Possibilities of Leafspray Mass Spectrometry in the analysis of fruits

by using soft fruits and star anise fruit

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Marijn Schrage
Supervision by Teris van Beek
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ABSTRACT

During the last decade, several ambient MS techniques like MALDI, DESI and DART are developed, making analysis on mass spectroscopy possible without or with low input sample pre-treatment. During this thesis, the relatively new ambient MS technique leafspray is explored. Experiments were performed on both soft fruits (kiwi, tomato and apple) in search of the content of ascorbic acid and on the hard fruit of anise star. Anise star knows two species which are morphologically the same; the edible Chinese one and the Japanese version which contains a strong neurotoxin. A safe way to distinguish both is by mass spectrometry.

Ascorbic acid content is not measureable due to the absence of a strong, consistent spray, anisatin however shows clear results, with differences from 200 to 300 times between Chinese and Japanese star anise. As a benchmark technique, DART is chosen, showing differences between the two species up to 1000.

Leafspray is used on orbitrap MS and on iontrap MS and shows a strong distinction between both star anises on both orbitrap MS and iontrap MS. Compared to DART, Leafspray technique is cheaper and easier in use. The signal is stronger, more consistent and shows less variation.

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1.1 AMBIENT TECHNIQUES

The origin of mass spectroscopy (MS) lays over a century ago. Back then, it was performed by Thomson and Aston, during their work about the existence of positively charged ions. Meanwhile, it has become a wide spread way to analyze all kind of compounds. (University of Bristol)

Though Mass spectroscopy is an effective and fine technique, sample preparation for MS takes a lot of time and also might influence the analytes of interest, or the sample in general. Since there is a need for fast results , for example during drug tests in sport events, or for measuring residues on fruits in a supermarket, a quick method for fast analysis without (much) sample preparation was needed.

This resulted in the development of the ambient techniques (Figure 1 shows an overview of ambient and ambient-like techniques).

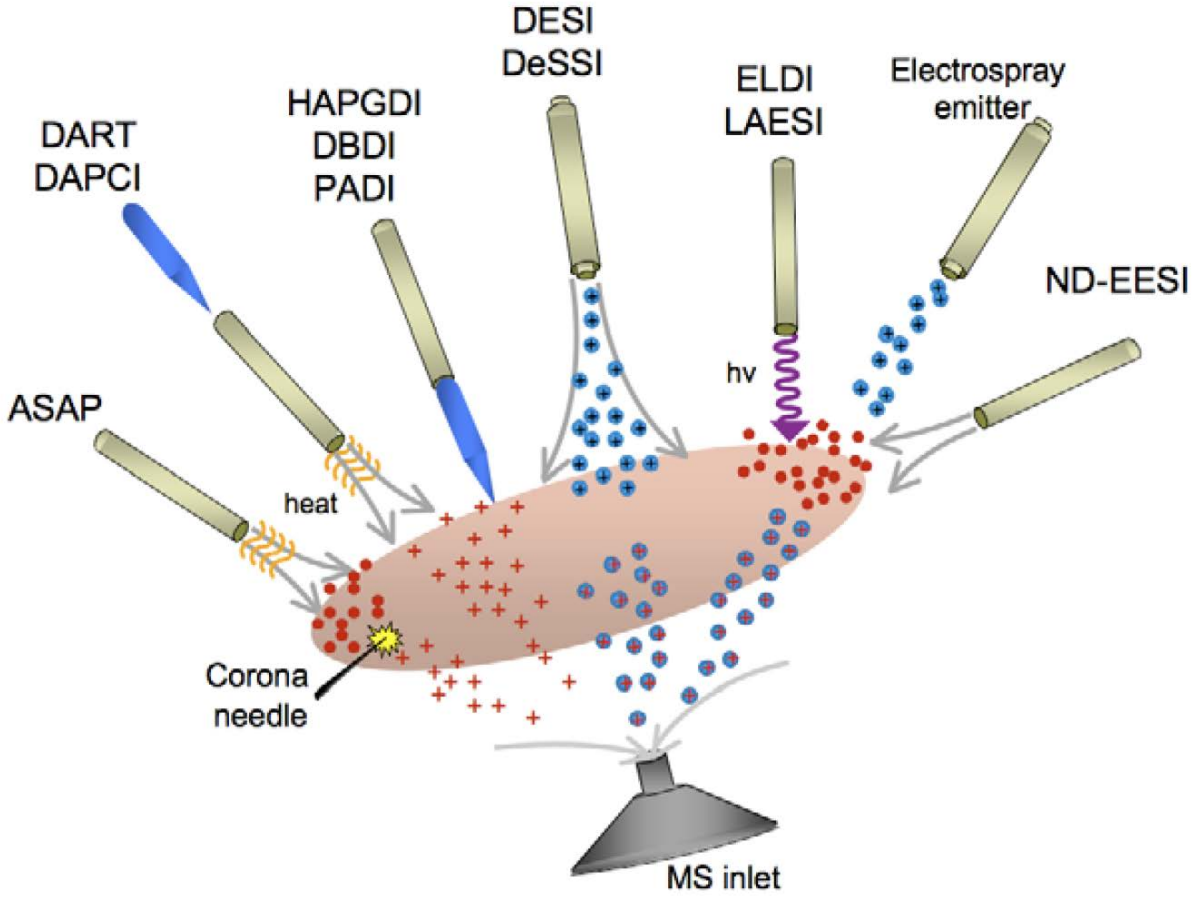

FIGURE 1: SEVERAL AMBIENT AND AMBIENT-LIKE TECHNIQUES. (Venter, Nefliu et al. 2008)

During the time, mass spectroscopy has developed several different ambient techniques. One example of an ambient-like technique is matrix-assisted laser desorption ionization short MALDI. For the MALDI technique, a stainless steel plate is present, horizontally and close to the MS inlet. The analyte is embedded on a matrix on this plate. A laser sends light in pulses towards this plate, resulting in ionization of the analyte. Now the analyte is ionized, it can enter the MS-inlet. An additional ambient-like technique is needle biopsy and spray ionization. For this

technique, a needle is fluid with body fluid, for example from the brains, liver, kidney, adrenal gland, stomach or spinal cord. In order to spray the fluid toward the MS-inlet, a high voltage is applied to the needle. Another example is desorption electro spray ionization, (DESI). DESI uses a surface, containing the solved analyte. A spray emitter with applied voltage produces small ionized droplets, which fall on the pre-wetted surface with the analyte dissolved. This results in new charged drops, originated from the surface with the solved analyte. The charged drops with analyte enter the MS-inlet and can be analyzed. One more method is the direct analysis in real time, DART technique.

1.1.1 DART

The DART technique has the advantage that there is no sample preparation needed at all and it gives very fast and adequate result. A helium gas stream derived from the DART add-on is directed toward the MS inlet, with the sample in between. Parts from the surface of the sample are directly ionized and react with the surrounding air and water molecules to form reactive ions, which are detected by the mass spectrometer. A DART add-on is required and attached to the MS, afterwards the sample can be held in between the add-on and the MS inlet. (Curtis, Minier et al. 2010)

1.1.2 PAPERSPRAY

A relatively new technique is paperspray. The paperspray method is used to analyze compounds in medical samples, for example urine, blood and tissue, though it can be also used for other compounds like residues on fruits for instance.

For this technique, paper is cut into a triangular shape and the analytes are applied on the paper (by dripping in case of urine or blood or by rubbing in the case of fruit residues). Next, solvent is added on the paper substrate. The paper is put in front of the MS inlet and a high voltage is applied. The analytes spray in the direction of the MS inlet and can be detected by the mass spectrometer.(Soparawalla, Tadjimukhamedov et al. 2011; Zhang, Xu et al. 2011)

1.1.2.1 POSSIBILITIES

Paperspray is a very fast technique, and the positive side of it, it does not need expensive additions to your MS machine, like a DART add-on. Besides, it has minimized sample preparation. Therefore, if this technique is combined with a portable MS system, it can for example be used in shops, for testing of residues on fruits (like oranges or apples), like Soparawalli et all describe in their paper. You only wipe the papers on the peel of orange and e.g. residues of the fungicide thiabendazole can be found. (Soparawalla, Tadjimukhamedov et al. 2011)

The technique can also be applied to detect therapeutic drugs in dried blood stains.(Zhang, Xu et al. 2011) Zhang et al explain in their article about the technique of paperspray combined with the analysis of therapeutic drugs in dried blood spots. Another advantage of the technique is that it is simple and therefore can also be performed by people that are not experienced chemists, which is the case in the traditional techniques with chromatography and analysis with mass spectrometry, ultraviolet, fluorescence or immunoassays.

1.1.3 LEAFSPRAY/FRUITSPRAY (LS/FS)

A new technique, originated from paperspray and named leafspray, was first described a couple of years ago and creates new possibilities. Several variations are put forward by literature, but the core principle stays the same. A high voltage is applied to pure plant material, resulting in a spray in the direction of the MS inlet (shown in Figure 2). One of the main advantages, apart from the absence of expensive ads, is the possibility to observe in living plants and living plant material.

For example Peng, Zhang et al. use a capillary combined with leafspray to obtain information about the bio-molecules in aloë-vera, grape, kumquat and cherry tomato. (Peng, Zhang et al. 2012)

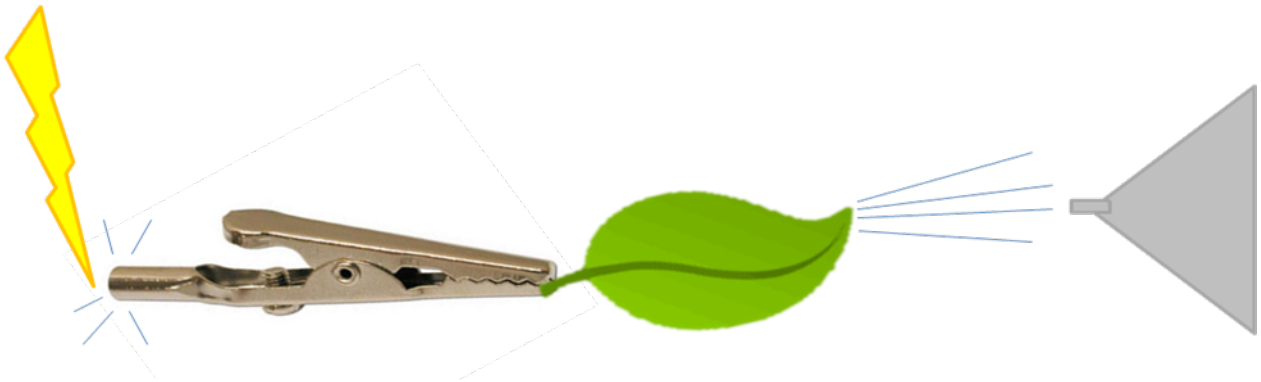

FIGURE 2: SCHEMATIC OVERVIEW OF THE LEAFSPRAY TECHNIQUE

Liu, Wang et al. cut a tip in the plant material, before applying the electrical potential, or use the natural tips of the plant (needles, sprouts or just a sharp/pointy fruit) to spray the charged droplet towards the mass spectrometer inlet. Using this technique they are capable of finding amino acids, carbohydrates, fatty acids, alkaloids and lipids. (Liu, Wang et al. 2011) + supplemental information)

The success of Liu, Wang et al. in 2011, seems to have given rise to more experimental use of the leafspray/fruitspray (LS/FS) technique. In 2012 Malaj, Ouyang et al. showed the leafspray technique to be capable of determining pesticide residues on fruit samples of apple, pear, lemon, orange and vegetables like cucumber, eggplant and potatoes. (Malaj, Ouyang et al. 2012) Also, Sarkar, Srimany and Pradeer could show increasing trend of ursolic and oleanolic acid in Tulsi (Holy Basill. *Ocimum sanctum* Linn) using Leafspray. (Sarkar, Srimany et al. 2012)

In comparison with other ambient techniques, the leafspray technique is simpler and needs less expensive equipment. For other techniques, there is still a little sample preparation needed, except in the case of DART, but for the DART technique, an expensive ion source is needed, where leafspray only needs a small crocodile clamp and a voltage which can be applied to the clamp.

1.1.3.1 TAYLOR-CONE

I order to receive a clear result for LS/FS, it is necessary to get a continuous spray. The best way to get this spray is to create a so called Taylor-cone, also called a cone-jet. In order to get a Taylor cone, a sharp tip is necessary. Back in 1964, Sir Geoffrey Ingram Taylor found that if an electric field is brought into contact with a not too large amount of conductive fluid, a cone is formed, with an angle of 98.6° and a round tip. If the right voltage is applied, the tip of the cone forms a small “jet”, resulting in little droplets leaving the tip. Enough of these drops leaving the tip results in a so called “plume” (showed in Figure 3). This Taylor cone, if formed in front of the MS inlet, allows the droplets get right into the MS inlet resulting in a continuous spray intake for the LS/FS technique. (Taylor 1964)

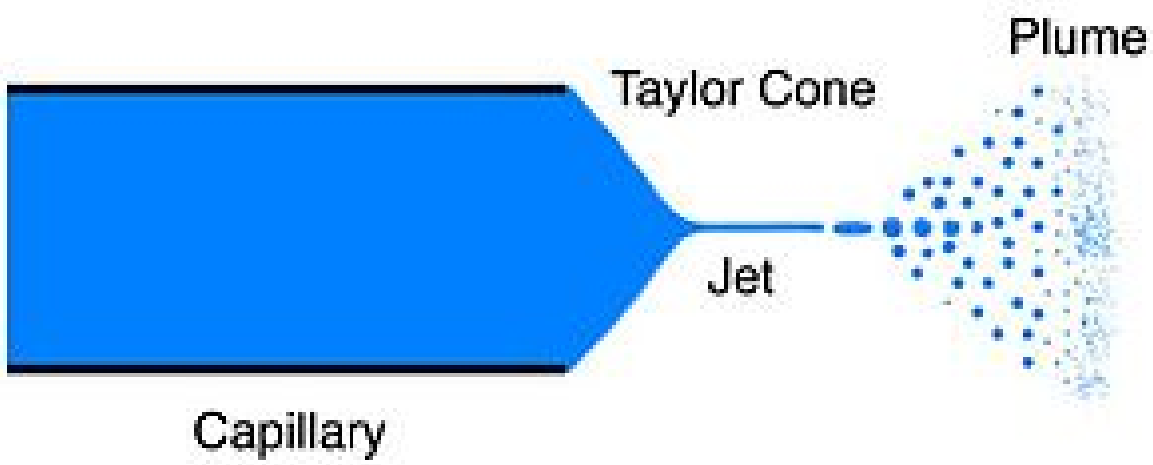

FIGURE 3: TAYLOR CONE

1.2 Fruits

1.2.1 Ascorbic acid as a content of soft fruits

Ascorbic acid, also called Ascorbic acid (structure shown in Figure 4) is an antioxidant which is believed to reduce risks of chronic diseases as cancer, cardiovascular diseases and cataract. Ascorbic acid is thought to influence cholesterol levels, with increase of HDL (high density lipoprotein) and decrease of LDL (low density lipoprotein) levels, lowering the risk of cardiovascular diseases. Being an antioxidant, Ascorbic acid can inhibit DNA oxidation and thus reduce risks of cancer. (Simon 1992)

Ascorbic acid is also believed to be a cofactor for several enzymes involved in the biosynthesis of collagen (main component of connective tissue), carnitine (essential for sending fatty acids to the mitochondria) and neurotransmitters. (Burri and Jacob 1997; Tsao 1997) Besides, a shortage of ascorbic acid results in fatigue and lethargy, tooth loss, joint pains, bone and connective tissue disorders and poor wound healing (symptoms of scurvy). Furthermore depression, hypochondria and mood changes are symptoms that occur during Ascorbic acid deficiency. (Burri and Jacob 1997)

Intake of Ascorbic acid is essential for humans and primates, since it is not produced by the body itself due to a mutation in the L-gulonolactone oxidase gene. (Woodall and Ames 1997) Especially (citrus) fruits and vegetables contain high amounts of ascorbic acid. During this thesis, we will focus on Kiwi, apple and tomato and we will check the amount of ascorbic acid in different parts of the fruits, i.e. near the peel, in the core and in the mesocarp.

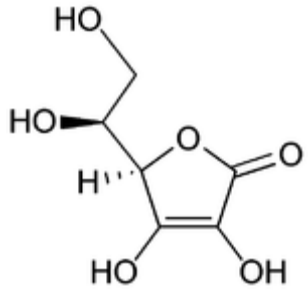

FIGURE 4: STRUCTURE OF ASCORBIC ACID (ASCORBIC ACID) MOLECULE FORMULE: C6H8O6

1.2.1.1 Amounts Ascorbic acid found in fruits

The American health website was checked for contents of ascorbic acid in Kiwi, Tomato and Apple. In Table 1, below, these data are shown.

TABEL 1: CONTENTS OF ASCORBIC ACID, NATRIUM AND POTASSIUM IN SOFT FRUITS.

	Ascorbic acid content in mg/100 g	Na (in mg/100 g)	K (in mg/100 g)
Kiwi	92.70	3.00	312.00
tomato, red, raw	12.70	5.00	237.00
apple without Peel	4.00	0.00	90.00
apple with peel	4.60	1.00	107.00

Though it must be mentioned the information about Ascorbic acid content at different websites and in articles varies. Vinci, Botre et al. mention for Kiwi 45.93 up to 67.23 mg/100g (Vinci, Botre et al. 1995). And the website of the United States Departure of Agriculture mentions 92.7 for green kiwi, 13.7 for tomato and 4.6 and 4 for apple respectively with and without peel.

The data for sodium and potassium show some variation, though the differences are not that high to be alarmed. (Health)

1.2.2. STAR ANISE

Star anise is a sweet smelling spice known for several applications. Its fruits (Figure5) are popular in the Asian kitchen and can also be used to make tea. In addition to this, star anise can be found in e.g. Christmas decorations as an adornment.

FIGURE 5: FRUITS OF STAR ANISE

However, there is a slight drawback to the use of this fruit, for two different kinds of star anise exist; Chinese star anise and Japanese star anise (shown in Figure 6).The last one contains a strong neurotoxin. Japanese star anise is hardly distinguishable from Chinese for they are morphologically almost the same. The neurotoxin anisatin that is found in Japanese star anise causes emesis, diarrhea, bradycardia, hallucinations, rhabdomyolysis, convulsions and even seizures. ((van Dijk 2002; Ize-Ludlow, Ragone et al. 2004; Upton 2006)In: (Howes, Kite et al. 2009) and therefore, Japanese can only be used as a decorative product.

FIGURE 6: LEFT: JAPANESE STAR ANISE, RIGHT: CHINESE STAR ANISE

In the last years, several cases of ingestion were reported, some with fatal ending". Therefore it is crucial to tell both star anise species apart. (Shen, van Beek et al. 2012)

The main goal of this thesis was to reproduce the results made on Leafspray by other labs, e.g. those of Liu, Wang, et al (2011). Leafspray experiments on green onion, ginger, cauliflower and Brussels sprout can be performed with and without added solvent. Also they can be performed in both negative and positive mode. Results can be compared to data from literature.

If this would succeed it is possible to do measurements of our own. These experiments can be performed on (soft) fruits as described in literature (Liu, Wang et al. 2011; Malaj, Ouyang et al. 2012; Sarkar, Srimany et al. 2012), but also on hard fruits, like star anise, by using a solvent to create a stable spray. When these experiments would result in qualitative results, it would be interesting to explore and optimize the technique of leafspray. For example using different solvents, changing the distance toward the MS inlet and the amount of solvent and the voltage applied can be optimized in order to make leafspray an adequate technique.

When qualitative results are possible, it can be interesting to take a look at the possibility of coupling the leafspray technique to quantitative results. In order to obtain quantitative results, a known technique has to be used as a control. For this purpose, for example, high-performance liquid chromatography (HPLC) can be used.

Next to the described leafspray from literature, it is possible to seek for different ways to analyze samples. For example by using a capillary to aim the spray, as described by (Liu, Wang et al. 2011) or a triangle shaped steel plate to increase precision and/or signal.

Subsequently, it would be interesting to experiment on different types of MS; both the LXQ and the exactive. This is mainly to increase the accessibility of the technique and to make it also available for laboratories without an (expensive) exactive MS.

1.4.1 ASCORBIC ACID AND ANALYSIS

1.4.1.1 PROTOCOL HPLC FOR HIGHLY POLAR GLUCOSINOLATES AND SO ASCORBIC ACID

Ascorbic acid is a highly polar molecule and has a negative charge, therefore, in order to analyze the compound; a protocol had to be found that is in collaboration with the chemical. During the late 90's Gabrys, Tjallingii and van Beek did a HPLC analysis in the Wageningen UR ORC lab for the highly polar and negatively charged chemical glucosinolates (figure 7) in cabbages. The protocol used for HPLC during this thesis will be similar to the protocol used by Gabrys, Tjallingii and van Beek.(Gabrys, Tjallingii et al. 1997)

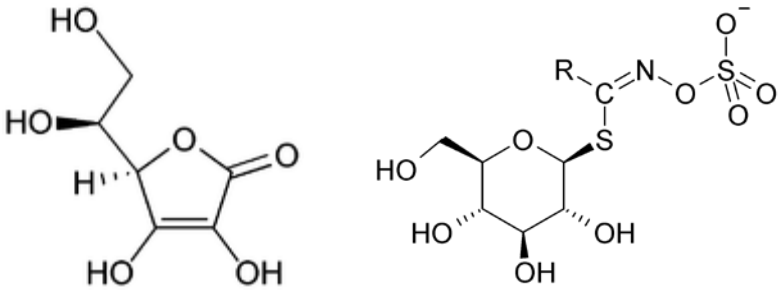

FIGURE 7: LEFT: CHEMICAL STRUCTURE ASCORBIC ACID
RIGHT: CHEMICAL STRUCTURE GLUCOSINOLATE

Ascorbic acid has a mass of 176,124, therefore peaks can be expect at: *m/z* $[M-H]^-$ = 175,12 *m/z* $[M+H]^+$ = 177,132 and *m/z* $[M+Na]^+$ = 199,114. Frenchich, Torres et al. did performed liquid chromatography on Ascorbic acid, resulting in the spectrum shown in Figure 8. Their measurements are in negative ion mode. Though it has to me mentioned that this spectrum has to be used with care, for Frenchis explains the compound at m/z = 169.2 as [M-OH], where M-OH would be expected at *m/z = 159*. And the *m/z = 169.2* is an unknown compound.

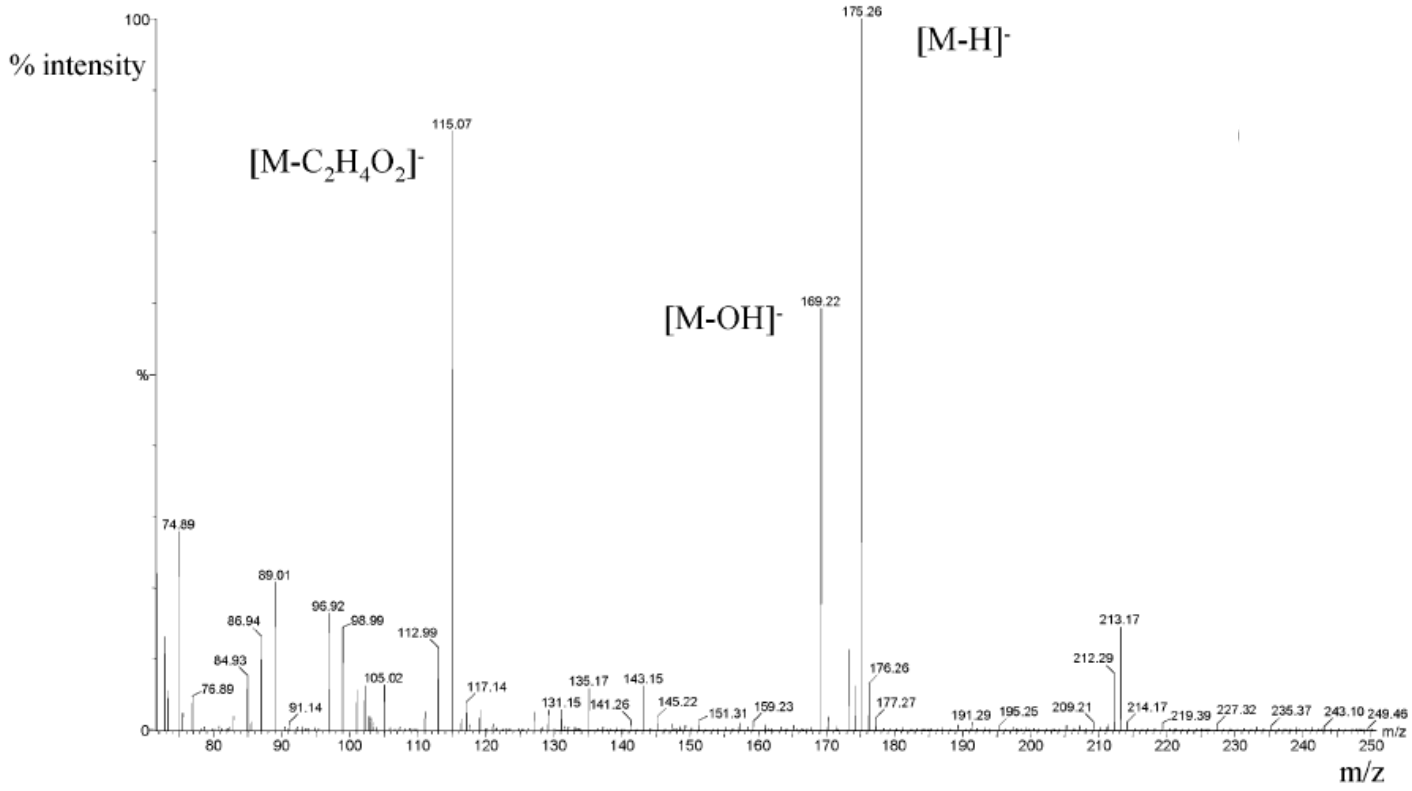

FIGURE 8: SPECTRUM FROM ASCORBIC ACID, NEGATIVE ION MODE, OBTAINED BY LIQUID CHROMATOGRAPHY.(FRENICH, TORRES ET AL. 2005)

During the execution of this thesis, the sample material was changed. Initially, the soft fruits kiwi, apple and tomato where analyzed on their content of ascorbic acid (ascorbic acid), but since there were problems with the spraying capacity of these fruits, resulting in un-satisfying and unclear results (shown in the result section) using both Leafspray and HPLC, the Leafspray technique was also applied on the more robust star anise fruits. Star anise was checked for the toxic compound anisatin that differentiates the two species from each other. Fruit juice can be produced from soft fruits and HPLC analyses can be performed on this, but the hard fruit from the star anise can be easier analyzed using a different technique.

1.4.2 ANISATIN AND ANALYSIS

The common method to distinguish Japanese from Chinese star anise is thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS) or UPLC (in biological sample)

Shen et van Beek (2011) suggested the procedure of using DART as a benchmark technique, for it is a very fast ambient technique, without sample preparation. For this reason, DART is used as a control to LS/FS.

LS/FS would be a cheaper alternative to DART to indicate anisatin presence and with that, differentiate Japanese from Chinese star anise. In order to do so, it is necessary to create a spray out of the dry, robust anise star fruit. This can be achieved by addition of a solvent, which will enter the MS inlet. For anisatin has a non-common mass, we choose to use a High resolution mass spectrometry (HRMS) for analysis.

1.4.2.1 STRUCTURE OF ANISATIN

Figure 9 shows the molecular structure (schematically) of anisatin.

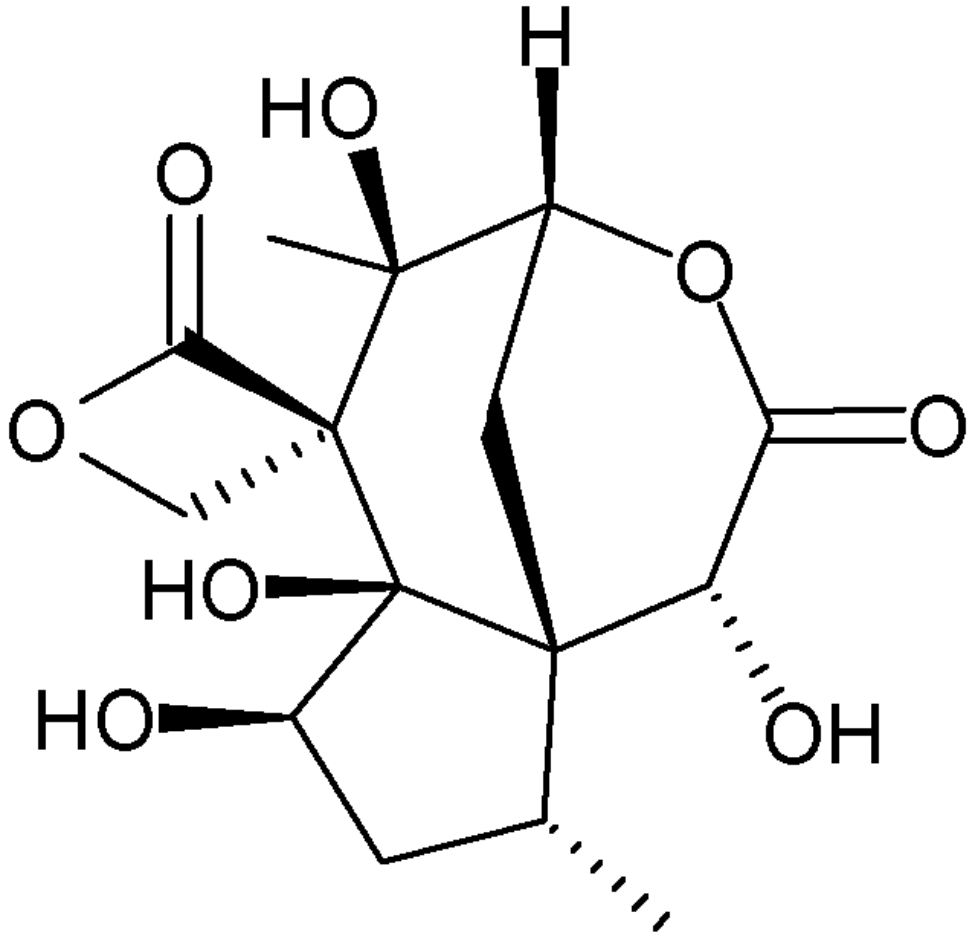

FIGURE 9: STRUCTURE OF ANISATIN, MOLECULE FORMULE: $C_{15}H_{20}O_8$

1.4.2.2 ANISATIN AND MASS SPECTROMETRY

Under DART circumstances, anisatin is expected to react with NH4 from air in positive mode, though, in LS circumstances, the anisatin might react with the Potassium (K) present in the plant material. Therefore, for DART and LS in positive mode, a different mass can be expected. M = 328,3, Therefore *m/z* [M+H] = 329,3, *m/z* [M-H] = 327,3, *m/z* [M+NH4] = 346,4 and *m/z* [M+K] = 367,4 can be expected.

2 MATERIALS AND METHODS

2.1 PAPERSPRAY

The technique of leafspray is based on the existing technique of paperspray. Therefore, a paperspray experiment will be performed.

2.1.1 MATERIAL AND REAGENTS

Ascorbic acid was available in pure form from Merck, Darmstadt, and F.R. Germany. MeOH was from J.T. Baker (Deventer, Netherlands) High-purity helium gas (grade 6.0) was provided by Linde Gas Benelux B.V.

2.1.2 PAPERSPRAY-LIONEAR IONTRAP MS

2.1.2.1 ANALYSIS CONDITIONS

The paperspray iontrap MS system consisted of a crocodile clamp, to which a voltage could be applied, coupled to a Thermo LXQ linear ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). LXQ was used in negative mode. Settings: capillary temperature 300 °C, capillary voltage −3 V, tube lens voltage −80 V and CID 25%.

2.1.2.2 QUALITATIVE AND QUANTITATIVE MEASUREMENTS OF ASCORBIC ACID

Ascorbic acid was dissolved in MeOH (0.5 mg/ml and 0.05 mg/ml). Triangle shaped papers (3 cm in length, 2 cm width) were dipped in the diluted solution and a voltage of 5 kV was applied. In order to keep the spray going, the paper was dripped with the solution of ascorbic acid in MeOH (0.5 mg/ml or 0.05 mg/ml).

2.2 REPRODUCTION OF THE RESULTS OF LIU ET AL.

Measurements of cauliflower, Brussels sprouts, green onion and ginger were done according to the protocol described by Liu, Wang et al. Fruits were obtained from the local market.

2.3 SOFT FRUITS

2.3.1 MATERIALS AND REAGENTS

Apple, kiwi and tomato samples were obtained from Albert Heijn. Also, some apple samples were provided by Teris van Beek his backyard.

High-purity helium gas (grade 6.0) was provided by Linde Gas Benelux B.V. Methanol was from J.T. Baker (Deventer, Netherlands). Ascorbic acid was available in pure form from Merck, Darmstadt, and F.R. Germany.

2.3.2 HPLC

2.3.2.1 ANALYSIS CONDITIONS

Juice was produced from soft fruits (figure 10), using a juice centrifuge obtained from “Blokker” shop, Wageningen, Netherlands. Juice was filtered through a micro filter and analyzed with HPLC according to the method described for glucosinolates by (Betz and Page 1988). A Zorbax XDSC18USKH column was used.

The eluting solvent was an isocratic mixture of 2% MeOH and 0.005 M TAS (tetrabutylammonium hydrogen sulfate)buffer. The flow rate was 1 ml/min and the run time 25 min, injection volumes were 5µl.

2.3.2.2 QUALITATIVE AND QUANTITATIVE MEASUREMENTES OF SOFT FRUITS

Based on the known concentration (0.5 mg/ml) of ascorbic acid a dilution line was made with concentration of 1, 0.5, 0.2, 0.1, 0.05, 0.02, 0.01, 0.005, 0.002 and 0.001. These concentrations where run under the protocol as described above, to obtain a standard curve. HPLC on Fruit samples was performed under the same protocol as the dilutions. HPLC outcomes were compared to the standard curve to obtain information about the concentration of ascorbic acid in soft fruits.

2.3.3 LS/FS – ORBITRAP MS

2.3.3.1 ANALYSIS CONDITIONS

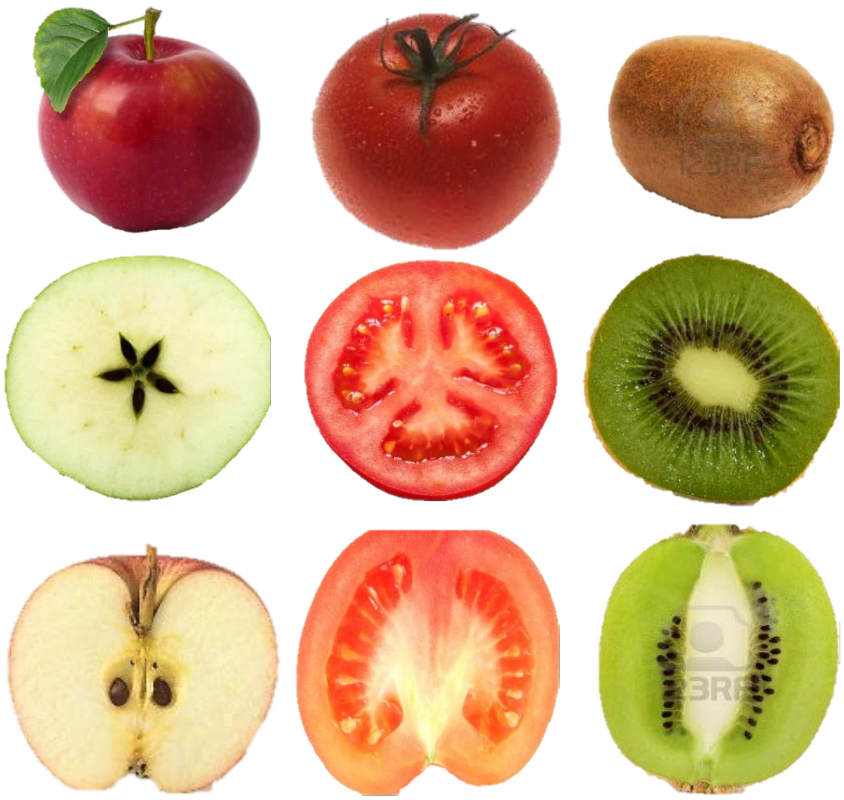

FIGURE 10: SOFT FRUITS TO MEASURE

The leafspray-orbitrap MS system consisted of a crocodile clamp, to which a voltage could be applied, coupled to an exactive high-resolution mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) (shown in figure 11). The mass spectrometer was calibrated each day before starting the analysis of the samples. For instrument control, data acquisition and data processing XCALIBUR software (v. 2.1) was used. Measurements were done in negative mode.

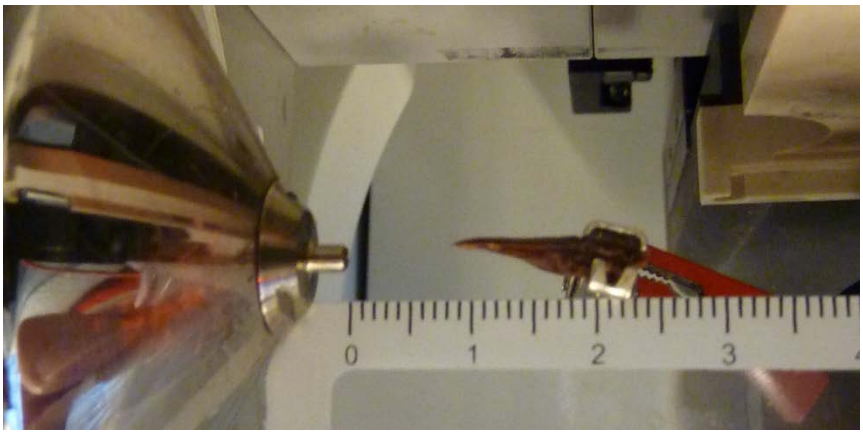

FIGURE 11: SET UP OF THE LEAFSPRAY-ORBITRAP MS SYSTEM

Voltage applied was 5 kV distance used was varying from 2 to 12 mm. New samples were wetted using 250 µl Methanol (MeOH), after each measurement, the sample was rewetted using ±100 µl.

Settings of the orbitrap (−)-mode: capillary voltage −60 V; tube lens voltage −125 V; skimmer voltage −28 V; capillary temperature: 250 °C. The resolution was set at “ultra high” and a scan rate of 1 Hz was used. The mass range was chosen very wide to also include possible dimers or other interesting components. The mass range was set at *m/z* = 150-750.

2.3.3.2 QUALITATIVE RESULTS OF SOFT FRUITS

For LS/FS, one triangle shaped piece of fruit was put in the crocodile clamp which put the tip of the sample 7 mm away from the MS inlet. The fruit was wetted with 250 µl MeOH. The LS/FS-MS measurement was started by recording the background. Then the voltage of 5 kV was applied. In the case that the spray was not initiated, more MeOH was applied. When still no spray had arisen, the distance was slightly varied and later put back at 7 mm.

2.3.3.3 QUANTITATIVE LS/FS-HRMS MEASUREMENTS

To obtain information about the quantity of ascorbic acid, a dilution line was made, based on a known concentration of ascorbic acid (0.5 mg/ml). The concentration was 1, 0.5, 0.2, 0.1, 0.05, 0.02, 0.01, 0.005, 0.002 and 0.001. A spectrum of these different concentrations can be obtained using electrospray ionization or paperspray.

With this data a standard curve can be developed for concentration of ascorbic acid and read out of MS. Data from MS on LS/FS can be compared to the standard curve to obtain information about the concentration of ascorbic acid. This information can be compared to control data obtained in the HPLC-experiments.

Due to non informative results of the LS technique in soft fruits, these measurements were not performed.

2.3.4 LS/FS – LINEAIR IONTRAP MS

2.3.4.1 ANALYSIS CONDITIONS

The leafspray - iontrap MS system consisted of a crocodile clamp, to which a voltage could be applied, coupled to a Thermo LXQ linear ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). . For instrument control, data acquisition and data processing, XCALIBUR software (v. 2.1) was used. Measurements were done in negative mode. Voltage applied was 5 kV distance used was varying from 2 to 12 mm. New samples were wetted using 250 µl Methanol (MeOH), after each measurement, the sample was rewetted using ±100 µl.

Settings of the LXQ (−)-mode: Settings: capillary temperature 300 °C, capillary voltage –3 V, tube lens voltage –80 V and CID 25%

2.3.4.2 QUALITATIVE AND QUANTITATIVE LS/FS-HRMS MEASUREMENTS OF SOFT FRUITS

For LS/FS, one triangle shaped piece of fruit was put in the crocodile clamp which put the tip of the sample 7 mm away from the MS inlet. The fruit was wetted with 250 µl MeOH. The LS/FS-MS measurement was started by recording the background. Then the voltage of 5 kV was applied. In the case that the spray was not initiated, more MeOH was applied. When still no spray had arisen, the distance was slightly varied and later put back at 7 mm.

Quantitative measurements can be performed similar to Orbitrap MS.

2.4 STAR ANISE FRUITS

2.4.1 MATERIALS AND REAGENTS

Chinese star anise (*Illicium verum* Hook. f.) samples were obtained from Taste&Tools Sterrenmix tea, batch 2013, purchased in the Netherlands, from a local market in Sumatra, Indonesia and from a Belgian supermarket (YadanO star anise). Japanese star anise (*Illicium anisatum* L.) samples were obtained from PhytoLab (Vestenbergsgreuth, Germany) and from RIKILT (Wageningen, Netherlands). High-purity helium gas (grade 6.0) for DART was provided by Linde Gas Benelux B.V. Methanol was from J.T. Baker (Deventer, Netherlands), acetone from Sigma-Aldrich (Germany), ethyl acetate from Biosolve (Valkenswaard, Netherlands) and methyl *tert*-butyl ether from Acros Organics (Belgium).

2.4.2. DART–ORBITRAP MS

2.4.2.1 ANALYSIS CONDITIONS

The DART–orbitrap MS system consisted of a DART ion source (model DART-SVP, IonSense, Saugus, USA) coupled to an exactive high-resolution mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The mass spectrometer was calibrated at the beginning of each day. XCALIBUR software (v. 2.1) was used for instrument control, data acquisition and data processing.

DART settings positive mode: He as ionizing gas, fixed flow of ∼3.5 L/min; gas beam temperature 350 ◦C; grid electrode voltage +350 V. MS: capillary voltage +60 V; tube lens voltage +100 V; skimmer voltage +20 V; capillary temperature: 250 ◦C. The resolution was set at “ultra high” and a scan rate of 1 Hz was used. The mass range was m/z 300–400.

DART settings negative mode: He as ionizing gas, fixed flow of ∼3.5 L/min; gas beam temperature 400 ◦C; grid electrode −350 V. MS: capillary voltage −60 V; tube lens voltage −125 V; skimmer voltage −28 V; capillary temperature: 250 ◦C. The resolution was set at “ultra high” and a scan rate of 1 Hz was used. The mass range was m/z 300–400, similar to Shen et al. (2012)

2.4.2.2 QUALITATIVE AND QUANTITATIVE DART–HRMS MEASUREMENTS OF DRY CHINESE STAR ANISE AND JAPANESE STAR ANISE FRUITS

DART measurements were performed, similar to Shen (2012). One carpel (typically 1/8th of an intact fruit) was taken using tweezers. Then the DART–MS measurement was started by recording background. Next the star anise was held in the DART sampling area and slightly moved until a clear signal related to star anise was observed. During 20–30s the star anise piece was held in this position. This was repeated twice more, i.e. an intermittent measurement of background and star anise. The ion at m/z 327.108 (negative mode) or m/z 346.148 (positive mode) was observed. For each of the 3 measurements, the average signal height over the middle 80% of one star anise measurement was taken as a measure of the amount of anisatin.

2.4.3 LS/FS – ORBITRAP MS

2.4.3.1 ANALYSIS CONDITIONS

The leafspray – orbitrap MS system consisted of a crocodile clamp, to which a voltage could be applied, coupled to an Exactive high-resolution mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The mass spectrometer was calibrated each day, before starting the analysis of the samples. For instrument control, data acquisition and data processing, XCALIBUR software (v. 2.1) was used. Measurements were done in both positive and negative mode.

Voltage applied was 5 kV distance used was 7 mm. New samples were wetted using 250 µl Methanol (MeOH), after each measurement, the sample was rewetted using ±100 µl.

Settings of orbitrap (+)-mode: capillary voltage +60 V; tube lens voltage +100 V; skimmer voltage +20 V; capillary temperature: 250 °C. Settings (−)-mode: capillary voltage −60 V; tube lens voltage −125 V; skimmer voltage −28 V; capillary temperature: 250 °C. The resolution was set at "ultra high" and a scan rate of 1 Hz was used. The mass range was chosen very wide to also include possible dimers or other interesting components. The mass range was set at *m/z* = 150-750.

2.4.3.2 QUALITATIVE AND QUANTITATIVE LS/FS-HRMS MEASUREMENTS OF DRY CHINESE STAR ANISE AND JAPANESE STAR ANISE FRUITS

Similar to DART, also for LS/FS, one carpel (typically 1/8th of an intact fruit) was taken by tweezers. This carpel was put in the crocodile clamp which put the tip of the sample 7 mm away from the MS inlet. The carpel was wetted with 250 µl MeOH. The LS/FS-MS measurement was started by recording the background. Then the voltage of 5 kV was applied. In the case that the spray was not initiated, more MeOH was applied. When still no spray had arisen, the distance was slightly varied and later put back at 7 mm. The procedure was repeated with the same carpel, rewetted with ±100 µl. The ions at m/z 327.108 (negative mode) and 367.079 (positive mode) was observed. For each of the 3 measurements, the average signal was measured and taken as a measurement of the amount of anisatin.

2.4.3.3 OPTIMIZING THE LS/FS HRMS TECHNIQUE

For optimizing the technique, several solvents were used i.e.: Methyl *tert*-butyl ether (MTBE), Ethanol (EtOAc), Acetone, Methanol (MeOH) and Methanol with Ammonium acetate. Also different distances of the sample towards the MS inlet were tested. Even so different voltage was applied and the ratio between distance and voltage was changed.

2.4.4 LS/FS – LIONAIR IONTRAP MS

2.4.4.1 ANALYSIS CONDITIONS

The leafspray - iontrap MS system consisted of a crocodile clamp, to which a voltage could be applied, coupled to a Thermo LXQ linear ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). . For instrument control, data acquisition and data processing, XCALIBUR software (v. 2.1) was used. Measurements were done in negative selective reaction (SRM) mode. The transition from *m/z* 327 to *m/z* 265 was monitored. Settings (−)-mode: capillary temperature 300 °C, capillary voltage −3 V, tube lens voltage −80 V and CID 25%.

2.4.4.2 QUALITATIVE AND QUANTITATIVE LS/FS-HRMS MEASUREMENTS OF DRY CHINESE STAR ANISE AND JAPANESE STAR ANISE FRUITS

Similar to Orbitrap-MS LS/FS, also for LXQ-LS/FS, one carpel (typically 1/8th of an intact fruit) was taken by tweezers. This carpel was put in the crocodile clamp which put the tip of the sample 7 mm away from the MS inlet. The carpel was wetted with 250 µl MeOH. The LS/FS-MS measurement was started by recording the background. Then the voltage of 5 kV was applied. In the case that the spray was not initiated, more MeOH was applied. When still no spray had arisen, the distance was slightly varied and later put back at 7 mm. The spray at *m/z* 327 was measured and SRM mode was initiated to record *m/z* 265. The procedure was repeated with the same carpel, rewetted with ±100 µl. The ions at *m/z* 327 (negative mode) and in SRM mode *m/z* = 265 were observed. For each of the 3 measurements, the average signal was measured and taken as a measurement of the amount of anisatin.

2.5 TRIANGLE-SHAPED STAINLESS STEEL PLATE

2.5.1 MATERIALS AND REAGENTS

Material and reagents were similar to LS/FS. Additionally a triangle-shaped small plate of stainless steel (4 cm in length, 2 cm width) was obtained from “ijzerhandel De Gij”, Wageningen, the Netherlands.

2.5.2 PLATESPRAY – ORBITRAP MS

2.5.2.1 ANALYSIS CONDITIONS

Conditions were similar to LS/FS with as an exception, instead of a piece of fruit, a triangle-shaped small plate of stainless steel was clamped in the crocodile clamp and the fruit-piece was put on top of the plate. 100 µm MeOH was added. The spray should arise from the tip of the triangle-shaped plate.

2.6 CAPILLARY USE

2.6.1 MATERIALS AND REAGENTS

Conditions were similar to LS/FS. Additionally a triangle-shaped small plate of stainless steel from “ijzerhandel De Gij”, Wageningen, the Netherlands and a capillary, 50 µm in diameter was obtained from the lab of ORC, Wageningen, the Netherlands.

2.6.2 LEAFSPRAY – ORBITRAP MS

2.6.2.1 ANALYSIS CONDITIONS

Conditions were similar to LS/FS with as an exception, instead of a piece of fruit, a triangle-shaped small plate of stainless steel was clamped in the crocodile clamp and the fruit piece was put on top of the plate. 100 um MeOH was added and the capillary was put in je MeOH-fruit juice mixture which was formed on the triangle-shaped plate. The capillary, containing fruit juice mixed with MeOH, can be compared to a sharp tip and sprays towards the MS-inlet, resulting in a continuous and stable spray.

Additional to this, the capillary can be put directly in the piece of fruit, which is clamped in the crocodile clamp. Now, even for soft fruits, like kiwi and tomato, without a sharp tip, there is the possibility to produce a stable, continuous spray.

3. RESULTS

3.1 PAPERSPRAY

Paperspray measurements showed a spray, as can be observed below in figure 12. Results are shown in table 2.

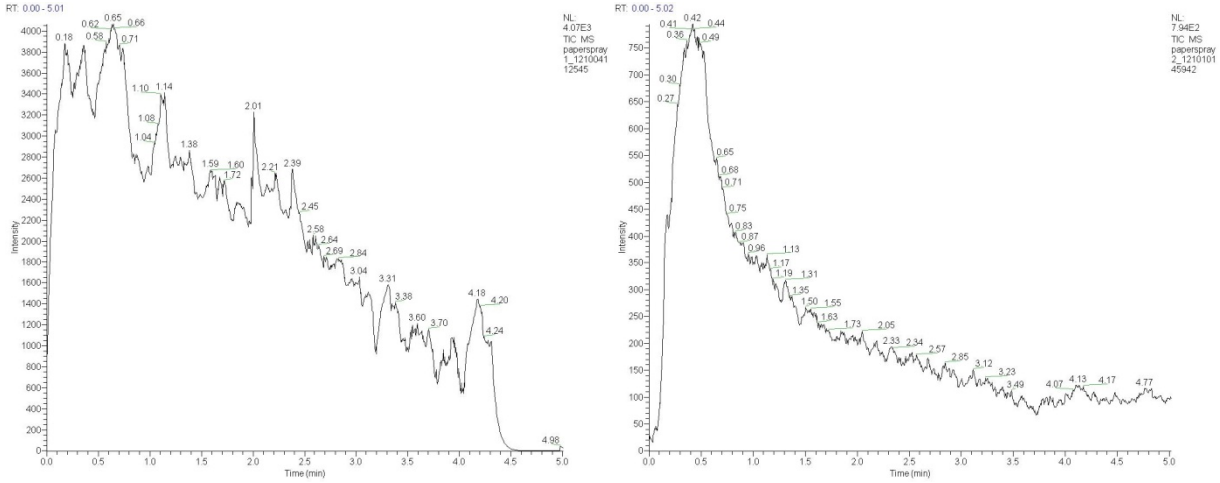

FIGURE 12: PAPERSPRAY OF ASCORBIC ACID IN MEOH ON LXQ LEFT: 0.5 MG/ML RIGHT: 0.05 MG/ML

TABLE 2

Concentration Ascorbic Acid	MS results for *m/z* = 175
0.5	2.56E3
0.05	2.33E2

A continuous spray is observed for several minutes. Because soft fruit measurements did not show significant results, these experiments were not further continued to produce a standard curve.

3.2.1. GREEN ONION

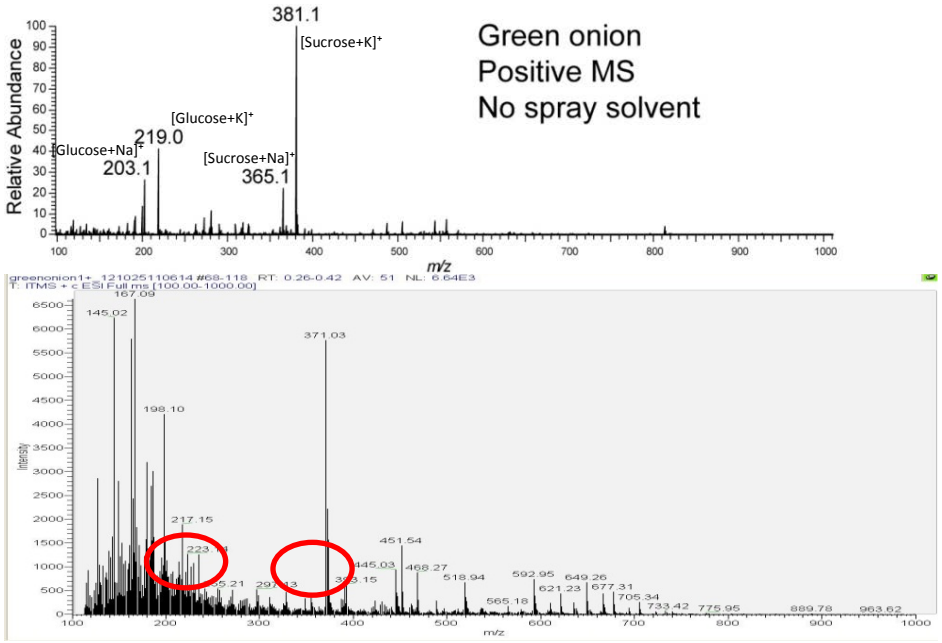

FIGURE 13: TOP: LEAFSPRAY OF GREEN ONION IN POSITIVE MS MODE, NO SPRAY SOLVENT ADDED, AS FOUND IN LITERATURE (LIU, WANG ET AL. 2011) *M/*Z 203=[GLUCOSE+NA]$^+$, *M/*Z 219=[GLUCOSE+K]$^+$, *M/*Z365=[SUCROSE+NA]$^+$ AND *M/*Z 381=[SUCROSE+K]$^+$ BOTTOM: OWN EXPERIMENT; GREEN ONION IN POSITIVE MS MODE, ON LXQ, NO SPRAY SOLVENT ADDED.

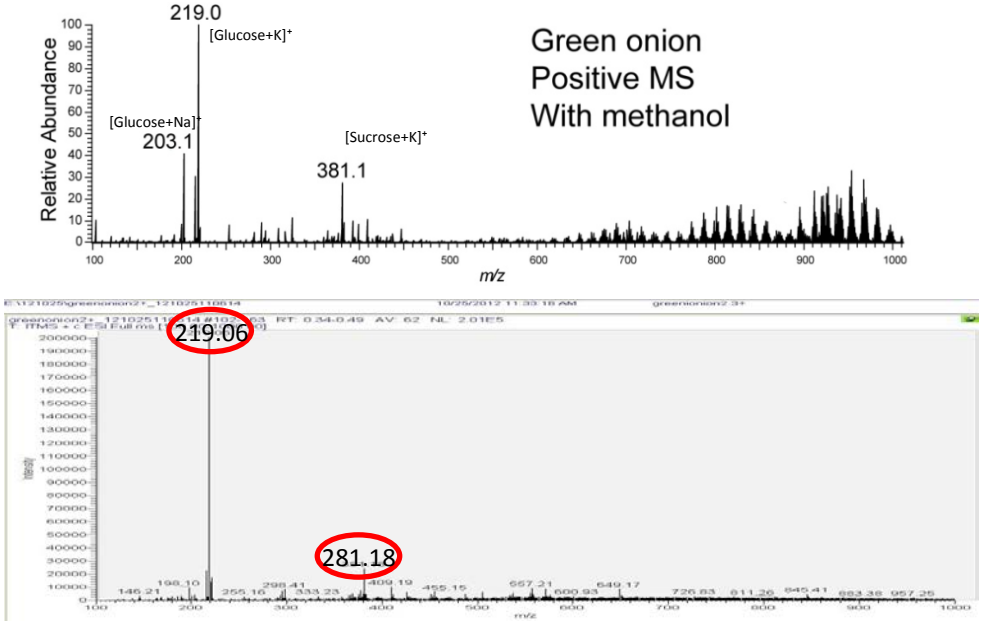

FIGURE 14: TOP: LEAF SPRAY OF GREEN ONION IN POSITIVE MS MODE, WITH MEOH ADDED (SPRAY SOLVENT) , AS FOUND IN LITERATURE (LIU, WANG ET AL. 2011) *M/*Z 203=[GLUCOSE+NA]$^+$, *M/*Z 219=[GLUCOSE+K]$^+$ AND *M/*Z 381=[SUCROSE+K]$^+$ BOTTOM: OWN EXPERIMENT; GREEN ONION IN POSITIVE MS MODE, ON LXQ, WITH SPRAY SOLVENT ADDED. *M/*Z 219=[GLUCOSE+K]$^+$ AND *M/*Z 381=[SUCROSE+K]$^+$.

As observed in Figure 13, the data obtained from literature is not in accordance with the data obtained by the performed experiment, however, in presence of the solvent MeOH, the data obtained and those originated from literature do match (*m/z* 219=[glucose+K]$^+$ and *m/z* 381=[sucrose+K]$^+$ are both observed)(Figure 14).

3.1.2 GINGER

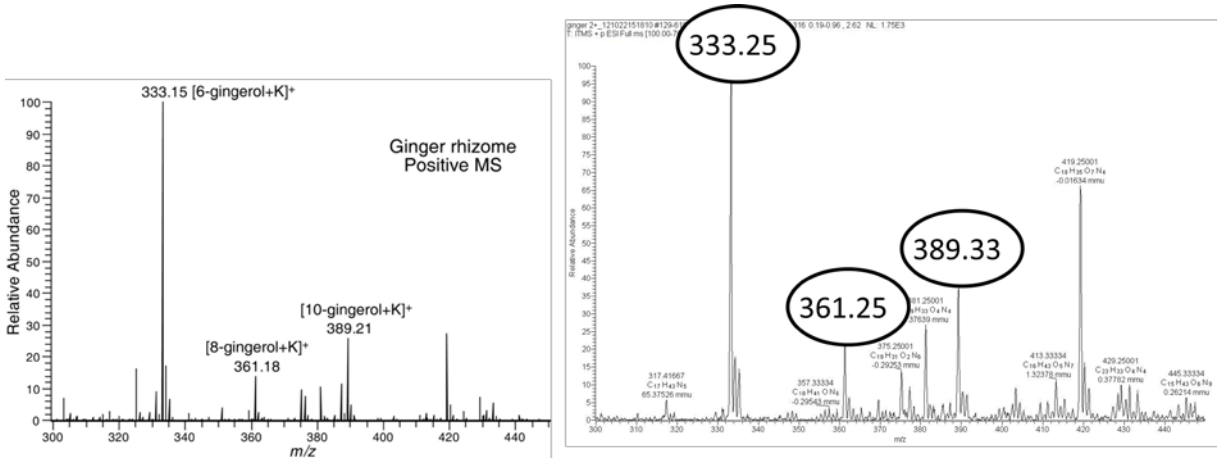

FIGURE 15: LEFT: LEAF SPRAY OF GINGER IN POSITIVE MS MODE, WITH MEOH ADDED (SPRAY SOLVENT) , AS FOUND IN LITERATURE (LIU, WANG ET AL. 2011) RIGHT: GINGER IN POSITIVE MS MODE, WITH SPRAY SOLVENT ADDED.

For ginger, the same observations are made as for green onion; in combination with the solvent, a similar result is obtained, compared to literature (Liu, Wang et al. 2011) (Figure 15). Though, for the same ginger without solvent added, a different result was found. This result was not similar to results found in literature. Also it is observed, that during the experiments, only a stable spray was observed when solvent was added (shown in figure 16).

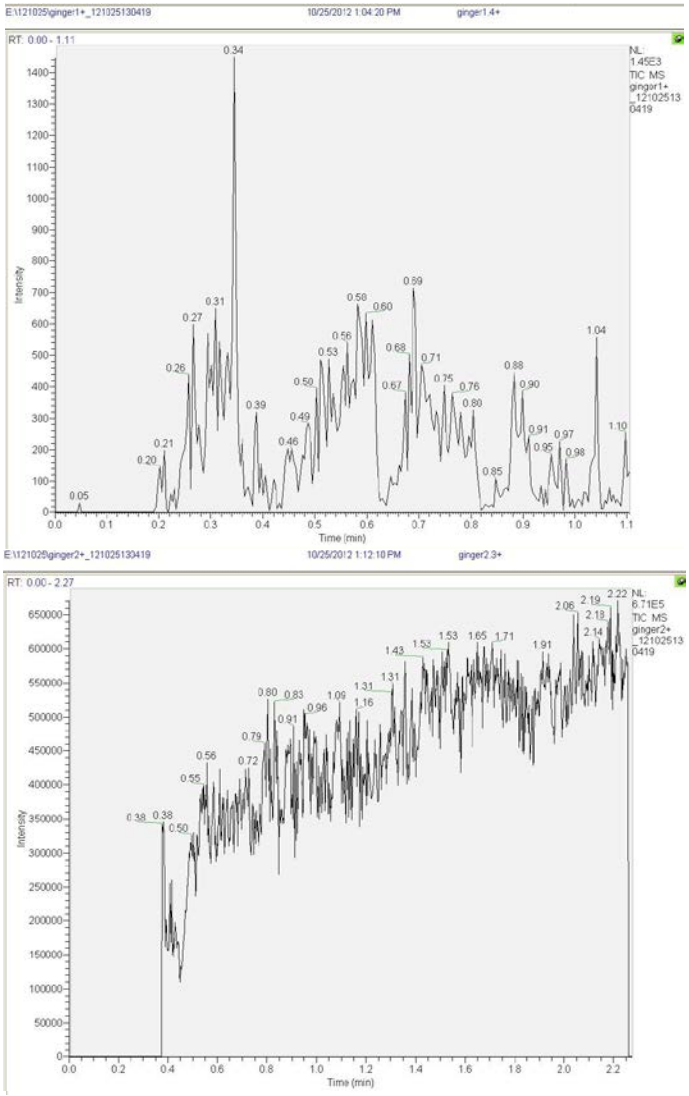

FIGURE 16: TOP: UNSTABLE LEAF SPRAY OF GINGER IN POSITIVE MS MODE, WITHOUT MEOH ADDED. BOTTOM: 'STABLE' SPRAY OF GINGER IN POSITIVE MS MODE, WITH MEOH ADDED.

For Brussels sprouts without and with solvent no (stable) spray result was obtained (figure 17) where literature (Liu, Wang et al. 2011), showed a stable spray for Brussels sprouts.

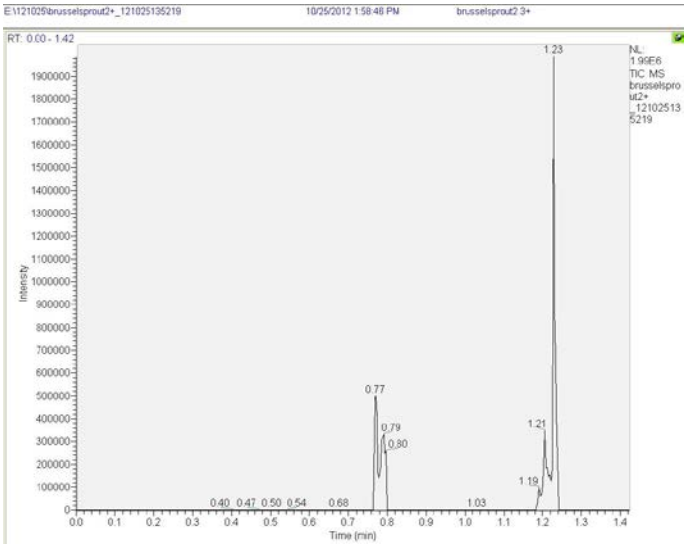

FIGURE 17: BRUSSEL SPROUTS, SOLVENT ADDED, POSITIVE MS MODE ON LXQ, SHOWING A VERY UNSTABLE/NO SPRAY.

3.3.1 HPLC

Because Ascorbic acid is a highly polar compound and ion pair chromatography was used, the analyte was fast eluted from the column.

The low resolution is showing in the results of the pure ascorbic acid samples (figure 18). Different compositions of eluent, faster flow and slower flow were tried, though non large improvements were booked, concerning the peak width. (Figure 18)

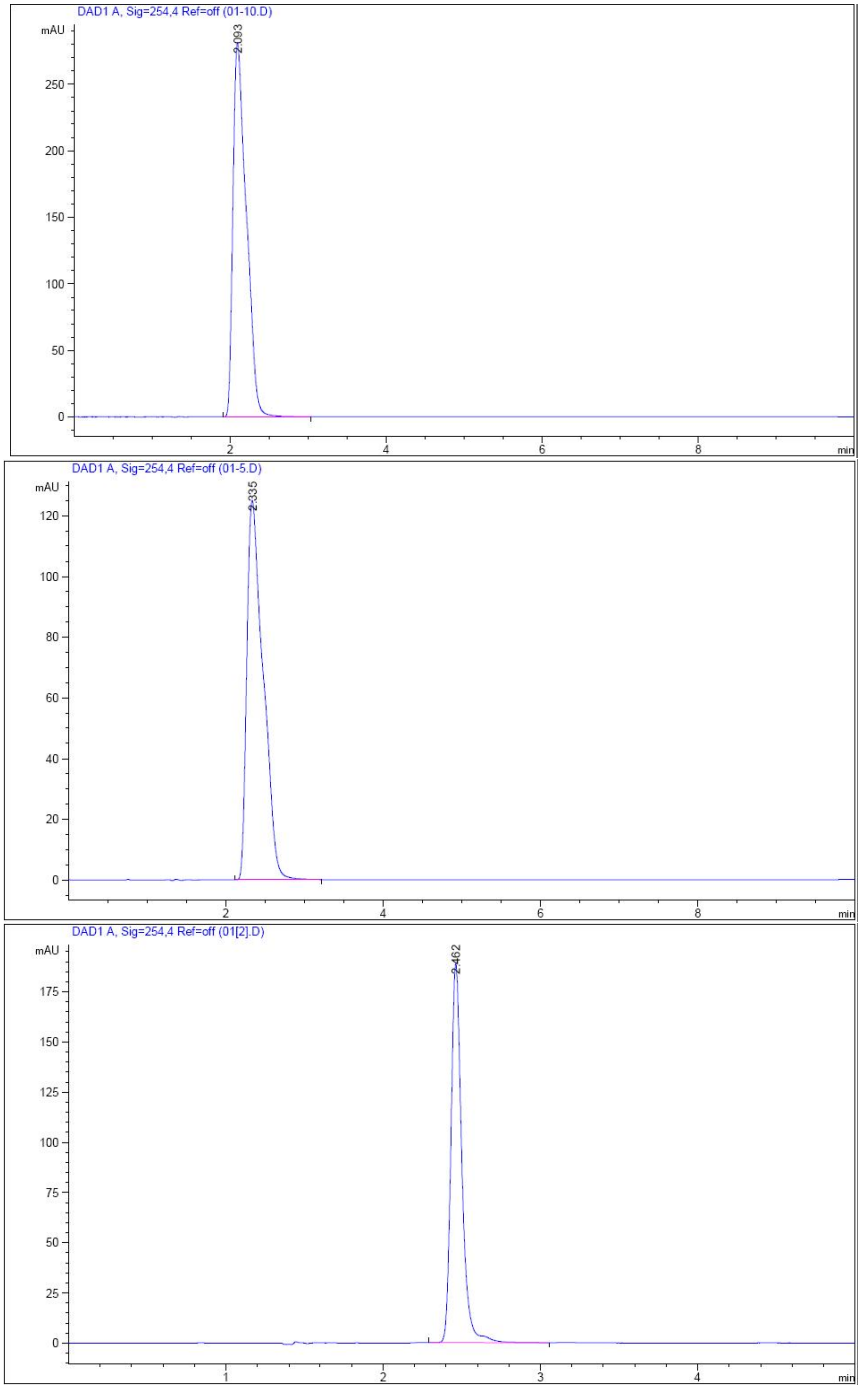

FIGURE 18: PURE ASCORBIC ACID, DILUTED. IN THE ELUENT IS RESPECTIVELY 10% MEOH, 5%MEOH AND 2% MEOH, COMBINED WITH TAS (TAS (TETRABUTYLAMMONIUM HYDROGEN SULFATE) BUFFER PRESENT.

The graphs from figure 18 show different retention times. The result at 2% MeOH shows the least broad peak (watch the axis with care). Therefore, 2 % MeOH was chosen to do the fruit measurements.

Kiwi-results also showed a very width peak (figure 19). Apple and Tomato HPLC outcomes where worse and did not show an ascorbic acid peak (data not shown).

Due to the fact that this thesis was not mainly about HPLC and to focus on leafspray improvements, HPLC measurements on Ascorbic acid were put on hold.

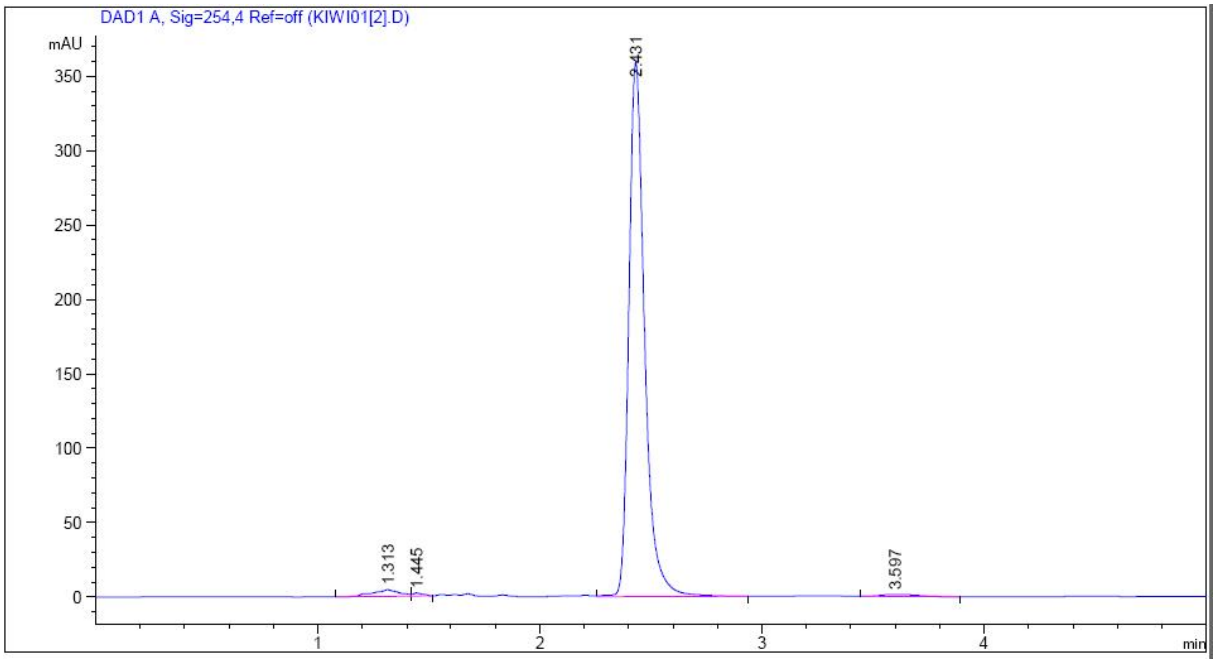

FIGURE 19: ASCORBIC ACID IN 10 TIMES DILUTED KIWI-JUICE, RETENTION PEAK AT 2.431 FOR ASCORBIC ACID. ELUENT: 2% MEOH IN TAS (TETRABUTYLAMMONIUM HYDROGEN SULFATE) BUFFER.

3.3.2 ELECTROSPRAY IONISATION

On LXQ MS, electrospray ionization(ESI) experiments as a comparing to LS/FS were performed on kiwi-, tomato- and apple juice. Some of the results are shown below (figure 20-22).

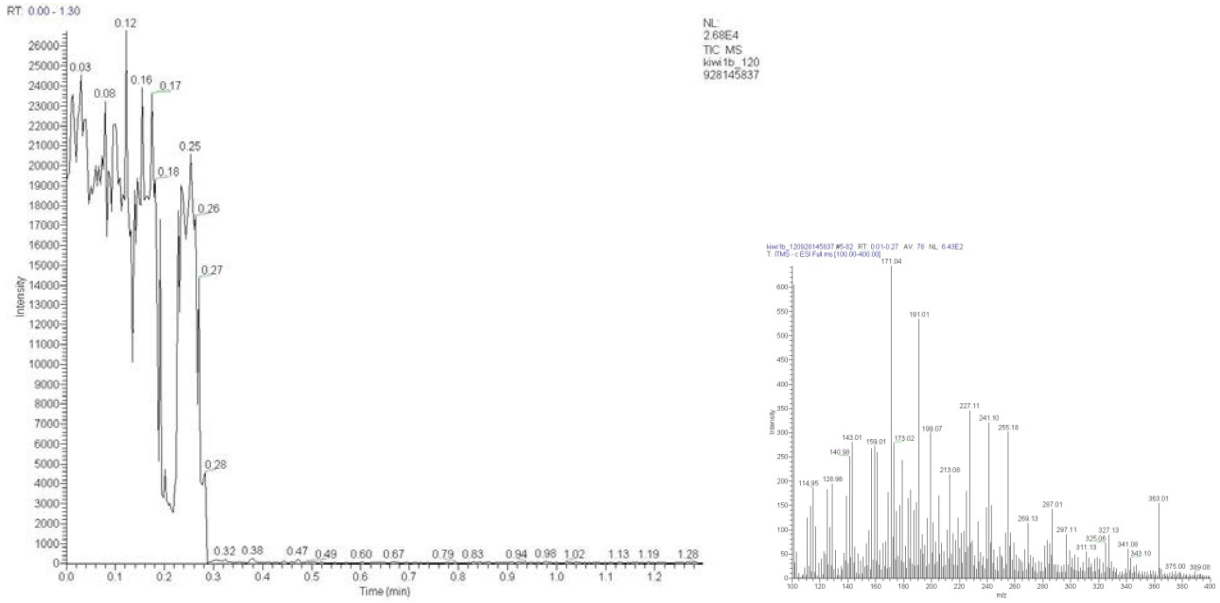

FIGURE 20: LS/FS ON LXQ, KIWI WITH MEOH. LEFT: SPRAY OF KIWI. RIGHT: SPECTRUM OF KIWI. NO DISTINCTIVE PEAK AT *M/Z* = 175 (ASCORBIC ACID) WAS OBSERVED.

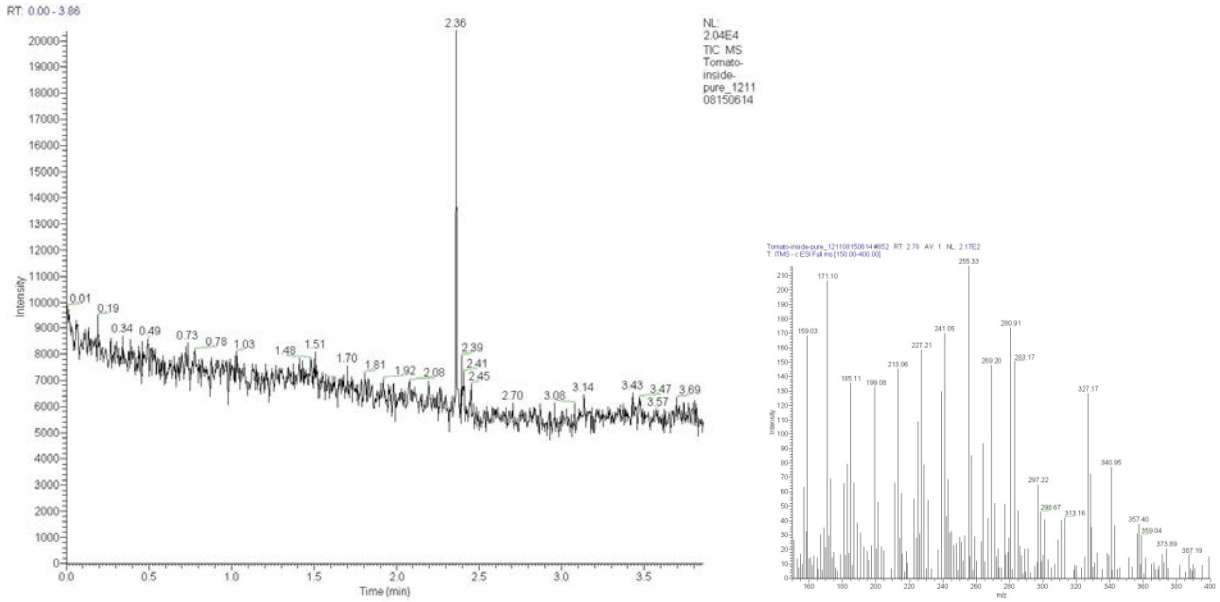

FIGURE 21: LS/FS ON LXQ, TOMATO WITH MEOH. LEFT: SPRAY OF TOMATO USING ESI RIGHT: SPECTRUM OF TOMATO. NO DISTINCTIVE PEAK AT *M/Z* = 175 (ASCORBIC ACID) WAS OBSERVED.

When observing the spray from kiwi and tomato, the absolute spray is 6.4E2 for Kiwi and 2.17E2 for tomato.

Apple showed a more clear spray. To obtain a stable signal, different solvents were tried. For apple and ascorbic acid, H2O, MeOH and a combination of both were used. (Figure 22)

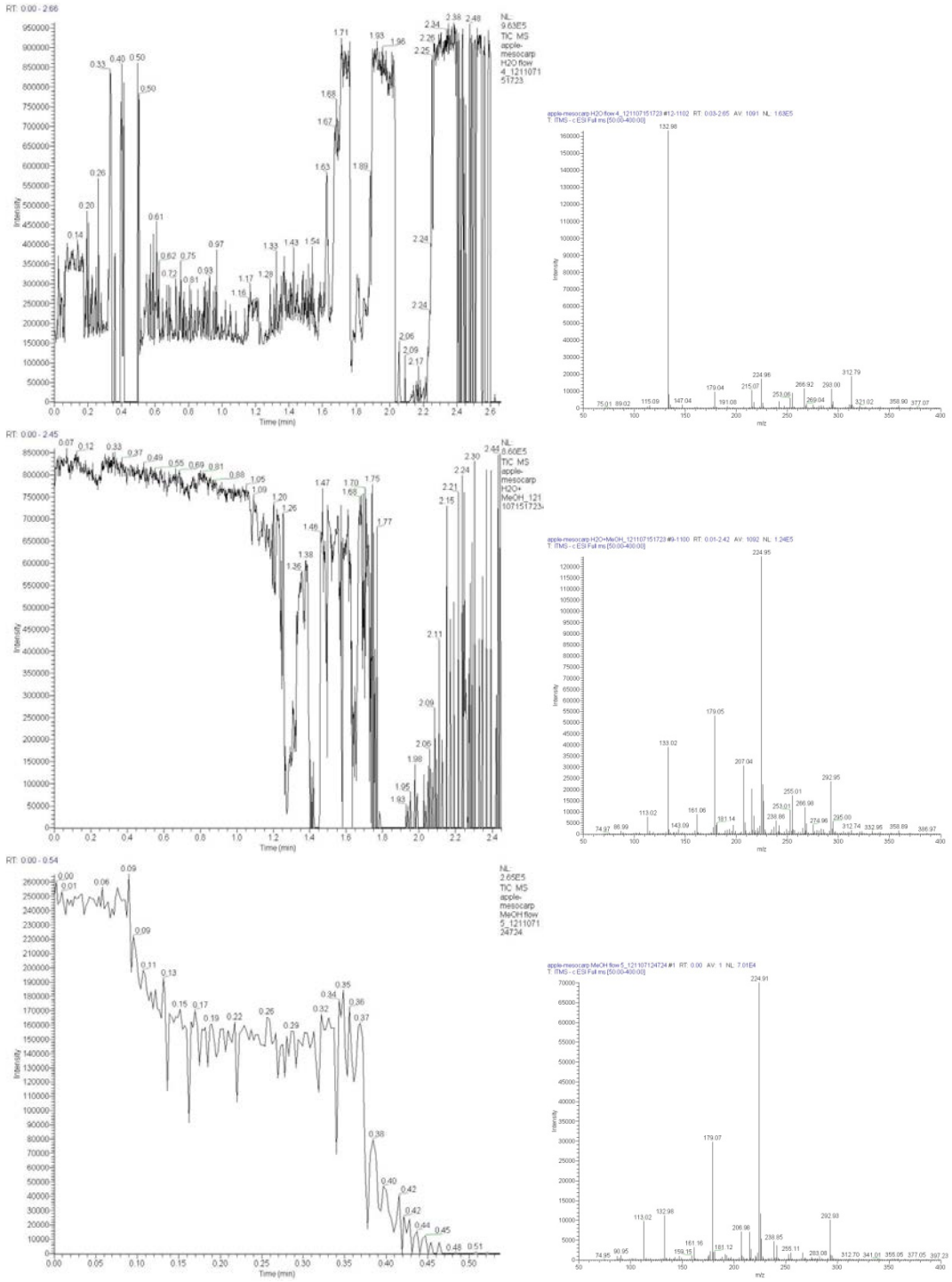

FIGURE 22: TOPLEFT: SPRAY FROM APPLE WITH H2O AS A SOLVENT, ON LXQ. TOPRIGHT: SPECTRUM OBTAINED FROM SPRAY WITH APPLE WITH H2O AS A SOLVENT. MIDDLELEFT: SPRAY FROM APPLE WITH H2O+MEOH AS A SOLVENT, ON LXQ. MIDDLERIGHT: SPECTRUM OBTAINED FROM SPRAY WITH APPLE WITH H2O+MEOH AS A SOLVENT. BOTTEMLEFT: SPRAY FROM APPLE WITH MEOH AS A SOLVENT, ON LXQ. BOTTEMRIGHT: SPECTRUM OBTAINED FROM SPRAY WITH APPLE WITH MEOH AS A SOLVENT.

A change in concentration is observed in the peaks in the spectra when a different solvent is used. Also the spray seems more stable with MeOH or a combination of MeOH and H_2O. Thought, also for apple no distinctive peak at *m/z* = 175 (ascorbic acid) was observed.

3.3.3 FRUIT SPRAY

On exactive MS, LS/FS for kiwi was performed. No continuous spray was produced. For apple, a short, quite continuous spray could be produced, but also using the exactive MS, the spectrum did not contain a distinctive peak for ascorbic acid.

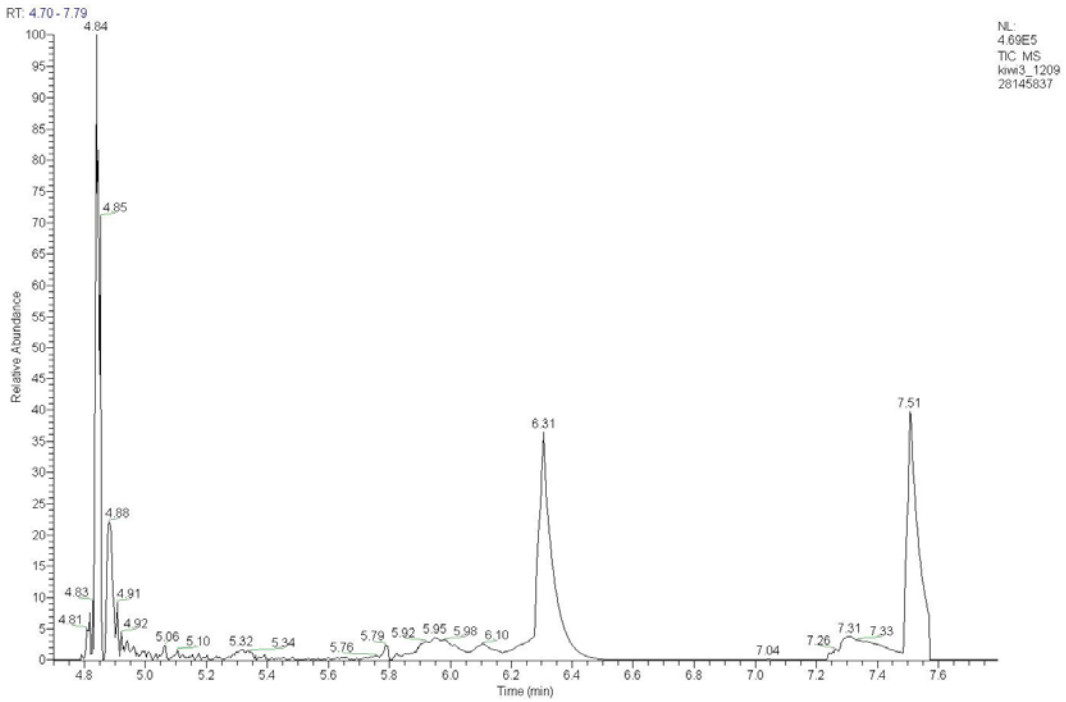

FIGURE 23: FRUITSPRAY OF KIWI ON EXACTIVE MS, WITH SPRAY SOLVENT (MEOH). NO (STABLE) SPRAY WAS OBSERVED.

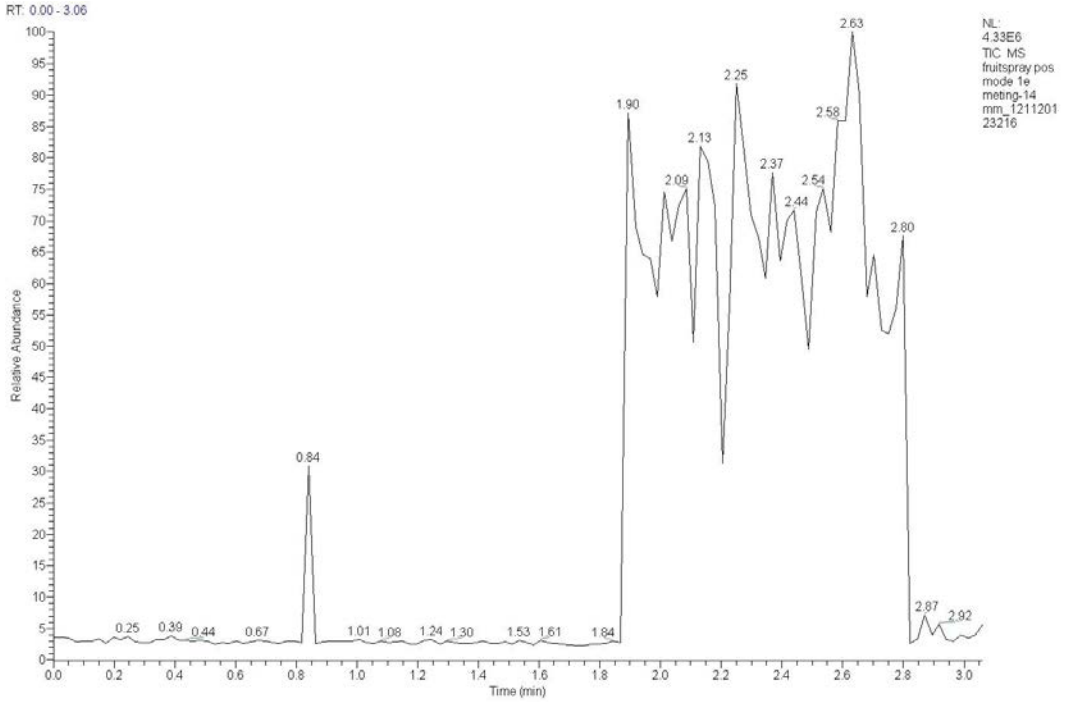

FIGURE 24: SLIGHTLY "STABLE" SPRAY WAS OBSERVED IN APPLE, WHICH HOLDS A SHAPER TIP COMPARED TO KIWI, THOUGH NO ASCORBIC ACID PEAK WAS FOUND IN THE SPECTRUM ORIGINATING FROM THE SPRAY ABOVE. ON EXACTIVE MS.

3.4 STAR ANISE FRUITS

3.4.1 DART

For positive DART analysis, the anisatin reacts with $NH4^+$ from the air, resulting in [M+NH4] *m/z* = 346.148. For negative DART analysis, the anisatin loses an H, resulting in a [M-H] *m/z* = 327.108. (Shen, van Beek et al. 2012)

Results from the DART analysis on exactive orbitrap MS for both Chinese (Figure 25 and 26) and Japanese (Figure 27 and 28) star anise are shown below.

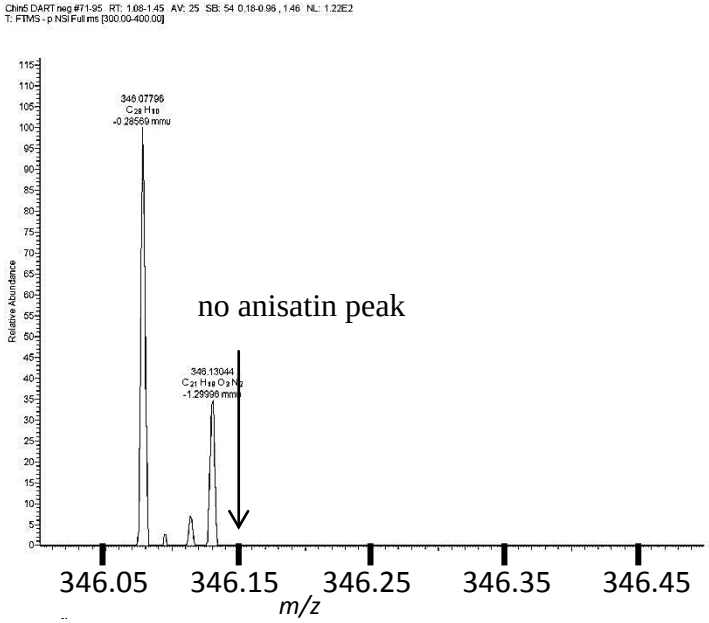

FIGURE 25: CHINESE STAR ANISE, ANALYZED USING DART, POSITIVE MODE, ON EXACTIVE. NO ANISATIN PEAK WAS FOUND.

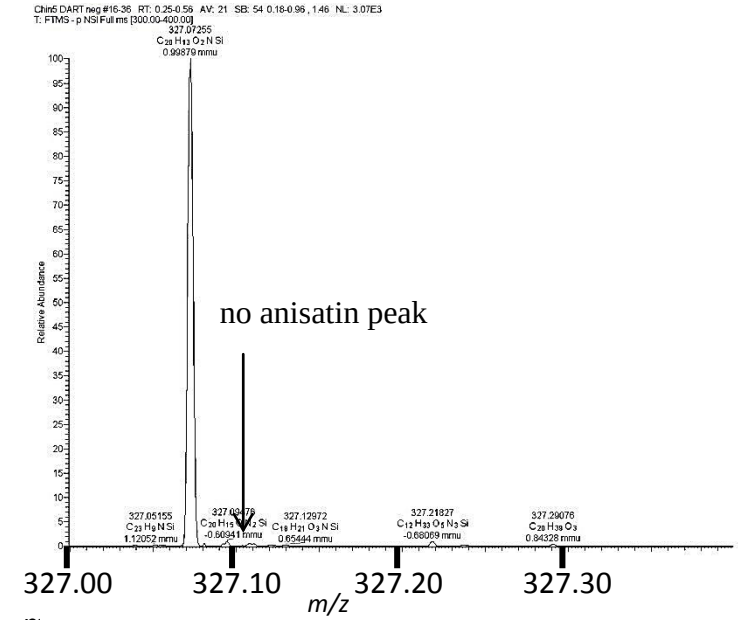

FIGURE 26: CHINESE STAR ANISE, ANALYZED USING DART, NEGATIVE MODE, ON EXACTIVE. NO ANISATIN PEAK WAS FOUND.

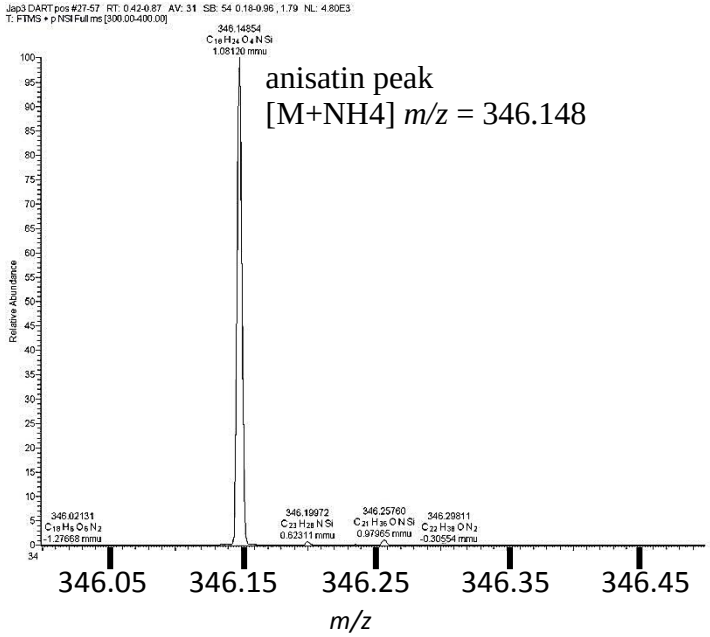

FIGURE 27: JAPANESE STAR ANISE, ANALYSED USING DART, POSITIVE MODE, ON EXACTIVE. ANISATIN PEAK WAS FOUND AT [M+NH4] M/Z= 346.148

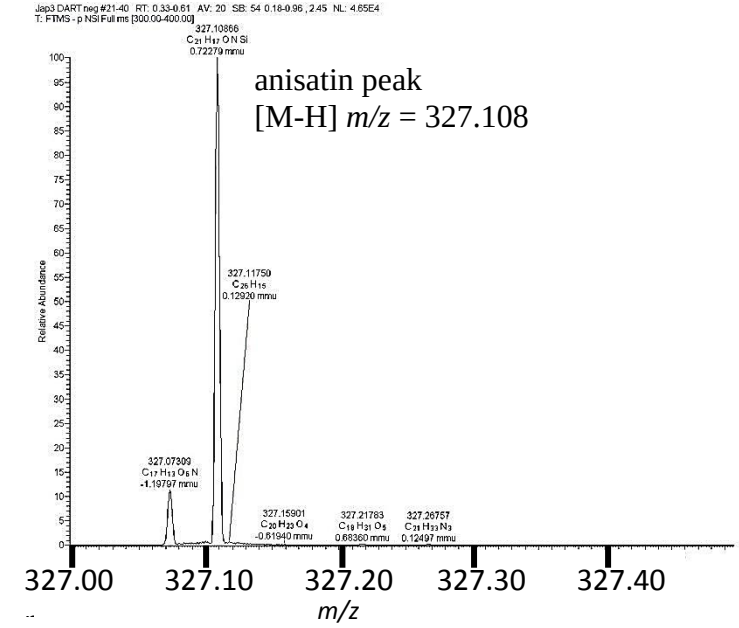

FIGURE 28: JAPANESE STAR ANISE, ANALYSED USING DART, NEGATIVE MODE, ON EXACTIVE. ANISATIN PEAK WAS FOUND AT [M-H] M/Z = 327.108

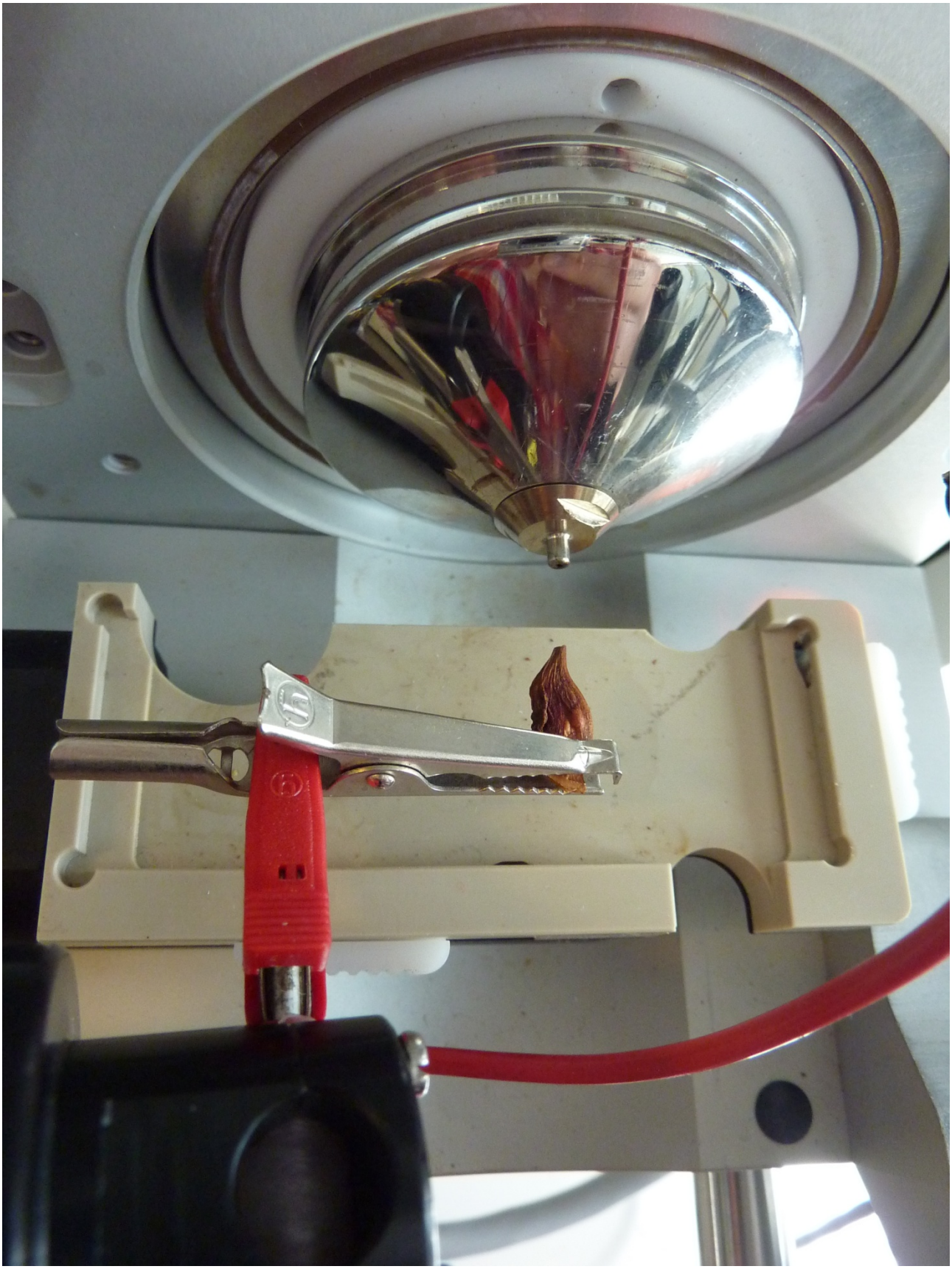

FIGURE 29: FRUITSPRAY TECHNIQUE SETUP WITH THE DOUBLE CROCODILE CLAMP FOR EASY SWITCHING BETWEEN SAMPLES.

3.4.2.1 OPTIMIZING THE TECHNIQUE

Star anise has a natural hard, sharp tip, but the dried fruits do not contain liquid. When MeOH is added, the sample contains a combination of a sharp tip and being moist. For these samples, when a spray is initiated, a small cone could be observed from the tip of the star anise fruit. This cone would maintain as long as MeOH was present. When a sample ran out of MeOH, the cone and signal would disappear. Though, when new MeOH was added, both cone and signal were resumed.

The distance of the sample towards the MS inlet did not affect the spray, although when the sample was to close (up to 3 mm) to the inlet, electric sparks were observed. When the sample was further away than 15 mm, the signal suddenly disappeared. Therefore, samples were put on a distance of 7mm towards the MS-inlet.

The voltage had to be set at 5kV or higher (figure 30). Voltage below 5kV did not result in any spray or result. Voltage above 5kV did not increase or changed the signal. When no signal was observed at 5 kV, increasing the voltage to 5.5 kV sometimes initiated a spray, though these cases were rare.

When the ratio between voltage and distance was changed, no change in signal was observed

Several solvents were used to obtain an optimum solvent. Methanol, acetone, ethyl acetate and methyl tert-butyl ether (MTBE) were picked, for anisatin is soluble in all these solvents. Only methanol showed a stable, lasting spray. MTBE did show a spray, however this was not stable. Acetone and ethyl acetate did not show a spray. Therefore, methanol is picked for all other experiments.

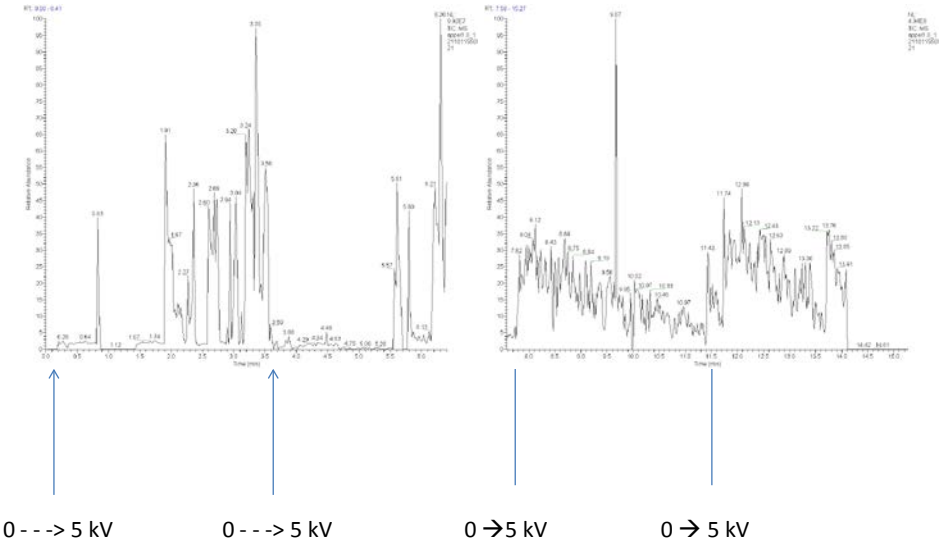

FIGURE 30: LS/FS OPTIMIZING CHANGING THE CHARGE. --- > INDICATES A SLOW INCREASE OF CHARGE, → INDICATED A DIRECT CHANGE.

For positive LS/FS analysis, the anisatin reacts with potassium (K) which are already present in the plant (anise star), resulting in [M+K] *m/z* = 367.079. For negative DART analysis, the anisatin loses an H, resulting in a [M-H] *m/z* = 327.108.

Results from the LS/FS analysis on exactive orbitrap MS for both Chinese (figure 31 and 32) and Japanese (Figure 33 and 34) are shown below.

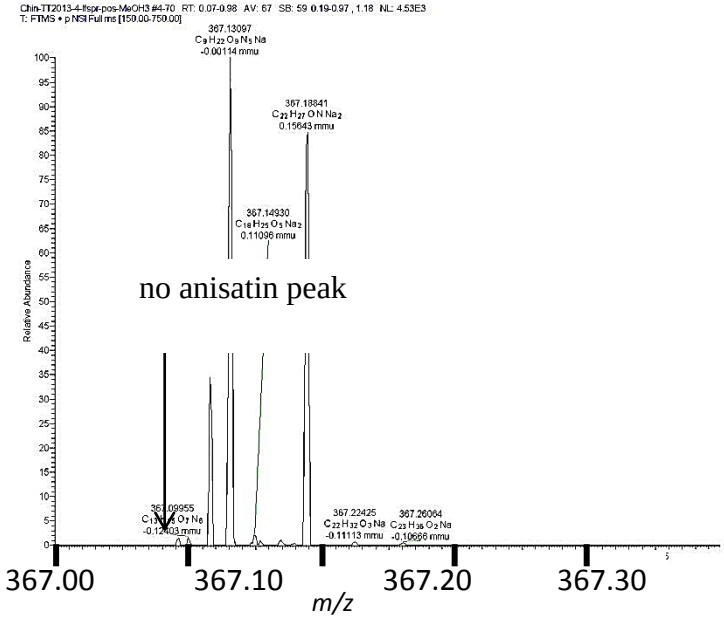

FIGURE 31: CHINESE STAR ANISE, ANALYSED USING LS/FS, POSITIVE MODE, ON EXACTIVE. NO ANISATIN PEAK WAS FOUND

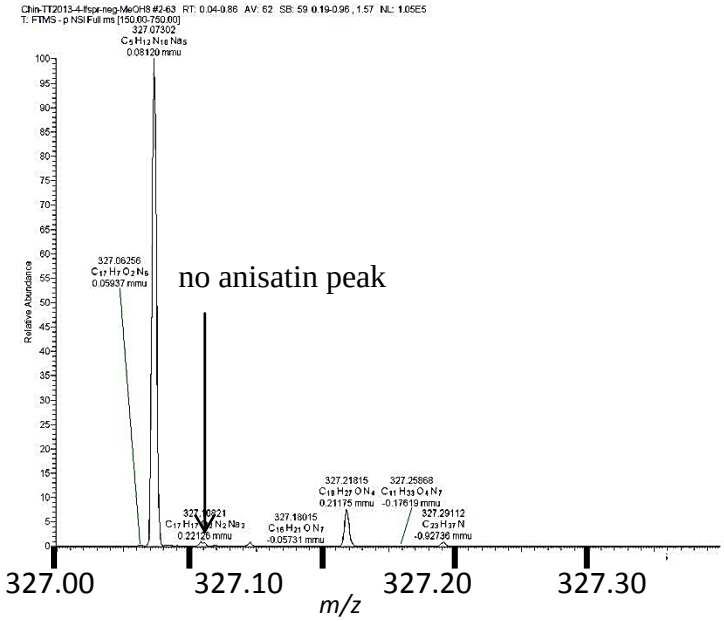

FIGURE 32: CHINESE STAR ANISE, ANALYSED USING LS/FS, NEGATIVE MODE, ON EXACTIVE. NO ANISATIN PEAK WAS FOUND

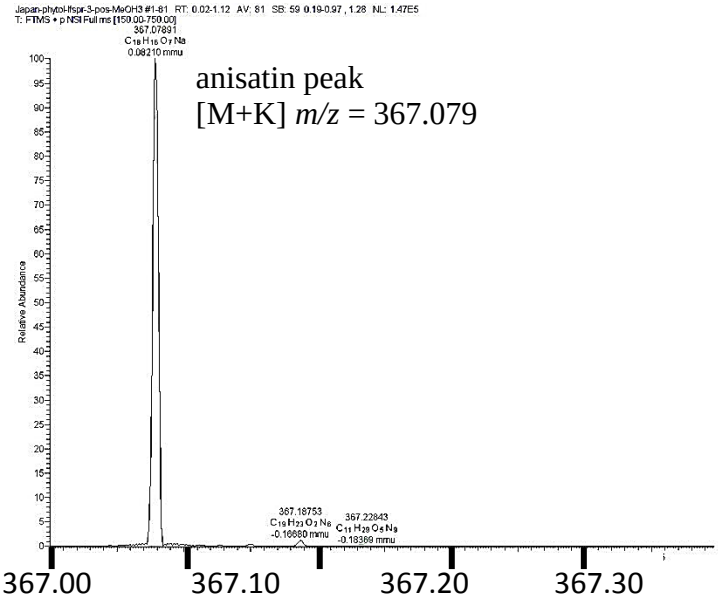

FIGURE 33: JAPANESE STAR ANISE, ANALYSED USING LS/FS, POSITIVE MODE, ON EXACTIVE. ANISATIN PEAK WAS FOUND AT [M+K] M/Z = 367.079

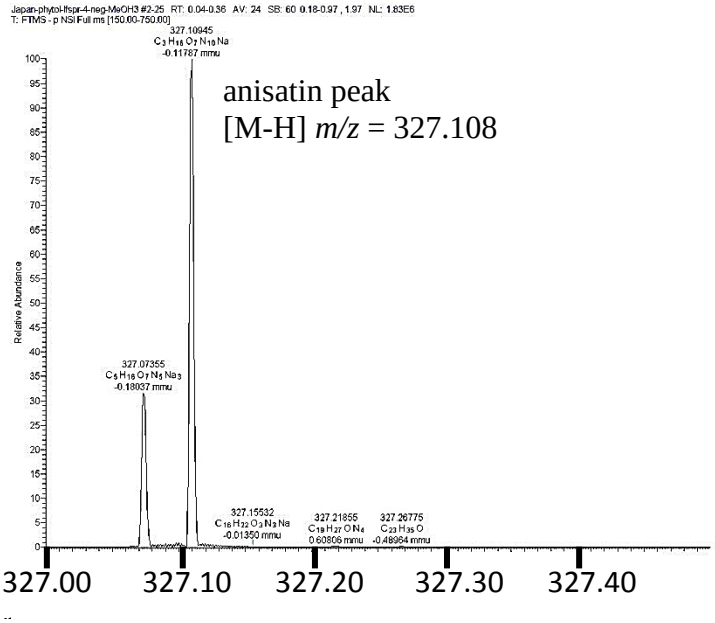

FIGURE 34: JAPANESE STAR ANISE, ANALYSED USING LS/FS, POSITIVE MODE, ON EXACTIVE. ANISATIN PEAK WAS FOUND AT [M-H] M/Z = 327.108

A table was made of all the measurement. Background was not significantly present in DART, or in LS/FS, so there is no compensation for the background. The results shown in the table below (Table 3)

TABLE 3: DART AND LS-FS RESULTS ON ORBITRAP. THE TABLE SHOWS THE AVERAGE SIGNAL HEIGHT AND STANDARD DEVIATIONS OF ANISATIN IN NEGATIVE AND POSITIVE MODE IN BOTH DRY FRUITS OF CHINESE AND JAPANESE STAR ANISE. EACH SAMPLE WAS MEASURED AT LEAST IN TRIPLICATE.

	Japanese					Chinese			
	Negative mode		Positive mode			Negative mode		Positive mode	
Sample	Leaf spray	DART	Leaf spray	DART	Sample	Leaf spray	DART	Leaf spray	DART
PhytoLab 1	$4.4\times10^{6} \pm 2.7\times10^{6}$	$1.1\times10^{5} \pm 0.4\times10^{5}$	$1.1\times10^{6} \pm 0.7\times10^{6}$	$1.2\times10^{4} \pm 0.2\times10^{4}$	T&T 1	$1.6\times10^{4} \pm 0.3\times10^{4}$	5 ± 9	$7.2\times10^{3} \pm 0.3\times10^{3}$	0 ± 0
PhytoLab 2	$3.5\times10^{6} \pm 1.6\times10^{6}$	$8.3\times10^{4} \pm 4.3\times10^{4}$	$3.6\times10^{6} \pm 1.2\times10^{6}$	$1.0\times10^{4} \pm 1.0\times10^{4}$	T&T 1	$1.1\times10^{4} \pm 0.1\times10^{4}$	17 ± 22	$6.7\times10^{3} \pm 0.3\times10^{3}$	0 ± 0
PhytoLab 3	$1.6\times10^{6} \pm 0.5\times10^{6}$	$2.1\times10^{5} \pm 2.2\times10^{5}$	$1.2\times10^{6} \pm 0.5\times10^{6}$	$1.2\times10^{4} \pm 0.1\times10^{4}$	T&T 1	$1.4\times10^{4} \pm 0.2\times10^{4}$	$1.4\times10^{2} \pm 0.8\times10^{2}$	$8.1\times10^{3} \pm 2.7\times10^{3}$	0 ± 0
RIKILT 1	$4.7\times10^{6} \pm 0.9\times10^{6}$	$1.7\times10^{5} \pm 0.6\times10^{5}$	$1.8\times10^{6} \pm 0.1\times10^{6}$	$1.8\times10^{4} \pm 2.2\times10^{4}$	Yadano	$2.6\times10^{4} \pm 0.3\times10^{4}$	$4.6\times10^{2} \pm 2.1\times10^{2}$	$2.6\times10^{4} \pm 0.3\times10^{4}$	2 ± 3
RIKILT 2	$4.1\times10^{6} \pm 0.7\times10^{6}$	$2.0\times10^{5} \pm 0.8\times10^{5}$	$2.2\times10^{6} \pm 0.8\times10^{6}$	$4.7\times10^{2} \pm 8.1\times10^{2}$	Sumatra	$1.6\times10^{3} \pm 0.4\times10^{3}$	$1.1\times10^{2} \pm 0.8\times10^{2}$	0 ± 0	0 ± 0

Next to experiments on the exactive orbitrap MS, measurements were done on the iontrap MS (LXQ)

Japanese star anise was tested on LXQ in negative SRM mode. CID of the $[M-H]^-$ peak at *m/z* = 327 ion yielded fragments at *m/z*= 309, 297, 283, 265 and 127(shown in figure 35). *m/z* = 265 and *m/z* = 127 showed the strongest peaks. The peak at *m/z* = 265 corresponding to $[M-H-H_2O-CO_2]^-$ was picked for analysis.

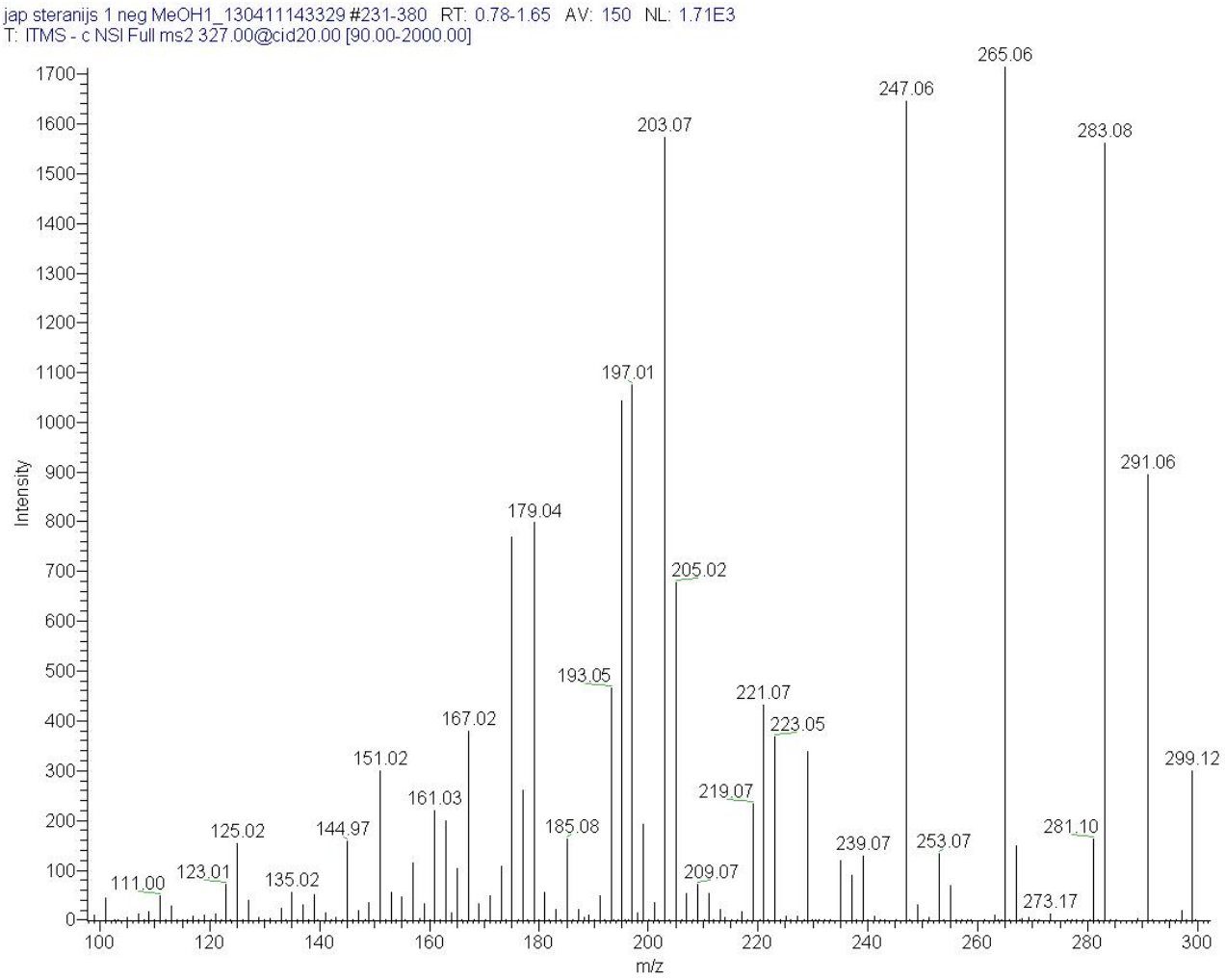

FIGURE 35: ION YIELDED FRAGMENTS OF CID OF THE $[M-H]^-$ PEAK AT M/Z = 327 ON LXQ MS SAMPLE FROM JAPANESE STAR ANISE.

Also, for the measurements on the iontrap MS (LXQ) a table was made (Table 4).

TABLE 4: DART AND LS-FS RESULTS ON IONTRAP MS (LXQ). THE TABLE SHOWS THE AVERAGE SIGNAL HEIGHT AND STANDARD DEVIATIONS OF ANISATIN IN NEGATIVE MODE IN BOTH DRY FRUITS OF CHINESE AND JAPANESE STAR ANISE. EACH SAMPLE WAS MEASURED AT LEAST IN TRIPLICATE.

Japanese			Chinese		
Sample	Leafspray	DART	Sample	Leafspray	DART
PhytoLab 1	1.45×10^{3} $\pm 0.4\times10^{3}$	1.2×10^{2} $\pm 0.2\times10^{2}$	T&T 1	57 $\pm$ 32	1.0 $\pm$ 1.3
PhytoLab 2	6.6×10^{2} $\pm 0.3\times10^{2}$	1.2×10^{2} $\pm 0.3\times10^{2}$	T&T 1	34 $\pm$ 11	1.1 $\pm$ 0.9
PhytoLab 3	3.3×10^{2} $\pm 1.1\times10^{2}$	2.0×10^{3} $\pm 0.6\times10^{3}$	T&T 1	16 $\pm$ 7	0.4 $\pm$ 0.3
RIKILT 1	1.8×10^{3} $\pm 0.8\times10^{3}$	6.8×10^{2} $\pm 4.0\times10^{2}$	Yadano	40 $\pm$ 15	0.6 $\pm$ 0.6
RIKILT 2	3.3×10^{2} $\pm 0.3\times10^{2}$	2.0×10^{2} $\pm 0.5\times10^{2}$	Sumatra	38 $\pm$ 29	2.1 $\pm$ 1.1

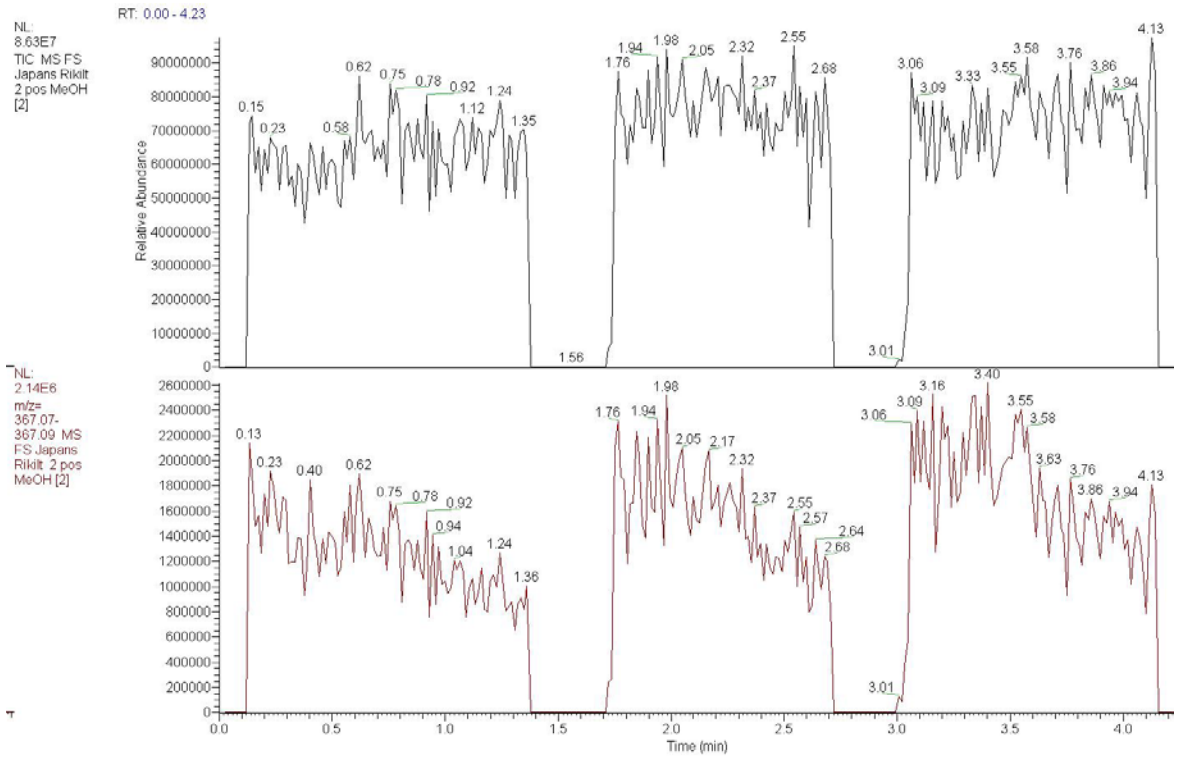

FIGURE 36: LS/FS OF JAPANESE RIKILT 2, POSITIVE MODE WITH MEOH, UP: TOTAL ION CURRENT (TIC) SIGNAL, BELOW: M/Z 367.07-367.09 (ANISATIN).

3.5 TRIANGLE-SHAPED STAINLESS STEEL PLATE

No continuous spray was obtained, therefore, no results were observed. Addition of extra solvent did not show any effect.

3.6 CAPILLARY

When the capillary was put into the fluid, the fluid would not fill the complete capillary and still air was present inside the capillary. No spray at all was observed. Also, the MS inlet contains a vacuum, which would "suck up" the capillary when close. Therefore the capillary had to remain at a certain distance from the MS. Shortening the capillary, or increasing the amount of liquid did not affect the outcome of the experiment.

4 DISCUSSION AND CONCLUSION

4.1 PAPERSPRAY

Paperspray did show a nice, stable spray and seemed to have a 10 times higher read out when the concentration was 10 times increased. Though it has to be mentioned the read outs of MS were rather low for a full spray, compared to later experiments on anise star. The technique works, but samples still have to be prepared before usage. It can be interesting to extend this technique, in order to make a standard curve and further research on quantitative properties of the leafspray technique.

4.2 REPRODUCING THE RESULTS FROM LIU ET AL.

If we observe the results from the reproduction of the results of Lui et al, it is noticeable that with the solvent, the results look reproducible, however without the solvent, there is not a continuous spray or no spray at all and therefore no signal.
For example in ginger, different kinds of gingerol combined with a potassium ion(at *m/z* = 333, 361 and 389) where observed in both literature (Liu, Wang et al. 2011) and the performed experiment. For Green onion, glucose and sucrose, both combined with a potassium ion, were observed in the obtained spectra (*m/z* = 219 and 281 respectively) and reported by Liu et al.

Brussels sprouts did not show a clear spray. Sprouts do contain lots of sugars, so the use of a polar solvent like MeOH cannot be a problem. The problem might be due to a wax layer surrounding the leaves. Because sprouts grow in winter, they have more tough leafs compared to green onion. MeOH might not be able to reach the components inside the leaf and with that not be able to spray these to the MS inlet.

Also, sprouts, compared to ginger and green onion, contain less fluid. Another possibility is therefore that is might be harder to dissolve the components.

A solution could be to put the leaves in a moist surrounding overnight and then repeat the experiment.

Because there is only a clear spray observed when a solvent (preferably MeOH) is added, it can be concluded that the adding of solvent is essential for a stable spray in LS/FS.

If the amount of liquid would be the only requirement, soft fruits, like kiwi and tomato, with a lot of liquid in them, should give a clear, continuous spray. This is for example shown in the ESI experiment, where the spray is produced by hand through injection of the diluted juice. However, in LS/FS this is not the case. Tomato and kiwi do not give a spray at all, only apple gives a spray result (shown in figure 24). The main difference between these fruits is the firmness of the fruit. Apple is more firm compared to the other fruits, and therefore easier to cut into a piece with a sharp tip. As mentioned in the introduction part, a sharp tip is important to obtain a Taylor cone. And a Taylor cone results in a continuous spray, which gives a continue signal. Kiwis and tomatoes were too juicy to obtain a sharp tip and therefore gave no strong signal.

To solve the tip-problem, the kiwi-parts have been frozen, however still no Taylor cone and no spray was observed. This is probably due to the lack of liquid in the frozen samples. Adding MeOH as a solvent defrosted the tip, resulting again in a non-sharp tip and absence of signal.

It can be concluded from these results that a sharp tip is necessary for LS/FS, to obtain a clear, continues signal.

In literature tomatoes did show a stable, strong spray (Liu, Wang et al. 2011). Thought, for tomatoes only the peel was used as a sample. The peel is more firm compared to the inside of the tomato. Also, for measurements on the inside of the tomato, a capillary was used to obtain a sharp tip. This option was also tested and the outcome is discussed later in this discussion.

In order to obtain results from soft fruits, a sharp tip is required. Another option, besides the use of a capillary could be an instant tip, made from a triangle-shaped stainless steel plate, with the fruit and its juice on top, mixed with MeOH and to use the tip of this device. The outcome of these experiments is discussed later in this discussion.

Also, another “fake” tip can be used. An injection needle can be used for instance, or another sharp object like a pin pointing downwards, leading the fluid forward its tip. Further research might reveal other options.

4.4 STAR ANISE FRUITS

4.4.1 DART ON STAR ANISE FRUITS

DART can be used as a benchmark technique for anise star (Shen, van Beek et al. 2012) and is therefore used as a control/compare organism during this study. For DART, only m/z 346.150 $[M+NH_4]^+$ was observed. The NH_4^+ originates in this product from air. DART measurements did seem to burn the anise star fruits, due to the heat from the DART add-on to produce the volatiles. Therefore, when comparing LS/FS to DART, LS/FS experiments were performed before DART experiments.

4.4.2 LEAFSPRAY ON STAR ANISE FRUITS

The spray that originated from the tip of the star anise as described in the result section can be a Taylor cone. Because a Taylor cone has a very continues and stable spray, as described in the introduction, this is the optimum circumstance for LS/FS measurements.

The electric sparks, produced when the sample was (to) close to the inlet is probably due to the energy sparking directly from the crocodile clamp towards the MS inlet is stead of sending an ionized spray towards the MS inlet. The fact that the sprays - from the experiments that showed sparks at close distances - were not continuous and irregular, contributes to this idea. Because the sprays where not continuous and irregular, their signal is not included in the results.

When the sample was far away from the MS inlet (from 15 mm upwards), no spray was observed. This is probably caused by the inlet and the charge being too far apart to create a spray at all or to reach the MS inlet.

The fact that the spray is only initiated at a certain voltage (5 kV) indicates there is a certain threshold for the Taylor spray, which is set at 5 kV for these experiments.

Because there is no change in signal observed when the ratio between voltage and distance is changed, it can be concluded that there is no effect of the ratio of voltage and distance, affecting the signal, The fact that the ratio between voltage and distance did not influence the signal, also supports the idea of a threshold for the Taylor spray to be initiated.

To improve the technique, we used different crocodile clamps for each sample. This speeds up the technique, for it was easy and fast to change the samples.

4.4.2.1 LS/FS TECHNIQUE ON ORBITRAP

When the LS/FS technique was used, combined with the orbitrap MS, both negative and positive modes could be used. For Japanese star anise (−)-mode a high signal at m/z 327.108 was found, nicely corresponding with $[M-H]^-$ and for (+)-mode, a slightly less high signal at m/z 367.08 was found, corresponding with $[M+K]^+$. The product does not occur in its natural form as an ion and does not contain an additional H. Potassium is present in plants and therefore also in their fruit. Because potassium is already present in the fruit itself, anisatin containing an additional K ion is probably more stable than anisatin containing an additional H ion. Which explains the high peak at m/z = 367.08. Also, the addition of Potassium was already reported before in LS/FS (Liu, Wang et al. 2011; Zhang, Li et al. 2012) and is therefore expected.

Signals obtained from Chinese star anise (1E4), where significantly lower compared to signals obtained from Japanese star anise (1E6), as can be observed in Tables 3 and 4. Important to note is that none of the Japanese star anise fruits had a lower signal than any Chinese star anise. And all Japanese star anise fruits showed a peak at *m/z* 327.108 and *m/z* 367.08 in

respectively negative and positive mode. The absence of false negatives is crucial in this case. This is because if a batch of anisatin containing anise star is not recognized as Japanese star anise, because of a false negative, there is a risk for human health.

4.4.2.2 LS/FS TECHNIQUE ON LXQ

LS/FS technique worked on orbitrap, but because LXQ is a cheaper MS, and more common present at (small) labs, the technique would be of more use if it is also to be used on an iontrap MS (LXQ).

When Japanese star anise was tested under CID, no strong signal was observed under conditions with collision energies of 20, 25 and 30. Therefore, no positive mode with selective reaction monitoring (SRM) could be performed on LXQ.

We observe less big differences between Chinese and Japanese on LXQ, this is probably due to part of Chinese star anise that split up into parts with the same mass as those which are collected in the iontrap after collision on anisatin.

The total ion current (TIC) signal does have a lot of influence on the amount of ions trapped and later measured. It could be interesting to define the ratio of the measured parts of anisatin and the TIC signal and compare these ratios. However, this is very hard to apply using the DART technique.

4.5 TRIANGLE-SHAPED STAINLESS STEEL PLATE

The idea of the triangle-shaped stainless steel plate was to create an artificial tip which would result in a continuous and stable spray. Unfortunately this did not work out. An explanation could be the electric charge which did not charge the fluid on top of the triangle, because it was not forced to pass the fruit-MeOH juice in order to reach the MS-inlet. Because the fluid did not contain a charge, there is no force to drive it toward the MS inlet and therefore no Taylor cone or spray at all is produced.

4.6 CAPILLARY

The use of a capillary did not have the expected effect. In literature (Liu, Wang et al. 2011) the use of a capillary is also mentioned. But in contrast to the capillary experiments that were performed during this thesis, in literature, a stable spray was observed. In order to obtain a stable spray using capillary several changes can be made to the used method. For once, a capillary with a smaller diameter can be used; in order to increase the capillary suction might cause the capillary to be full of fluid. Next to this, a short capillary can be used. This would decreases the distance between the point of voltage applied and the MS inlet, which decreases the resistance and might result in a (stable) spray. Another option is to put a solution of analyte and MeOH in the capillary and clamp only the capillary itself into the crocodile clamp, however, the fluid will be gone fast and the spray will be very short.

Next to starting the spraying, a solution has to be found for the suction of the capillary into the MS inlet. One way would be to put a clamp on the capillary to keep it in place in the fruit piece. Another way would be as mentioned above; clamping the capillary directly towards the crocodile clamp. Also a barbed capillary could help. The little hooks will keep the capillary from exiting the fruit piece.

When LS/FS using a capillary is a working technique, not only soft fruits can be used for measurements, also different parts of the fruits can be easily measured. For example the outer layer versus the inner layers of a kiwi or tomato fruit.

4.7 LS/FS TECHNIQUE COMPARED TO DART

The differences between DART and LS/FS experiments can be explained by the way the different techniques work. DART uses ions from the air, resulting in a combination of the product and NH_4^+ where using the LS/FS the charge added has to be originated from the product itself.

TABLE 5: PRO'S AND CONS OF LS/FS AND DART

PRO	CON
Higher signal relative to DART	Soft fruits are hard to measure/not measureable
Costs (relatively low)	Sharp tip is required
Less impact on sample compared to DART	DART shows larger differences between Japanese and Chinese star anise
More stable compared to DART	
Parameters not very critical voltage distance	
Fast and Simple	
Solvent has some influence	

Comparing the outcomes of the experiments on star anise qualitatively, both techniques are perfectly capable of telling apart Japanese from Chinese star anise. Japanese star anise signals obtained by the DART technique, is about 1000 times larger than Chinese. For LS/FS this amount is lower, Japanese star anise gives a 200-300 times higher signal than Chinese star anise. However, for DART the relative standard deviations (RSDs) are two to three times higher. For LS/FS RSDs were 28% (for (−)mode) and 26% (for (+)mode), were for DART (−)mode showed 55% RSDs and (+)mode even 86%.

Also, for LS/FS the spray is very stable (Figure 36), were in DART, it is hard to obtain a stable, continuous signal.

Both DART and LS/FS therefore can be very valuable for measuring whether a batch of star anise is Japanese or Chinese.

There are no strong results to prove quantitatively measuring is possible using leafspray technique. Though, because the spray is stable and not influenced by voltage or distance, it is possible to do semi-quantitative measurements. For example like the comparing of the Chinese and Japanese star anise.

5 FUTURE PROSPECTIVE

Leafspray/fruitspray is a very new technique, easy to use. Because no expensive sources are needed to put on a MS, the technique is relatively cheap. Hand-MS spectrometers exist(Soparawalla, Tadjimukhamedov et al. 2011), and using these, it might be possible to test samples on location, especially because Leafspray is not specifically bound to a certain kind of MS (both orbitrap and iontrap are possible). In order to work with this technique on local scale, more research is necessary, to obtain information about the products of interest and to optimize the technique on small scale.

Also, perhaps the semi-quantitatively can be tested using ESI as a reference technique, or by exploring the paperspray technique. Next to this, experiments can be performed on different compounds.

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Betz, J. M. and S. W. Page (1988). "Effect of processing on the glucosinolate composition of brassica spp." Abstracts of Papers of the American Chemical Society **196**: 192-AGFD.

Burri, B. and R. Jacob (1997). "Human metabolism and the requirement for

ascorbic acid." Packer L, Fuchs J, eds. Ascorbic acid in health and disease. New York: Marcel Dekker Inc: 341–366.

Curtis, M., M. A. Minier, et al. (2010). "Direct analysis in real time (DART) mass spectrometry of nucleotides and nucleosides: elucidation of a novel fragment [C5H5O]+ and its in-source adducts." J Am Soc Mass Spectrom **21**(8): 1371-1381.

Frenich, A. G., M. E. H. Torres, et al. (2005). "Determination of Ascorbic Acid and Carotenoids in Food Commodities by Liquid Chromatography with Mass Spectrometry Detection." Journal of Agricultural and Food Chemistry **53**(19): 7371-7376.

Gabrys, B., W. F. Tjallingii, et al. (1997). "Analysis of EPG Recorded Probing by Cabbage Aphid on Host Plant Parts with Different Glucosinolate Contents." Journal of Chemical Ecology **23**(7): 1661-1673.

Health, A. (21/03/2012). " Health nutrition data, Know what you eat." from HTTP://NUTRITIONDATA.SELF.COM/.

Howes, M. J. R., G. C. Kite, et al. (2009). "Distinguishing Chinese Star Anise from Japanese Star Anise Using Thermal Desorption - Gas Chromatography - Mass Spectrometry." Journal of Agricultural and Food Chemistry **57**(13): 5783-5789.

Ize-Ludlow, D., S. Ragone, et al. (2004). "Neurotoxicities in infants seen with the consumption of star anise tea." Pediatrics **114**(5): E653-E656.

Liu, J., H. Wang, et al. (2011). "Leaf Spray: Direct Chemical Analysis of Plant Material and Living Plants by Mass Spectrometry." Analytical Chemistry **83**(20): 7608-7613.

Malaj, N., Z. Ouyang, et al. (2012). "Analysis of pesticide residues by leaf spray mass spectrometry." Analytical Methods **4**(7): 1913-1919.

Peng, Y. e., S. Zhang, et al. (2012). "In Vivo Nanoelectrospray for the Localization of Bioactive Molecules in Plants by Mass Spectrometry." Analytical Chemistry **84**(7): 3058-3062.

Sarkar, D., A. Srimany, et al. (2012). "Rapid identification of molecular changes in tulsi (Ocimum sanctum Linn) upon ageing using leaf spray ionization mass spectrometry." Analyst **137**(19): 4559-4563.

Shen, Y., T. A. van Beek, et al. (2012). "Rapid control of Chinese star anise fruits and teas for neurotoxic anisatin by Direct Analysis in Real Time high resolution mass spectrometry." Journal of Chromatography A **1259**: 179-186.

Simon, J. A. J. (1992). "Ascorbic acid and cardiovascular disease: a review." J Am Coll Nutr **11**(2): 107-125.

Soparawalla, S., F. K. Tadjimukhamedov, et al. (2011). "In situ analysis of agrochemical residues on fruit using ambient ionization on a handheld mass spectrometer." Analyst **136**(21): 4392-4396.

Taylor, G. (1964). Disintegration of Water Drops in an Electric Field Proceedings of the Royal Society of London. Series A, Mathematical and Physical Sciences. **280:** 383-397

Tsao, C. (1997). "An overview of ascorbic acid chemistry and biochemistry." Packer L, Fuchs J, eds. Ascorbic acid in health and disease. New York: Marcel Dekker Inc.: 25–58.

University of Bristol, S. o. c., mass spectrometry "The history of mass spectrometry."

Upton, R., Ed. (2006). "Differentiation between Star Anise (Illicium verum) and the Toxic Adulterant Shikimi (Illicium anisatum)." American Herbal Pharmacopoeia, United States.

van Dijk, K. G. J. (2002). "Rhabdomyolysis due to false star anise." Neuromuscular Disorders **12**(7-8): 731-731.

Venter, A., M. Nefliu, et al. (2008). "Ambient desorption ionization mass spectrometry." TrAC Trends in Analytical Chemistry **27**(4): 284-290.

Vinci, G., F. Botre, et al. (1995). "Ascorbic-acid in exotic fruits-a liquid chromatographic investigation." Food Chemistry **53**(2): 211-214.

Woodall, A. and B. Ames (1997). "Diet and oxidative damage to DNA: the importance of ascorbate as an antioxidant. ." Packer L, Fuchs J,eds. Ascorbic acid in health and disease. New York: Marcel Dekker Inc.: 193–203.

Zhang, J. I., X. Li, et al. (2012). "Direct analysis of steviol glycosides from Stevia leaves by ambient ionization mass spectrometry performed on whole leaves." Analyst **137**(13): 3091-3098.

Zhang, Z., W. Xu, et al. (2011). "Silica Coated Paper Substrate for Paper-Spray Analysis of Therapeutic Drugs in Dried Blood Spots." Analytical Chemistry **84**(2): 931-938.