The extracts were spiked at the same concentration level to compare the matrix effect between them. Figures 18 and 19 show the results obtained for theobromine and gossypol and as you can see, the matrix effect was quite similar for all of them, and the differences are mainly related on the variability of the DART-HRMS method.





Figures 18 and 19. Matrix effect observed when analysing different types of feed samples.

6.2.2. Method limits of detection (mLOD)

The method limits of detection were estimated by spiking blank feed samples. Table 9 summarizes the results obtained.

Table 9. DART-HRMS/MS mLODs of analytes in feed and the legislated maximum allowed levels.

	Theobromine	**Gossypol**
iLOD [mg/L, extract feed solution]	0.0050	0.50
mLOD [mg/kg, feed sample]	0.80	2.5
Legislated maximum allowed level [mg/kg]	50	20
Legislated maximum allowed level/mLOD	50	8
Final concentration of the method [mg/L]	0.63	4.0

As can be seen, the DART-HRMS method allowed the detection of theobromine in feed samples at a concentration level of 1.0 mg/kg and gossypol at 2.5 mg/kg. The mLOD was 50 times lower than the legislated maximum allowed level for theobromine (50 mg/kg). However, for gossypol the DART-HRMS method only provided a mLOD 8 times lower than that legislated limit.

The use of MS/MS did not improve the mLOD of gossypol, but it certainly reduced the number of undesired signals. With theobromine, however, it did reduce the mLOD by orders of magnitude (in full scan, the LOD was around 0.10 mg/L in solution), proving that MS/MS significantly improves the analysis.

6.2.3. Analysis of positive samples

In order to evaluate the feasibility of the DART-HRMS method, ten different feed samples that were previously analysed by HPLC-MS were now analysed using the new DART-HRMS method proposed in this project. Some of these samples were positive in theobromine and others were reported negative with the HPLC-MS reference method used in the LAC.

As an example, Figure 20 shows the results of three samples (Samples: MTR01, 02 and 03, MTR meaning that is a real sample of theobromine). MTR01 was a feed sample with a concentration of theobromine above the mLOD but below the legislated limit (30 mg/kg); sample MTR02 contained theobromine close to the legislated level (51 mg/kg), and MTR03 was a positive sample with theobromine clearly above the legislated limit (293 mg/kg). Theobromine was easily detected in these samples at these concentration levels demonstrating that the method can be proposed for the screening of feed samples.

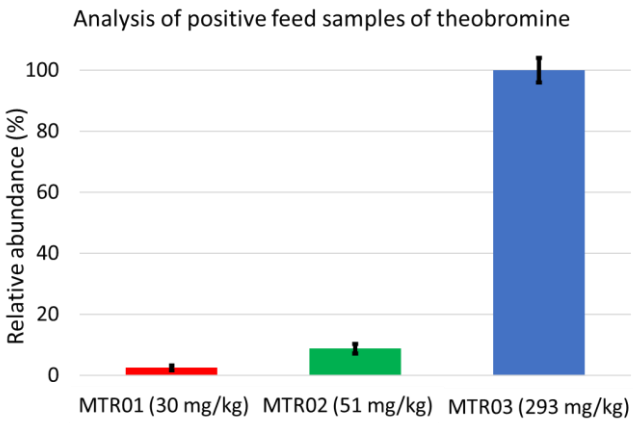


Figure 20. Comparison of three different feed samples that tested positive in theobromine.

The analysis of positive samples could not be made with gossypol, since no positives feed samples were available. In Figure 21, the MS/MS spectrum of MTR02 can be seen, where the parent ion of theobromine, along with the two fragments used for confirmation can be seen clearly.

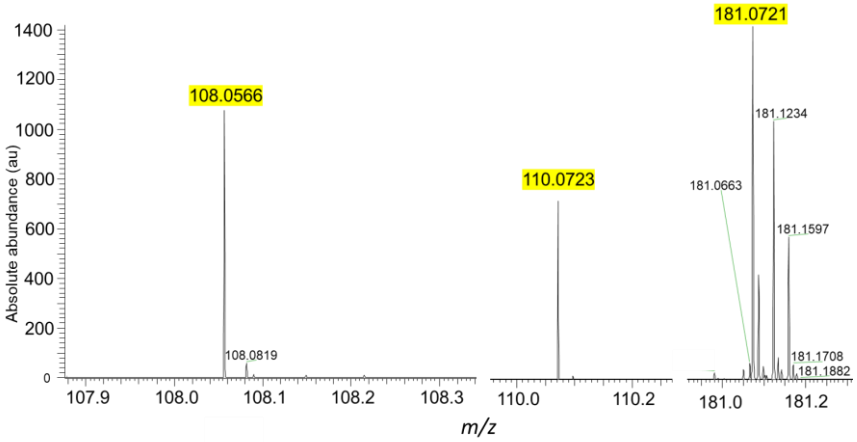


Figure 21: MS/MS spectrum of MTR02 (51 mg/kg).

7. CONCLUSIONS AND FUTURE STUDIES

The general conclusion of this TFG project is that DART-HRMS can be used as promising method for the screening of theobromine and gossypol in feed samples. The new DART-HRMS method has proven to be fast and sensitive enough to detect these analytes in feed samples with minimal manipulation of the samples and in a short analysis time, which would improve the productivity of agrifood control laboratories.

As specific conclusions:

- Theobromine and gossypol ionize with DART generating the protonated and deprotonated molecules, respectively.

- The DART conditions have been optimized and the optimal signal were obtained using nitrogen as DART gas, a DART-to-MS inlet distance of 2.6 mm, scanning mode at 0.8 mm/s and DART gas temperature of 400 °C for gossypol and 500 °C for theobromine.

- Low (LRMS) and high-resolution mass spectrometry (HRMS) have been evaluated and to achieve the best mLODs, HRMS is required to overcome the important isobaric interferences observed in the analysis of feed samples.

- Fragmentation studies were of great importance to detect the most characteristic product ions for selective detection and confirmation purposes. The combination of the mass spectral information provided by tandem in-time (MS^n) and in-space (MS/MS) allowed to establish the fragmentation pathway of these compounds.

- The sample manipulation was kept as minimal as possible, and the procedure proposed was a compromise between the matrix effect observed and the required detection limit.

- Although the DART-HRMS iLODs were very low (0.005 mg/L and 0.5 mg/L for theobromine and gossypol respectively), to mLODs down to 0.8 and 2.5 mg/kg, that were 50 and 8 times lower than the legislated level of theobromine and gossypol, respectively, it was necessary to use HRMS/MS to improve sensibility and selectivity.

Future studies should be focused on improving gossypol mLODs and controlling/correcting the signal variability. This TFG project was a “proof of concept” of the potential of the DART-HRMS/MS method as screening method for gossypol and theobromine. Further studies aim to transfer this method to agrifood control laboratories for routine analysis.

8. REFERENCES AND NOTES

[1] Cody; R.B., Laramée; J.A., Durst; H.D., "Versatile new ion source for the analysis of materials in open air under ambient conditions", *Analytical Chemistry*, **2005**, *vol. 77, no. 8*, 2297–2302.

[2] Javanshad; R., Venter; A.R., "Ambient ionization mass spectrometry: Real-time, proximal sample processing and ionization", *Analytical Methods*, **2017***, vol. 9, no. 34,* 4896–4907.

[3] Domin; M., Cody; R., "Ambient Ionization Mass Spectrometry", 2nd ed., *Royal Society of Chemistry*, **2015**.

[4] Chernetsova; E.S., Morlock; G.E., Revelsky; I.A., "DART mass spectrometry and its applications in chemical analysis", *Russian Chemical Reviews*, **2011**, *vol. 80, no. 3*, 235–255.

[5] Tsagkaris; A.S., Hrbek; V., Dzuman; Z., Haislova; J., "Critical comparison of direct analysis in real time orbitrap mass spectrometry (DART-Orbitrap MS) towards liquid chromatography mass spectrometry (LC-MS) for mycotoxin detection in cereal matrices", *Food Control*, **2022**, *vol. 132*, *art. 108548*.

[6] Gross; J. H, "Mass Spectrometry", 3rd ed., *Springer International Publishing*, **2017**.

[7] Perry; R.H., Cooks; R.G., Noil; R.J., "Orbitrap mass spectrometry: Instrumentation, ion motion and applications," *Mass Spectrometry Reviews*, **2008**, *vol. 27, no. 6*, 661–699.

[8] A. Makarov and M. Scigelova, "Coupling liquid chromatography to Orbitrap mass spectrometry", **2010**, *Journal of Chromatography A*, *vol. 1217, no. 25*. 3938–3945.

[9] Yan; X. Ting, Zhang; Y., Zhou; Y., Hui Li; G., Feng; X. Song, "Technical Overview of Orbitrap High Resolution Mass Spectrometry and Its Application to the Detection of Small Molecules in Food", *Critical Reviews in Analytical Chemistry*, **2020**.

[10] Smit; H. J., "Theobromine and the Pharmacology of Cocoa", *Handbook of Experimental Pharmacology*, **2011**, 201–234.

[11] Merck Veterinary Manual - Toxicology – Chocolate, https://www.msdvetmanual.com/ (accessed Mar 04, 2022).

[12] "Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed".

[13] Dodou; K., "Investigations on gossypol: past and present developments", *Expert Opinion on Investigational Drugs*, **2005**, *vol. 14, no. 11*, 1419–1434.

[14] O'Neil; M.J., "The Merck Index: An Encyclopaedia of Chemicals, Drugs, and Biologicals", 14th ed., *Reference Reviews*, **2013,** *vol. 21, no. 6*, 1719.

[15] Hazardous Substances Data Bank. PubChem. https://pubchem.ncbi.nlm.nih.gov/source/hsdb/7872 (accessed Mar 12, 2022).

[16] Protto; A. L., "Màster Universitari en Química Analítica: Direct analysis in real time-mass spectrometry for the analysis of banned dyes in food", *Universitat de Barcelona*, **2021.**

9. ACRONYMS

AcCN: Acetonitrile

MeOH: Methanol

MS: Mass Spectrometry

LRMS: Low Resolution Mass Spectrometry.

HRMS: High resolution Mass Spectrometry.

DART: Direct Analysis in Real Time.

LC-MS: Liquid Chromatography – Mass Spectrometry.

ESI: Electro Spray Ionization

LOD: Limit of Detection.

iLOD: Instrumental Limit of Detection

mLOD: Method Limit of Detection

Theo: Theobromine.

Gos: Gossypol.

LAC: *Laboratori Agroalimentari de Cabrils*

AIMS: Ambient Ionization Mass Spectrometry

APCI: Atmospheric-Pressure Chemical Ionization

APPENDICES

APPENDIX 1: COLLISION CURVES AND FRAGMENTATION ROUTES

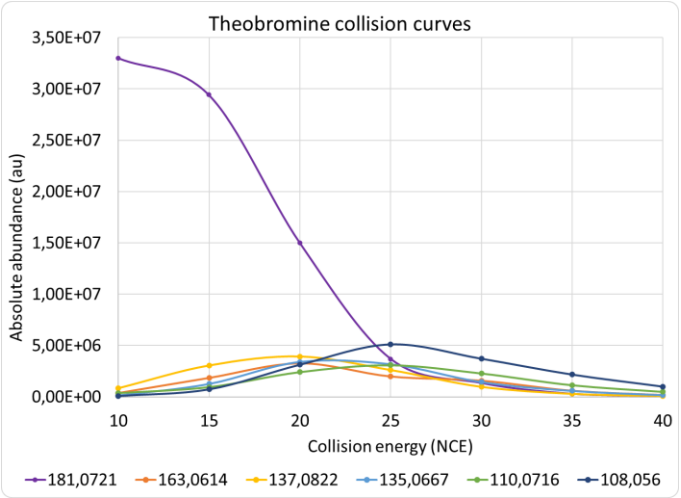


Figure A1. Collision curves of theobromine. The parent ion is shown in purple.

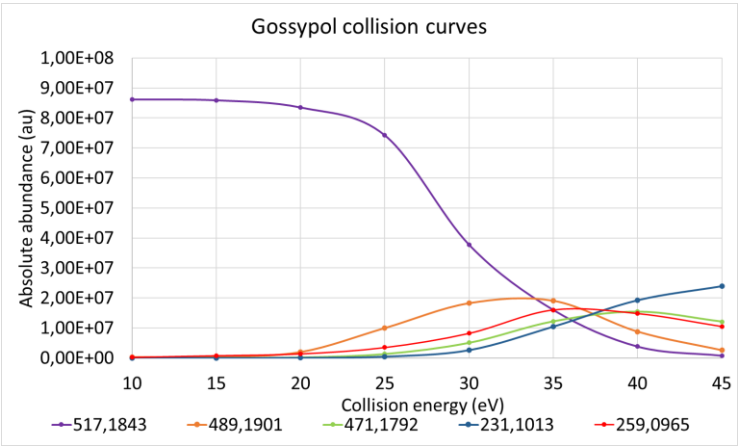


Figure A2. Collision curves of gossypol. The parent ion is shown in purple.

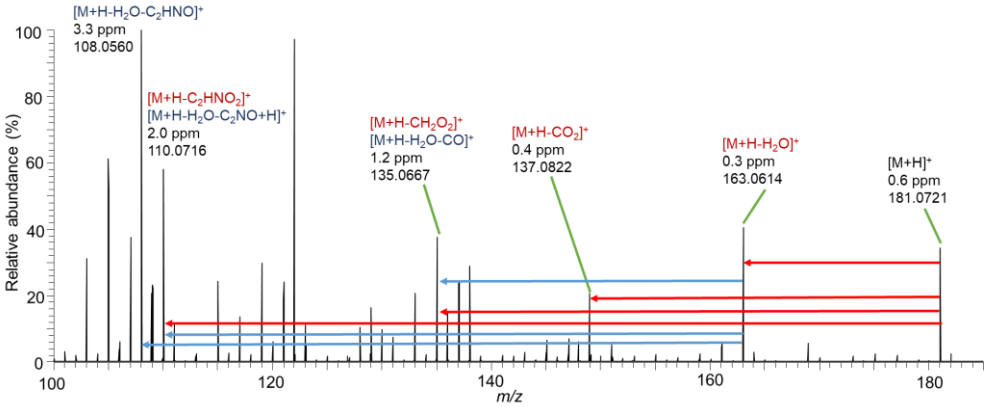


Figure A3. Fragmentation routes of theobromine. The measured m/z is shown along with the formula of the fragment and the accuracy in ppm. In red are the primary fragmentations, and in blue the secondary ones.

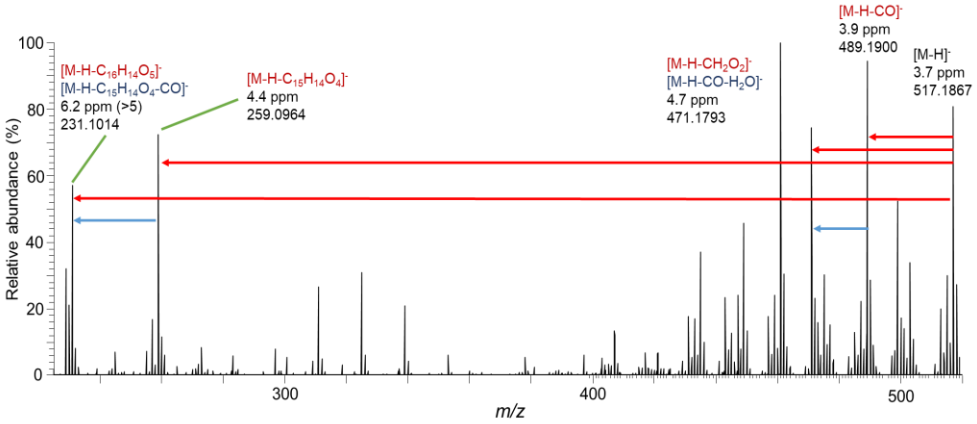


Figure A4. Fragmentation routes of gossypol. The measured m/z is shown along with the formula of the fragment and the accuracy in ppm. In red are the primary fragmentations, and in blue the secondary ones.