EUBerry

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The sustainable improvement of European berry production, quality and nutritional value in a changing environment: Strawberries, Currants, Blackberries, Blueberries and Raspberries

Work Package No.3

FRUIT QUALITY CHARACTERIZATION AND DETERMINATION

P3: the James Hutton Institute

METHODOLOGIES

For fruit internal quality –complex chemistries, (micro)nutrients and neutraceuticals

INDEX

Extraction of Polyphenolics from Berries

1. Introduction

1.1 Purpose

To describe the procedure for extracting polyphenolics from berries. This SOP is to be used for training of new and substitute staff, for auditing purposes and for general reference.

1.2 Scope

The method can be used for fresh or frozen berries. The extract can be used for determining total anthocyanins, total phenols and polyphenol composition by LC-MS.

1.3 Overview

Extraction is carried out by homogenizing fresh or frozen berries with 0.5% formic acid in acetonitrile followed by centrifugation.

2. Methods

2.1 Inputs to process

2.1.1 Apparatus and reagents required

Apparatus
Balance (3 place or better)
Microcentrifuge (Eppendorf 5415D or equivalent)
Borosilicate glass mortars (homogenisers), 5 mL capacity
1.5ml and 2ml microfuge tubes
8ml glass vials
96 well microfuge racks
Scalpel
Eppendorf P1000 pipette or equivalent
Handystep electronic pipette (Brand)

Chemicals
Acetonitrile
Formic acid
Double distilled water

2.2. Procedures

2.2.1 Extraction of polyphenolics

2.2.1.1 Using scalpel cut 3 fresh or frozen berries in half (quarters for strawberry) and weigh in a weighing boat. If it is not possible to obtain whole fruit, accurately weigh out approximately 3 g.

2.2.1.2 Transfer berries to homogeniser and add volume of 0.5% formic acid in acetonitrile equal to the weight of berries. Break up with spatula, allow frozen berries to thaw, crush with pestle and transfer to 2 mL microfuge tubes.

2.2.1.3 Centrifuge samples at 13200 rpm for 5 min and transfer supernatants to a 2 mL micorfuge tube using automatic pipette.

2.2.1.4 Centrifuge samples at 13200 rpm for 3 min and combine supernatants in an 8 mL vial.

2.2.1.5 Mix sample and transfer 500 ul aliquots to 1.5 mL microfuge tubes using Handystep.

2.2.1.6 Store samples in -20°C freezer

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Quantification of Ascorbic Acid in Berries

1. Introduction

1.1 Purpose

To describe the procedure for measuring the ascorbic acid concentration in berries. This SOP is to be used for training of new and substitute staff, for auditing purposes and for general reference.

1.2 Scope

The method can be used for fresh or frozen berries. Either ascorbic acid only or a total of ascorbic acid and dehydroascorbate can be measured.

1.3 Overview

The berries are homogenized and the pectin is degraded using a pectinase enzyme. The juice is recovered by centrifugation and filtration. If only the ascorbic acid concentration is to be measured, the juice is diluted with 5% metaphosphoric acid (MPA), filtered and analysed by reversed-phase HPLC using a gradient of potassium phosphate buffer (pH 2.8) and acetonitrile. If the total of ascorbic acid and dehydroascorbic acid is required, the filtered juice is diluted with phosphate buffer (pH 5.6) and treated with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) to reduce the dehydroascorbic acid to ascorbic acid. Following dilution, centrifugation and filtration, the total ascorbic acid is measured by reversed phase HPLC.

2. Methods

2.1 Inputs to process

2.1.1 Apparatus and reagents required

Apparatus
2 and 4 place balances
250 mL beaker
Waring blender
High-speed centrifuge
250 ml centrifuge pots
Microcentrifuge with chilling capability (e.g. Eppendorf 5415R)
Whatman No. 1 filter paper
Measuring cylinder
1.5 mL microfuge tubes
Vortex mixer
Disposable syringe

0.2 μm filter (Whatman 25 mm GD/X)
10 mL volumetric flask
HPLC system consisting of binary (or tertiary, quaternary) pump, autosampler, PDA detector, column heater and data system.
Synergi 4 μ Hydro-RP 80A HPLC column (250 mm x 4.6 mm; 4 μm) (Phenomenex)
Synergi 4 μ Hydro-RP 80A guard column (5 mm x 4.6 mm; 4 μm) (Phenomenex)

Chemicals
Pectinex 5X (Novozymes A/S)
Metaphosphoric acid (60% + 40% sodium phosphate stabiliser)
Sodium dihydrogen phosphate, monohydrate
Disodium hydrogen phosphate heptahydrate
tris(2-carboxyethyl)phosphine hydrochloride (TCEP)
Ascorbic acid (>99% purity)
Acetonitrile
Formic acid
Double distilled water

Preparation of solutions and buffers
5% MPA containing 5 mM TCEP. 2.5 g MPA in 25 mL water in volumetric flask and 35.83 mg TCEP, sonicate to dissolve, aliquot 1 mL into microfuge tubes and freeze -80°C).
100mM sodium phosphate buffer (pH 5.6).1.309 g monosodium phosphate, monohydrate and 0.138 g disodium phosphate heptahydrate in 100 mL water.

2.2. Procedures

2.2.1 Sample preparation

2.2.1.1 Remove frozen fruit from storage (-20°C freezer) and weigh 150 g into a beaker.
Allow to thaw at room temperature for approximately 4 h.

2.2.1.2 Add 150 μl Pectinex 5X to the berries and homogenize using a Waring blender for 60 s.

2.2.1.3 Cover beaker with aluminium foil and leave at room temperature overnight.

2.2.1.4 Transfer sample to centrifuge pot and centrifuge at 5000 x g for 20 min at 1°C for 20 min.

2.2.1.5 Filter sample into measuring cylinder using Whatman No. 1 filter paper. Note the volume of juice. Decant juice into 1.5 mL microfuge tubes and freeze at -20°C. Prior to analysis thaw tube at room temperature.

2.2.1.6 For measurement of ascorbic acid only, add 20 μl of filtered sample to 980 □l 5% (wt/vol) metaphosphoric acid (MPA) in a 1.5 ml microfuge tube, vortex and centrifuge 16000 x g for 5 min at 1°C.

2.2.1.7 Using a disposable syringe, put sample through 0.2 μm filter into microfuge tube. Transfer 200 μl of filtered sample to an autosampler vial. The sample is now ready for HPLC analysis.

2.2.1.8 For measurement of total of ascorbic and dehydroascorbic acid, 50 μl juice prepared in 2.2.6 was diluted with 450 μl 100 mM phosphate buffer (pH 5.6) in a 1.5 ml microfuge tube and, after vortexing, 100 μl was transferred to another microfuge tube. Add 2 μl 5% MPA containing 5 mM TCEP and leave for 18 h at room temperature.

2.2.1.9 Add 100 μl 10% MPA, centrifuge 16000 x g for 5 min at 1°C and filter as in 2.2.8. The sample is now ready for HPLC analysis.

2.2.1.10 Standard solutions are prepared by first preparing a 10 mg/mL solution of ascorbic acid; accurately weigh approximately 100 mg ascorbic acid into a 10 mL volumetric flask, make up to the mark with 5% MPA containing 5 mM TCEP and shake until dissolved. Prepare 1 mg/mL solution by adding 100 μl to 900 μl 5% MPA containing 5 mM TCEP in a microfuge tube and vortexing. In another 10 mL volumetric flask, prepare 0.1 mg/mL solution by adding 1.0 mL of the 1mg/mL solution and make up to the mark with 5% MPA containing 5 mM TCEP. In microfuge tubes,10, 20, 40, 60, 80 and 100 μg/mL solutions are prepared by adding 100, 200, 400, 600, 800 and 1000 μl of the 0.1 mg/mL solution to 900, 800, 600, 400, 200 and 0 μl 5% MPA containing 5 mM TCEP, respectively. Transfer 200 μl of each standard to an autosampler vial.

2,2,2 HPLC analysis

2.2.1 The samples and standards are injected in duplicate via an autosampler on to a reversed-phase HPLC column linked to a PDA detector. A pump capable of binary gradients is used. Parameters for the autosampler, mobile phase and PDA are as follows:-

Injection volume: 20 μl.

Autosampler temperature: 5°C

Column type:Synergi 4 μ Hydro-RP 80A HPLC column (250 mm x 4.6 mm; 4 □m)
Guard column:Synergi 4 μ Hydro-RP 80A guard column (5 mm x 4.6 mm; 4 □m)

Column temperature: 30°C.

Mobile phase: Solvent A – 0.1% aqueous formic acid
Solvent B – acetonitrile

Flow rate: 2.5 mL min^{-1}

Gradient :

Time (min)	A%	B%
0	100	0
0.50	100	0
0.75	20	80
1.25	20	80
1.50	100	0
3.00	100	0

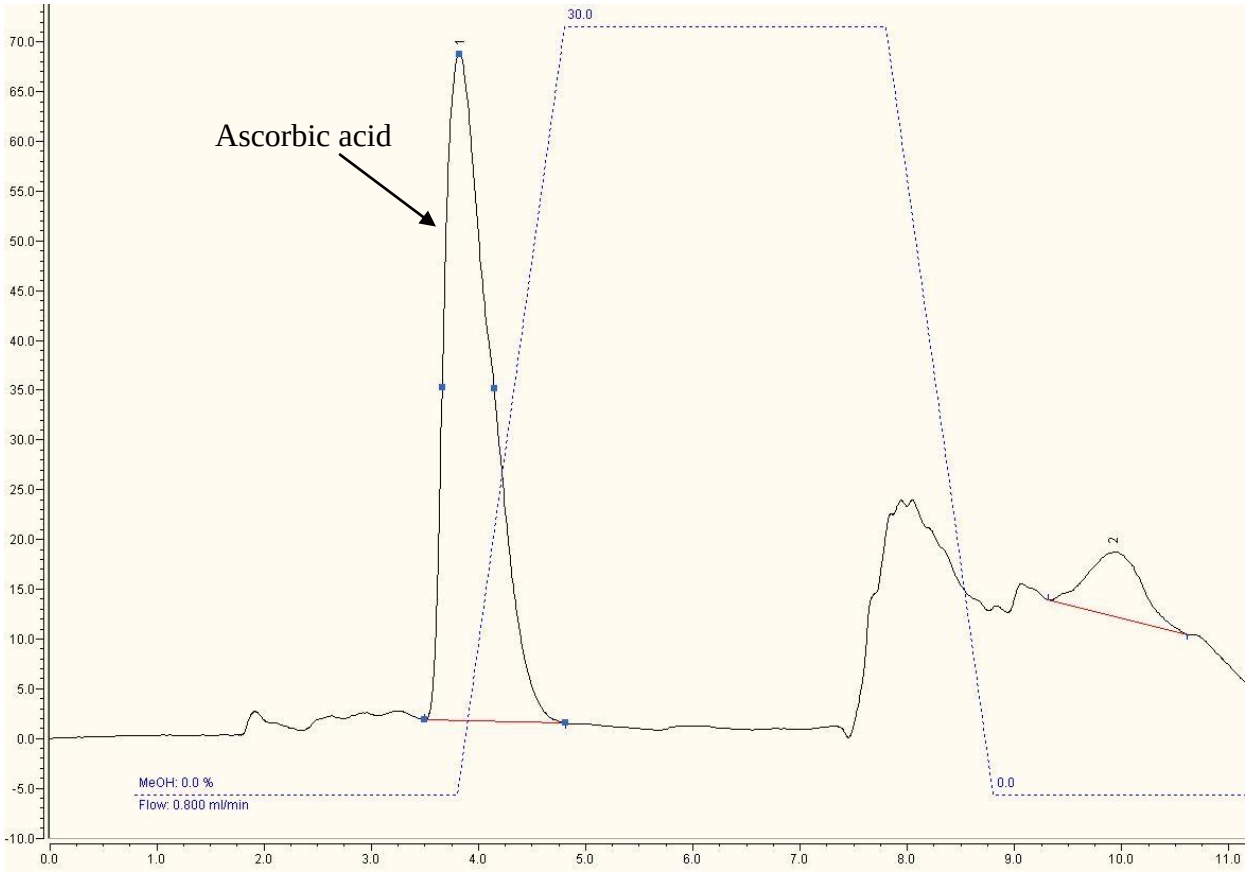
PDA range: 200-600 nm.

PDA channel: 245 nm

2.2.3 Quantification

The areas at 245 nm of the ascorbic acid peaks in the calibration standards are plotted against the concentration (μg/mL) and the equation of the best-fit line determined in Excel. The identity of the ascorbic acid peak in the samples is determined by comparison of retention time to that of the standards, and the areas are measured. Concentrations (μg/mL) in the samples are determined using the calibration equation. The concentrations (μg/mL) in the juice extracts are calculated by multiplying by the dilution factors – x50 for ascorbic acid determination and x20 for total of ascorbic acid and dehydroascorbic acid determination. The concentration (μg/g) in the original berries can then be determined by multiplying by the total volume (mL) of juice and dividing by the mass of berries (g).

Appendix HPLC profile of ascorbic acid in blackcurrant



Quantification of Organic Acids in Berries

1. Introduction

1.1 Purpose

To describe the procedure for measuring the concentration of individual and total organic acids in berries. This SOP is to be used for training of new and substitute staff, for auditing purposes and for general reference.

1.2 Scope

The method can be used for fresh or frozen berries. Often only citrate, malate and oxalate are measured but the method can be extended to include other organic acids such as fumarate, isocitrate, succinate and tartarate depending on the berry.

1.3 Overview

The berries are homogenized and the pectin is degraded using a pectinase enzyme. The juice is recovered by centrifugation and filtration. After suitable dilution of the juice, concentration of individual organic acids are analysed on an anion exchange HPLC column and with a gradient of 10% methanol and 100 mM sodium hydroxide in 10% methanol. Calibration standards of the relevant organic acids are run and the concentration of individual or total organic acids in the juices are thereby determined.

2. Methods

2.1 Inputs to process

2.1.1 Apparatus and reagents required

Apparatus
2 and 4 place balances
250 mL beaker
Waring blender
High-speed centrifuge
250 ml centrifuge pots
Microcentrifuge with chilling capability (e.g. Eppendorf 5415R)
Whatman No. 1 filter paper
Measuring cylinder
1.5 mL microfuge tubes
Vortex mixer
0.2 μm filter (Whatman 25 mm GD/X)
10 mL volumetric flask
Dionex high-performance anion exchange chromatographic (HPAEC) consisting of pump, autosampler, column heater, electrochemical detector and data system.

Dionex IonPac AS11-HC (250 mm x 4 mm) column.
Guard column of Dionex IonPac AS11-HC (50 mm x 4 mm) column.

Chemicals
Pectinex 5X (Novozymes A/S)
Sodium hydroxide
Methanol (HPLC grade)
Ultrapure water
Citric acid
Malic acid
Oxalic acid
Double distilled water

Preparation of mobile phase
100 mM sodium hydroxide in 10% methanol prepared by dissolving 4 g of sodium hydroxide in 900 mL ultrapure water and adding 100 mL methanol

2.2. Procedures

2.2.1 Juice extraction

2.2.1.1 Remove frozen berries from storage (-20°C freezer) and weigh 150 g into a beaker.
Allow to thaw at room temperature for approximately 4 h.
2.2.1.2 Add 150 µl Pectinex 5X to the fruit and homogenize using a Waring blender for 60 s.
2.2.1.3 Cover beaker with aluminium foil and leave at room temperature overnight.
2.2.1.4 Transfer sample to centrifuge pot and centrifuge at 5000 x g for 20 min at 1°C for 20 min.
2.2.1.5 Filter sample into measuring cylinder using Whatman No. 1 filter paper. Note the volume of juice. Decant juice into 1.5 mL microfuge tubes and freeze at -20°C. Prior to analysis thaw tube at room temperature.

2.2.2 Sample preparation

2.2.2.1 Make a 50 fold dilution of the filtered sample by adding 30 µl to 1200 µl ultrapure water in a 2 ml microfuge tube and vortex.
2.2.2.2. Centrifuge 16000 x g for 5 min at 1°C and transfer 200 µl of sample to HPLC vials ready for HPLC analysis.
2.2.2.3 Standard solutions are prepared of individual organic acids (normally citric acid, malic acid and oxalic acid) by first preparing a solution of each organic acid at 5 mg/mL; accurately weigh approximately 50 mg organic acid into a 10 mL volumetric flask, make up to the mark with ultrapure water and shake until dissolved. Prepare a mixture of each organic acid at 1 mg /mL solution by adding 2.0 mL of each solution to another 10 mL volumetric flask, make up to

the mark with ultrapure water and shake. In microfuge tubes, 20, 50, 100, 400, 700 and 1000 μg/mL solutions are prepared by adding 20, 50, 100, 400, 800 and 1000 μl of the 1 mg/mL solution to 908, 950, 900, 600, 200 and 0 μl ultrapure water, respectively. Transfer 200 μl of each standard to an autosampler vial.

2,2.3 HPLC analysis

The samples and standards are injected in duplicate via an autosampler on to an anion exchange HPLC column linked to an electrochemical detector. A pump capable of binary gradients is used. Parameters for the autosampler, mobile phase and electrochemical detector are as follows:-

Injection volume: 25 μl.

Autosampler temperature: 5°C

Column type: Dionex IonPac AS11-HC (250 mm x 4 mm)

Guard column: Dionex IonPac AS11-HC (50 mm x 4 mm)

Column temperature: 30°C.

Mobile phase: Solvent A – 10% aqueous methanol
Solvent B – 100 mM NaOH in 10% aqueous methanol

Flow rate: 1.5 mL min^{-1}

Gradient :

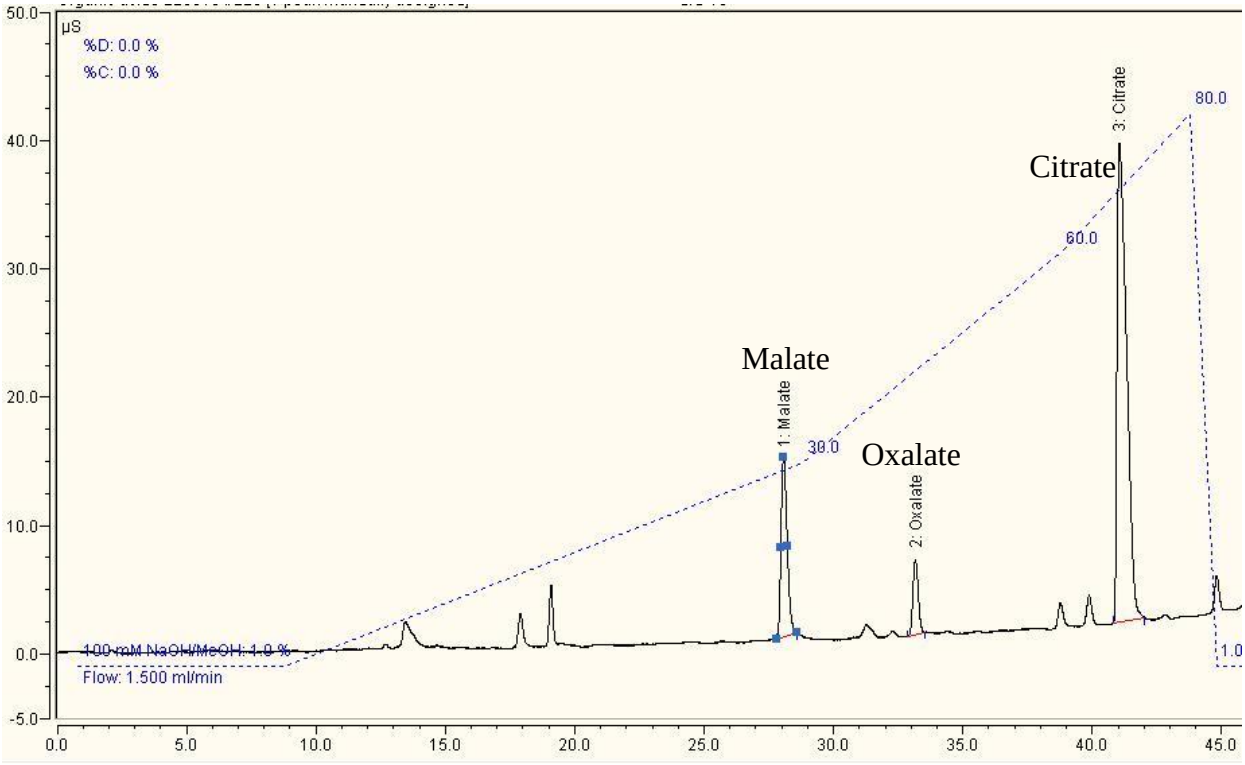
Time (min)	A%	B%
0	99	1
8	99	1
28	70	30
38	40	60
43	20	80
44	99	1
50	99	1

Detector: Electrochemical detector. The detector was preceded by a 4 mm ASRS 300 anion self-generating suppressor used in the external mode to suppress the background conductivity of the mobile phase. Ion suppression was undertaken at 240 mA with ultrapure water at 2 mL min^{-1}.

2.2.4 Quantification

The areas of the organic acid peaks in the calibration standards are plotted against the concentration (μg/mL) and the equation of the best-fit line determined in Excel. The identities of the organic acid peaks in the samples are determined by comparison of retention times to those of the standards, and the areas are measured. Concentrations (μg/mL) in the samples are determined using the calibration equation. Considering that the juices were diluted 50 fold, multiply this value by 0.05 to give the concentration in mg/mL in the undiluted juice extract. The concentration (mg/g) in the original fruit can then be determined by multiplying by the total volume (mL) of juice and dividing by the mass of berries (g). Results are expressed as concentration of individual or total organic acids.

Appendix HPLC profile of organic acids in blackcurrant



Quantification of Sugars in Berries

1. Introduction

1.1 Purpose

To describe the procedure for measuring the concentration of individual and total sugars in berries. This SOP is to be used for training of new and substitute staff, for auditing purposes and for general reference.

1.2 Scope

The method can be used for fresh or frozen berries. The method measures glucose, fructose and sucrose concentrations.

1.3 Overview

The berries are homogenized and the pectin is degraded using a pectinase enzyme. The juice is recovered by centrifugation and filtration. After suitable dilution of the juice concentration of individual sugars are analysed on an anion exchange HPLC column and an isocratic mobile phase of 200 mM sodium hydroxide. Calibration standards of the relevant sugars are run and the concentration of individual or total sugars in the juices are thereby determined.

2. Methods

2.1 Inputs to process

2.1.1 Apparatus and reagents required

Apparatus
2 and 4 place balances
250 mL beaker
Waring blender
High-speed centrifuge
250 ml centrifuge pots
Microcentrifuge with chilling capability (e.g. Eppendorf 5415R)
Whatman No. 1 filter paper
Measuring cylinder
1.5 mL microfuge tubes
Vortex mixer
0.2 μm filter (Whatman 25 mm GD/X)
10 mL volumetric flask
Dionex high-performance anion exchange chromatographic (HPAEC) consisting of pump, autosampler, column heater, pulsed amperometer detector and data system.
Dionex Carbopac PA-100 (250 mm x 4 mm) column.

Chemicals
Pectinex 5X (Novozymes A/S)
Sodium hydroxide
Glucose
Fructose
Sucrose
Double distilled water

Preparation of mobile phase
200 mM sodium hydroxide prepared by dissolving 8 g of sodium hydroxide in a litre of distilled water.

2.2. Procedures

2.2.1 Juice extraction

2.2.1.1 Remove frozen berries from storage (-20°C freezer) and weigh 150 g into a beaker.
Allow to thaw at room temperature for approximately 4 h.

2.2.1.2 Add 150 µl Pectinex 5X to the fruit and homogenize using a Waring blender for 60 s.

2.2.1.3 Cover beaker with aluminium foil and leave at room temperature overnight.

2.2.1.4 Transfer sample to centrifuge pot and centrifuge at 5000 x g for 20 min at 1°C for 20 min.

2.2.1.5 Filter sample into measuring cylinder using Whatman No. 1 filter paper. Note the volume of juice. Decant juice into 1.5 mL microfuge tubes and freeze at -20°C. Prior to analysis thaw tube at room temperature.

2.2.2 Sample preparation

2.2.2.1 Add 100 µl of filtered sample to 900 µl distilled water in a 1.5 ml microfuge tube and vortex. Add 100 µl of this dilution to 900 µl distilled water in another microfuge tube. Repeat similar 10 fold dilutions twice more and then add 200 µl of the final dilution to 800 µl distilled water in another microfuge tube so that a final dilution of 1 in 5000 of the juice is achieved. Incubate final dilution at 100°C in an oven for 5 min.

2.2.2.2. Centrifuge 16000 x g for 5 min at 1°C and transfer 200 µl of sample to HPLC vials ready for HPLC analysis.

2.2.2.3 Standard solutions are prepared of individual sugars (normally fructose, glucose and sucrose) by first preparing a solution of each sugar at 2 mg/mL; accurately weigh approximately 20 mg sugar into a 10 mL volumetric flask, make up to the mark with distilled water and shake until dissolved. Prepare a mixture of each sugar at 200 µg /mL solution by adding 1.0 mL of each solution to another 10 mL volumetric flask, make up to the mark with distilled water and shake. In

another 10 mL volumetric flask prepare 20 μg /mL solution by adding 1.0 mL of the 200 μg /mL solution and making up to the mark with distilled water. In microfuge tubes, 2, 4, 8, 12, 16 and 20 μg/mL solutions are prepared by adding 100, 200, 400, 600, 800 and 1000 μl of the 200 μg /mL solution to 900, 800, 600, 400, 200 and 0 μl distilled water, respectively. Transfer 200 μl of each standard to an autosampler vial.

2,2.3 HPLC analysis

The samples and standards are injected in duplicate via an autosampler on to a anion exchange HPLC column linked to a pulsed amperometer detector. A pump capable of isocratic delivery is used. Parameters for the autosampler, mobile phase and detector are as follows:-

Injection volume: 25 μl.

Autosampler temperature: 5°C

Column type: Dionex Carbopac PA-100 (250 mm x 4 mm)

Column temperature: 30°C.

Mobile phase: 200 mM sodium hydroxide (isocratic)

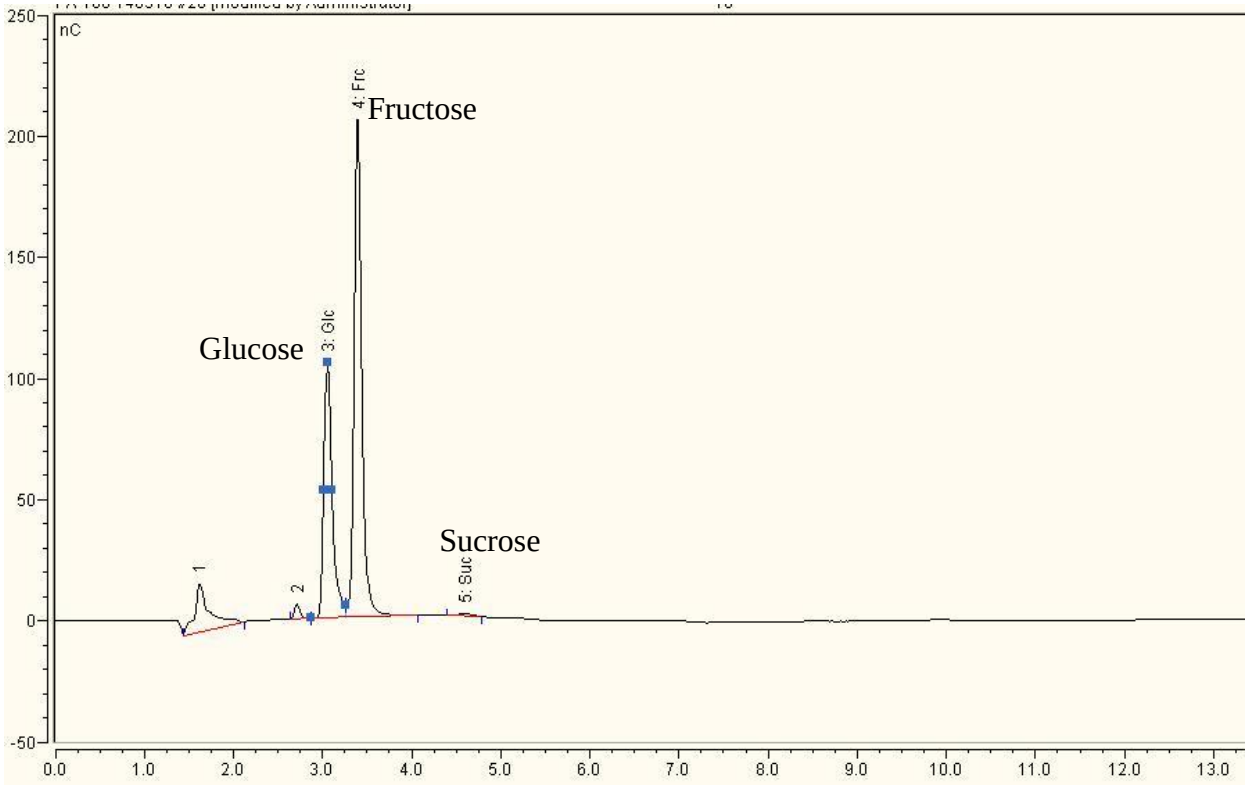
Flow rate: 1.0 mL min^{-1}

Detector: Pulsed amperometer in standard quad waveform mode

2.2.4 Quantification

The areas of the sugar peaks in the calibration standards are plotted against the concentration (μg/mL) and the equation of the best-fit line determined in Excel. The identities of the sugar peaks in the samples are determined by comparison of retention times to those of the standards, and the areas are measured. Concentrations (μg/mL) in the samples are determined using the calibration equation. Considering that the juices were diluted 1000 fold, this value is the concentration in mg/mL in the undiluted juice extracts. The concentration (mg/g) in the original fruit can then be determined by multiplying by the total volume (mL) of juice and dividing by the mass of berries (g). Results are expressed as concentration of individual or total sugars.

Appendix HPLC profile of sugars in blackcurrant



Measurement of Total Anthocyanins in Berries by pH Differential Method

1. Introduction

1.1 Purpose

To describe the procedure for measuring the concentration of total anthocyanins in berries This SOP is to be used for training of new and substitute staff, for auditing purposes and for general reference.

1.2 Scope

The method can be used for fresh or frozen berries or fruit juices.

1.3 Overview

The berries are extracted with 0.5% formic acid in acetonitrile and then diluted with pH 1 and pH 4.5 buffers. The absorbances ($A_{510} - A_{700}$) are measured at each pH and the concentration is calculated in cyanidin glucoside equvalents.

2. Methods

2.1 Inputs to process

2.1.1 Apparatus and reagents required

Apparatus
Cuvettes
Automatic pipettes; 10-200 and 100-1000 □l
50 mL beakers for buffers
Ice box
3 place balance
500 mL volumetric flasks for buffers
50 mL beakers for buffers
Ice box
Spectrophotometer

Chemicals
Concentrated hydrochloric acid (37%)
Potassium chloride
Sodium acetate trihydrate
Double distilled water

Preparation of buffers

pH 1.0 buffer. Add 10 mL to distilled water in a 500 mL volumetric flask and make up to 500 mL mark with distilled water. Add 7.456 g KCl to distilled water in a 500 mL volumetric flask and make up to 500 mL mark with distilled water. Add 335 mL of HCl solution to 165 mL KCl solution.
pH 4.5 buffer. Dissolve 6.804 g sodium acetate trihydrate in distilled water in 500 ml volumetric flask and make up to the mark with distilled water.

2.2. Procedures

2.2.1 Assay protocol

2.2.1.1 Berries are extracted with 0.5% formic acid in acetonitrile to give a "50% juice" as described in SOP Extraction of Polphenolics from Berries.

2.2.1.2 For each sample, add 960 μl of pH1.0 and pH 4.5 buffers to each of 3 cuvettes

2.2.1.3 Add 40 μl sample to each cuvette and mix using pipette tip using a clean tip for each buffer.

2.2.1.4 Add 1 mL of each buffer to 2 cuvettes to act as references.

2.2.1.5 15 min after diluting samples in buffer read the absorbances of the diluted juices in each buffer, against the appropriate reference, at 510 and 700 nm; the spectrophotometer may be capable of giving a $A_{510} - A_{700}$ reading directly. Absorbance values up to 2.0 are acceptable. If higher values are obtained the assay should be repeated using a sample at an appropriate dilution.

2.2.2 Calculation of anthocyanin concentration

2.2.2.1 In an Excel spreadsheet calculate $(A_{510} - A_{700})$pH 1.0 - $(A_{510} - A_{700})$pH 4.5 for each of the 3 values. This value is A

2.2.2.2 The molarity, in cyanidin-3-glucoside equivalents, of the diluted solution is then calculated by dividing A by 26900 (the extinction coefficient of cyanidin-3-glucoside). This value is B.

2.2.2.3 The molarity, in cyanidin-3-glucoside equivalents, of a "50%" juice is then calculated by multiplying B by 25. This value is C.

2.2.2.4 The concentration of anthocyanins in mg per100 mL "50% juice" is therefore C x 449 x100 where 449 is the molecular weight of cyandin-3-glucoside.

2.2.2.5 The concentration in the berries in mg 100 g^{-1} is calculated by multiplying by the volume of extract (mL) and dividing by the mass of berries. The average of the 3 values can then be calculated.

Measurement of Total Phenols in Berries

1. Introduction

1.1 Purpose

To describe the procedure for measuring the concentration of total phenols in berries. This SOP is to be used for training of new and substitute staff, for auditing purposes and for general reference.

1.2 Scope

The method can be used for fresh or frozen berries or fruit juices.

1.3 Overview

The berries are extracted with 0.5% formic acid in acetonitrile and then diluted with water. After addition of half strength Folin Ciocalteu reagent followed by saturated sodium carbonate solution, the absorbance at 750 nm is read. The phenol content is calculated from a standard curve of phloroglucinol.

2. Methods

2.1 Inputs to process

2.1.1 Apparatus and reagents required

Apparatus
Cuvettes
Automatic pipettes; 2-20, 10-100 and 100-1000 □l
500 ml reagent bottles
Eppendorf tubes (1.5 mL)
Filter paper (Whatman GF/D)
Magnetic stirrers and stirring plate
Ice box
3 place balance
4 place balance
Spectrophotometer

Chemicals
Folin Ciocalteu reagent (Sigma F9252)
Sodium carbonate
Phloroglucinol

Preparation of reagents
Half strength Folin Cioclateu reagent prepared by diluting Folin Ciocalteu reagent with distilled water (50:50 vol/vol).
Saturated sodium carbonate solution prepared by adding 13 g to 100 mL distilled water and mixing with a magnetic stirrer. On day of measurements filter sufficient solution for use on that day.

2.2. Procedures

2.2.1 Assay protocol

2.2.1.1 Berries are extracted with 0.5% formic acid in acetonitrile to give a "50% juice" as described in SOP Extraction of Polphenolics from Berries.

2.2.1.2 Prepare a "1% juice" by adding 20 μl of "50% juice" extract to 980 μl distilled water in an Eppendorf tube. Keep samples on ice until used.

2.2.1.3 Prepare a gallic acid standard at a concentration of 1 mg mL^{-1} by accurately weighing 1 mg into an Eppendorf tube and adding the appropriate volume of methanol. Dilute this 100 fold by adding10 μl to 990 μl distilled water

2.2.1.4 In triplicate add 0.25 mL of "1% juice" and diluted gallic acid solution to a cuvette. Add 0.25 mL water to another cuvette to act as a reference. Add 0.25 mL half strength Folin Ciocalteu reagent to cuvettes.

2.2.1.5 After 3 min add 0.5 mL filtered saturated sodium carbonate solution to each cuvette and mix using a pipette tip using a clean tip for each sample.

2.2.1.6 After 1 hour read absorbances at 750 nm relative to reference. Acceptable absorbances are between 0.3 and 2.0. Values outside this range are unacceptable and if such values are obtained the juice should be reanalysed at an appropriate dilution to give a value within the acceptable range.

2.2.2 Calculation of phenol concentration

2.2.2.1 The phenol concentration in the juice is calculate from a standard curve of phloroglucinol that is prepared with each new batch of half strength Folin Ciocalteu reagent and each new saturated sodium carbonate preparation. For this approximately 1 mg of phloroglucinol is accurately weighed directly in an Eppendorf tube (4 place balance) and dissolved in methanol to give a 1 mg mL^{-1} solution. Concentrations 10, 20, 30, 40, 60 and 80 μg $mL^{-1}$are prepared in Eppendorf tubes by adding 10, 20, 30, 40, 60 and 80 μl of the 1 mg mL^{-1} solution to 990, 980, 970, 960, 940 and 920 μl distilled water. Aliquots of the dilutions are treated as above and the absorbances at 750 nm are plotted against the concentration in Excel and the equation of the best fit line is used to calculate the phenol concentration in the juices in μg mL^{-1}.

2.2.2.2 The phenol concentration (μg mL^{-1}) in the "50% juice extract" is calculated by multiplying the measured concentration in the "1% juice " extract by 50

and the concentration ($\mu g\ g^{-1}$) in the berries is calculated by multiplying by the volume of extract (mL) and dividing by the mass of berries (g). Dividing this value by 10 gives the concentration in mg 100 g^{-1}. The measured concentration ($\mu g\ mL^{-1}$) of the1 mg mL^{-1} gallic acid standard is calculated by multiplying the value obtained for the diluted sample by 100. This value is used to check that the assay is performing correctly.

Analysis of Polyphenolics in Raspberry, Blackberry, Blueberry, Black Currant and Strawberry by High Pressure Liquid Chromatography-Photodiode Array-Mass Spectrometry (HPLC-PDA-MS)

1. Introduction

1.1 Purpose

To describe the procedure for analysing the polyphenolic compounds from raspberry, blackberry, blueberry, black currant or strawberry by high pressure liquid chromatography-photodide array-mass spectrometry (HPLC-PDA-MS). This SOP is to be used for training of new and substitute staff, for auditing purposes and for general reference.

1.2 Scope

The method can be used for fresh, frozen or freeze-dried raspberry, blackberry, blueberry, black currant or strawberry.

1.3 Overview

The method is for analysing polyphenolics from raspberry, blackberry, blueberry, black currant or strawberry by HPLC-PDA-MS. Following extraction, the solvent is removed by Speed Vac and then freeze-dried to remove any remaining water. The sample is re-dissolved in 0.5% formic acid in 5% aqueous acetonitrile and is analysed by reversed phase HPLC-PDA-MS using a mobile phase of 0.1% formic acid in water with increasing proportions of acetonitrile.

2. Methods

2.1 Inputs to process

2.1.1 Apparatus and reagents required

Apparatus
Balance (3 place or better)
Microcentrifuge (Eppendorf 5415D or equivalent)
Borosilicate glass mortars (homogenisers), 5 mL capacity
1.5ml and 2ml microfuge tubes

8ml glass vials
96 well microfuge racks
Scalpel
Freeze-drier
Speed vac SPD131DA (Savant) or equivalent, with refridgerated vapour trap and pump

Eppendorf P1000 pipette or equivalent
Handystep electronic pipette (Brand)
Single StEP^{TM} filter vial 0.45 □m PTFE (Thomson)
Thermo LCQ fleet ion trap mass spectrometer with Accela 600 pump, autosampler and PDA detector or equivalent
Synergi 4μ Hydro-RP 80A (150 mm x 2.0 mm; 4 □m reversed-phase HPLC column (Phenomenex)

Chemicals
Acetonitrile
Formic acid
Double distilled water

2.2 Procedures

2.2.1 Sample preparation

2.2.1.7 Berries are extracted with 0.5% formic acid in acetonitrile to give a "50% juice" as described in SOP Extraction of Polphenolics from Berries.
2.2.1.8 Store samples in -20°C freezer until ready to be taken to dryness.
2.2.1.9 Thaw samples in cold room (4°C) and leave on Speed Vac at 45°C for 3 h. Freeze samples in -20°C freezer.
2.2.1.10 Freeze-dry samples overnight using side arm of freeze-drier and keep in -20°C freezer.

2.2.2 Analysis of polyphenolics by HPLC-PDA-MS

2.2.2.1 Prior to use, dissolve sample in 0.5% formic acid in water/acetonitrile (95:5, vol/vol) in same volume (usually 500 ul) as prior to freeze-drying, vortex to disrupt pellet and centrifuge at 13200 rpm for 3 min. Transfer 0.4 mL sample to filter vial and push filter into vial.
2.2.2.2 The sample is injected via an autosampler on to a HPLC column linked to a PDA system (Thermo Accela) and then an electrospray ionization ion trap mass spectrometer (Thermo LCQ Fleet). The important parameters for the autosampler, mobile phase, PDA and mass spectrometer are as follows:-

Injection volume: 8 μl.

Column type: Synergi 4μ Hydro-RP 80A (150 mm x 2.0 mm; 4 □m reversed-phase HPLC column (Phenomenex)

Autosampler temperature: 6°C

Column temperature: 30°C.

Mobile phase: Solvent A – 0.1% (vol/vol) aqueous formic acid
Solvent B – 0.1% (vol/vol) formic acid in acetonitrile/water (50:50, vol/vol).

Flow rate: 200 μl min^{-1}

Gradient :

Time (min)	A%	B%
0	95	5
4	95	5
28	50	50
32	0	100
34	0	100
36	95	5
45	95	5

PDA range: 200-600 nm.

PDA channels: A. 280 nm (general polyphenols).
B. 365 nm (flavonols).
C 520 nm (anthocyanins).

Mass range: *m/z* 100-2000.

Source parameters: Capillary temperature 275°C, sheath gas and auxillary gas 35 and 5 units and spray voltage 3.8 kV

Scan events: Alternative full scan MS and MS/MS (data-dependant scan).
The sample is run twice – in positive ion and negative ion modes.

2.3 Polyphenol composition

2.3.1 Identification

Identification of polyphenols is based on PDA characteristics and mass spectra including MS and MS/MS data.

2.3.1.1 Raspberry polyphenols

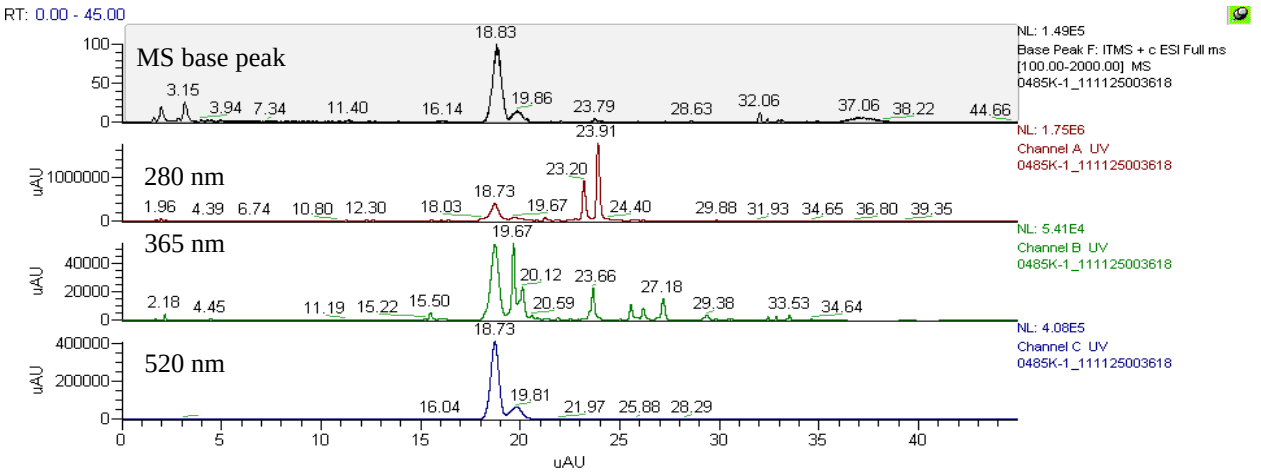
Raspberry contains mainly anthocyanins (analysed in positive ion mode) and ellagitannins (negative ion mode) with flavonols (negative and sometimes positive ion modes) as minor compounds.
The following table gives a list of polyphenols found in raspberry and expected masses.

Compound	**MS**	**MS2**

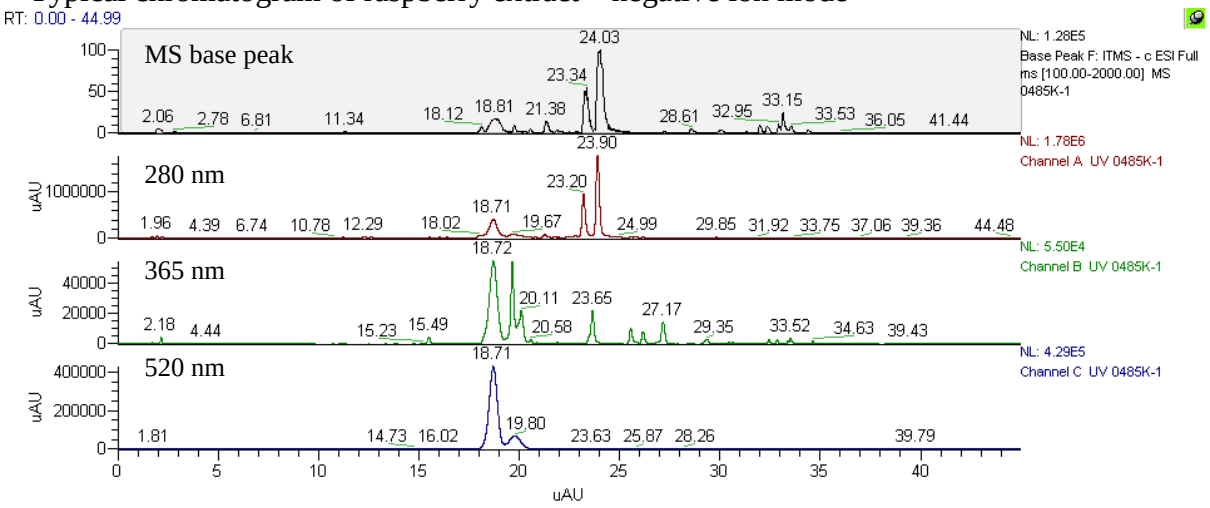
Anthocyanins	**$[M+H]^+$**	
Cyanidin 3-sophoroside	611	287
Cyanidin 3,5-diglucoside	611	449, 287
Cyanidin 3-(2'-glucosyl)rutinoside	757	287
Cyanidin 3-sophoroside-5-rhamnoside	757	611, 433, 287
Cyanidin 3-sambubioside	581	287
Cyanidin 3-(2'-xylosyl)rutinoside	727	287
Cyanidin 3-glucoside	449	287
Cyanidin 3-sambubioside-5-rhamnoside	727	581, 433, 287
Cyanidin 3-rutinoside	595	449, 287
Pelargonidin 3-sophoroside	595	271
Pelargonidin 3-(2'-glucosyl)rutinoside	741	271
Pelargonidin 3-sambubioside	565	271
Pelargonidin 3-glucoside	433	271
Pelargonidin 3-rutinoside	579	433, 271
Flavonols	**$[M-H]^-$**	
Quercetin galactosylrhamnoside/rutinoside	609	301
Quercetin (2'-glucosyl)rutinoside	771	301
Quercetin galactoside	463	301
Quercetin glucoside	463	301
Quercetin glucuronide	477	301
Quercetin diglucoside	625	301
Kaempferol glucuronide	461	285
Methylquercetin glucuronide	491	315
Ellagitannins/ellagic acid derivatives	**$[M-H]^-$**	
Sanguiin H-2	$[1103]^{-1}$ $[551]^{-2}$	301
Sanguiin-H6	$[1869]^{-1}$ $[935]^{-2}$	1697, 1567, 1407,897, 783, 633, 301
Sanguiin-H10 isomers	$[1567]^{-1}$ $[783]^{-2}$	935, 633, 301
Lambertianin C	$[1401]^{-2}$	2019, 1869, 1567, 1402, 935, 897, 633, 301
Ellagic acid pentoside 1	433	301
Ellagic acid pentoside 2	433	301
Ellagic acid acetyl arabinoside	475	301
Ellagic acid 4-acetyl xyloside	475	301
Ellagic acid	301	
Methyl ellagic acid pentoside	447	315

The following chromatograms show typical raspberry (cultivar 0485K-1) profiles and identification of some major components.

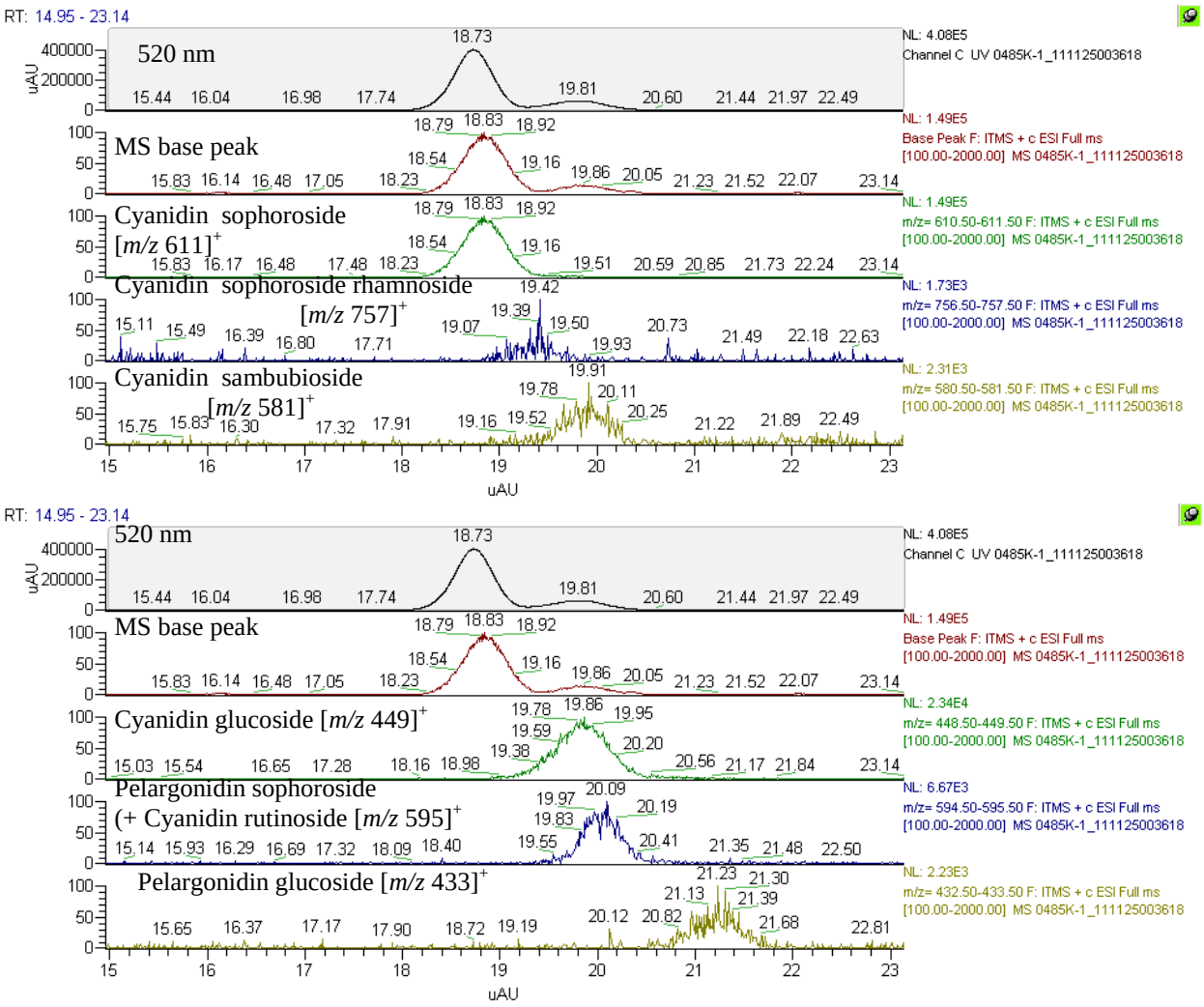
Typical chromatogram of raspberry extract – positive ion mode



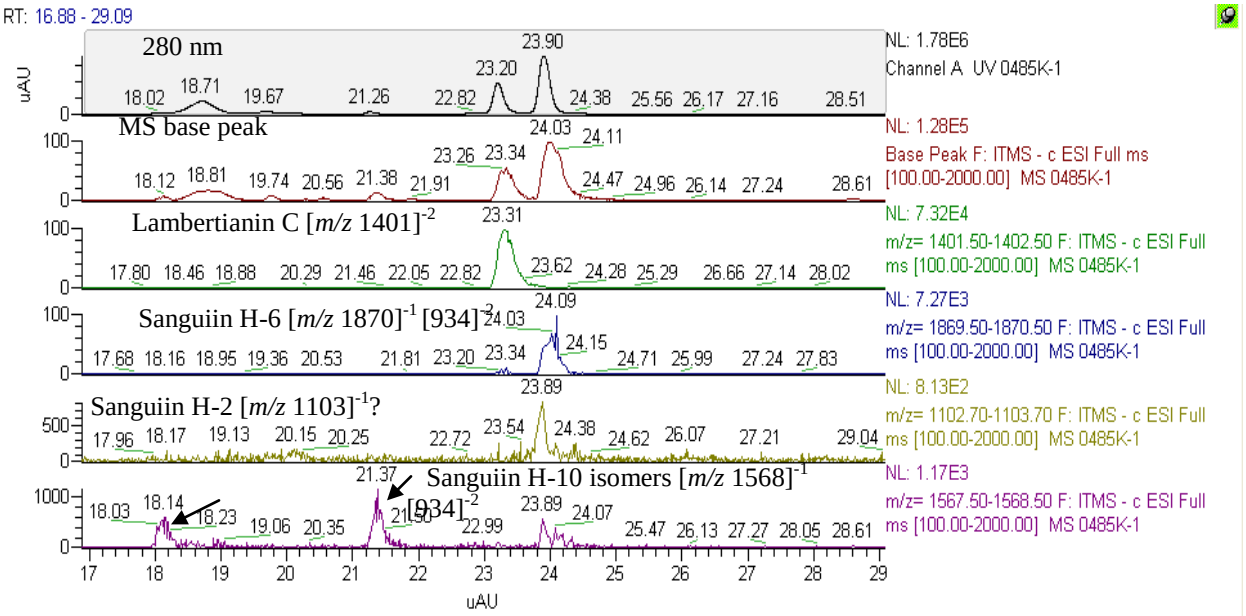
Typical chromatogram of raspberry extract – negative ion mode



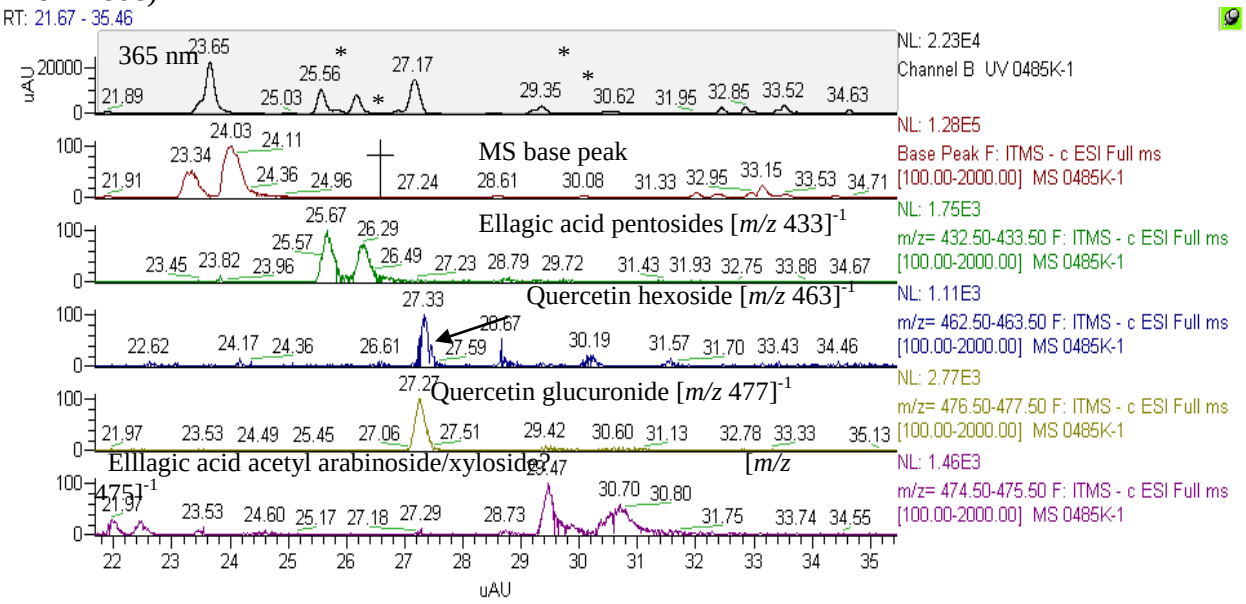
Mass chromatograms of raspberry anthocyanins (positive ion mode)



Mass chromatograms of raspberry ellagitannins (negative ion mode)



Mass chromatograms of raspberry flavonols/ellagic acid derivatives (negative ion mode)



2.3.1.2 Blackberry polyphenols

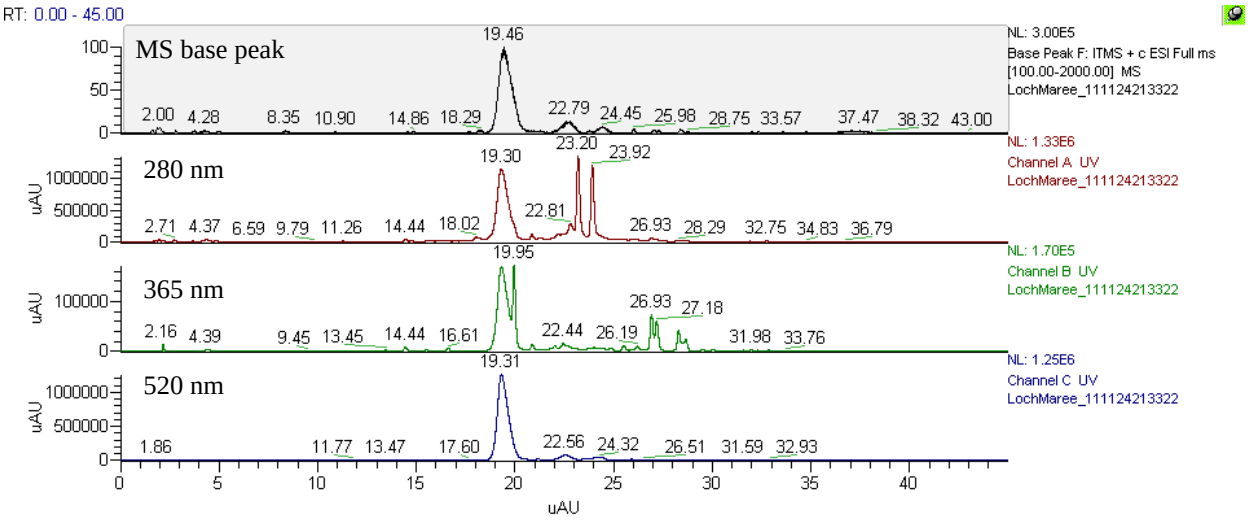
Blackberry contains mainly anthocyanins (analysed in positive ion mode) and ellagitannins (negative ion mode) with flavonols (negative and sometimes positive ion modes) as minor compounds.

The following table gives a list of polyphenols found in blackberry and expected masses.

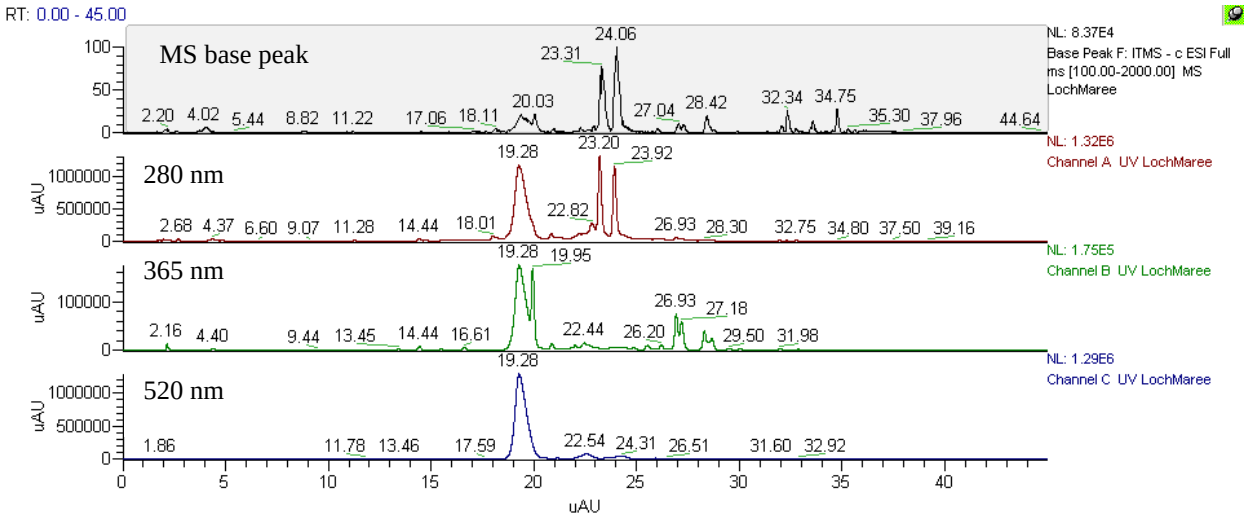
Compound	**MS**	**MS2**
Anthocyanins	**$[M+H]^+$**	
Cyanidin 3-glucoside	449	287
Cyanidin 3-arabinoside	419	287
Cyanidin 3-rutinoside	595	449, 287
Pelargonidin 3-glucoside	433	271
Cyanidin 3-(3'-malonyl)glucoside	535	287
Peonidin 3-glucoside	463	301
Cyanidin 3-xyloside	419	287
Cyanidin 3-(6'-malonyl)glucoside	535	449, 287
Cyanidin 3-dioxalylglucoside	593	287
Cyanidin 3-[6''-(3-hydroxy-3-methylglutaroyl) galactoside	593	287
Flavonols	**$[M-H]^-$**	
Quercetin 3-rutinoside	609	463, 301
Quercetin 3-galactoside	463	301
Quercetin 3-methoxyhexoside	493	463, 301
Quercetin 3-glucoside	463	301
Quercetin 3-pentoside 1	433	301
Quercetin 3-pentoside 2	433	301
Quercetin glucuronide	477	301
Quercetin 3-[6''-(3-hydroxy-3-methylglutaroyl) galactoside	607	463, 301
Quercetin acetylhexoside	505	301
Quercetin 3-glucosylpentoside	595	433, 301
Quercetin 3-oxalylpentoside	505	433, 301
Quercetin	301	
Kaempferol hexoside	447	285
Kaempferol 3-[6''-(3-hydroxy-3-methylglutaroyl) galactoside	591	285, 447
Ellagitannins/ellagic acid derivatives	**$[M-H]^-$**	
Sanguiin H-2	$[1103]^{-1}$ $[551]^{-2}$	301
Sanguiin-H6	$[1869]^{-1}$ $[935]^{-2}$	1697, 1567, 1407,897, 783, 633, 301
Sanguiin-H10 isomers	$[1567]^{-1}$ $[783]^{-2}$	935, 633, 301
Lambertianin C	$[1401]^{-2}$	2019, 1869, 1567, 1402, 935, 897, 633, 301
Ellagic acid	301	
Methyl ellagic acid glucuronide	491	315
Methyl ellagic acid pentoside	447	315

The following chromatograms show typical blackberry (cultivar Loch Maree) profiles and identification of some major components.

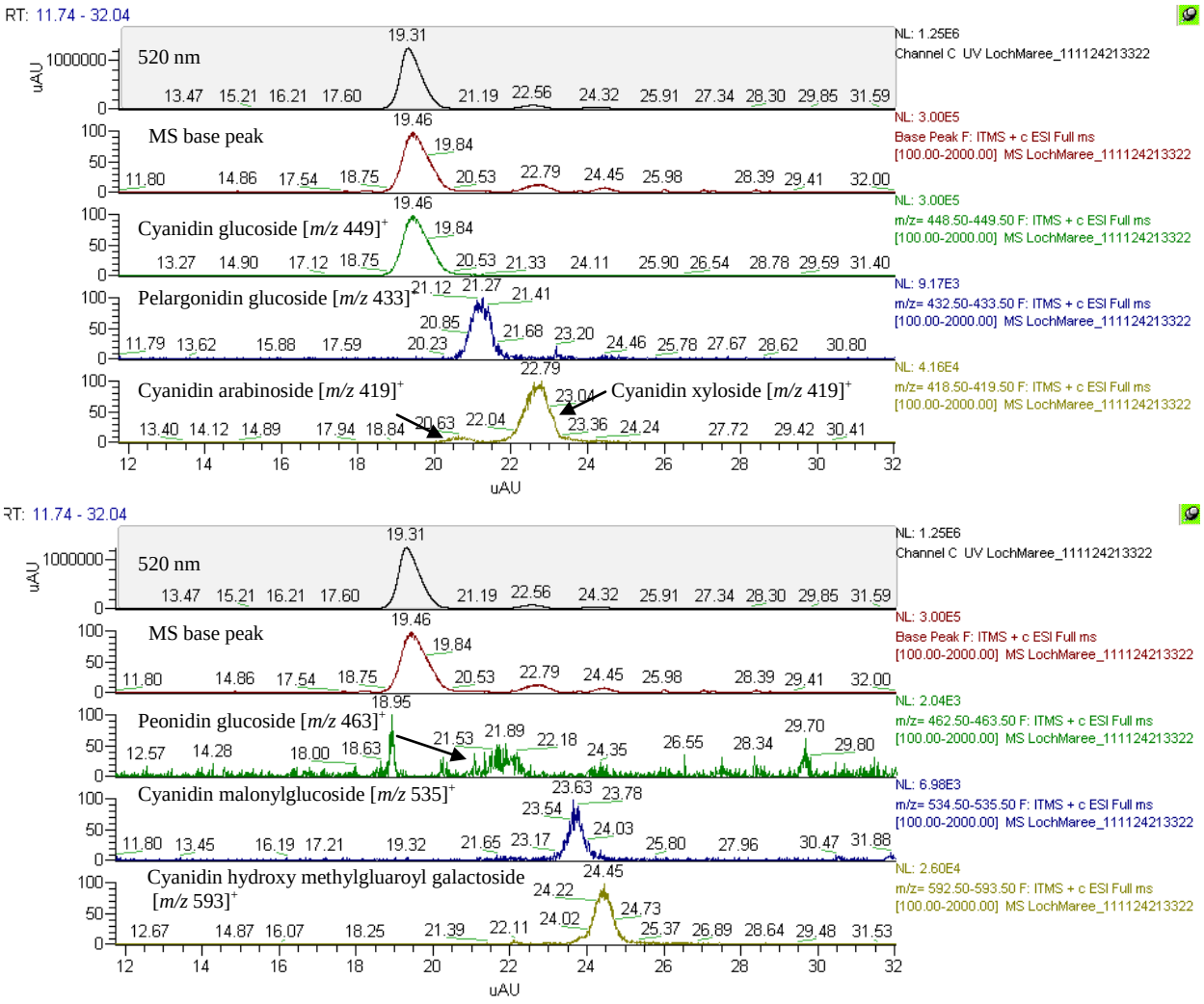
Typical chromatogram of blackberry extract – positive ion mode



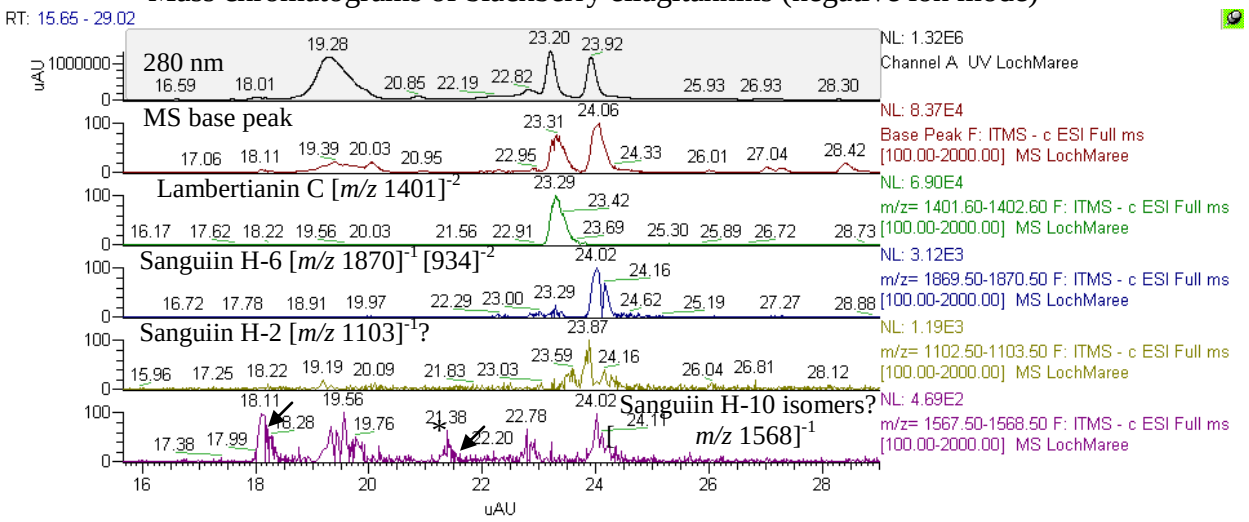
Typical chromatogram of blackberry extract – negative ion mode



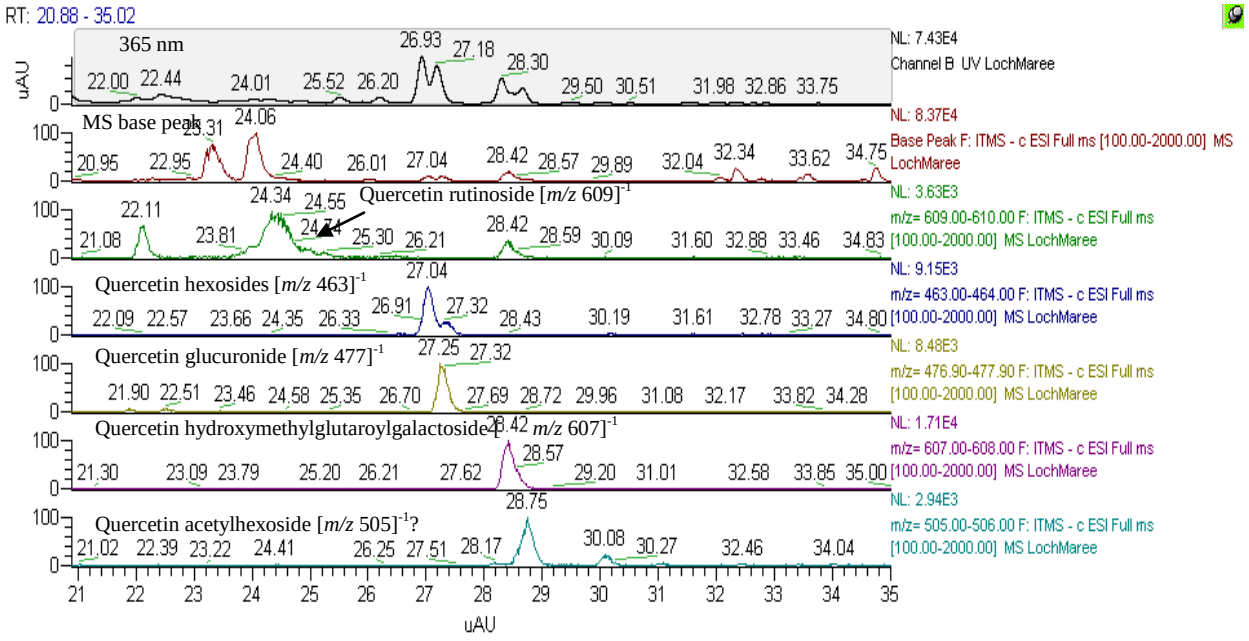
Mass chromatograms of blackberry anthocyanins (positive ion)



Mass chromatograms of blackberry ellagitannins (negative ion mode)



Mass chromatograms of blackberry flavonols (negative ion mode)



2.3.1.3 Black currant polyphenols

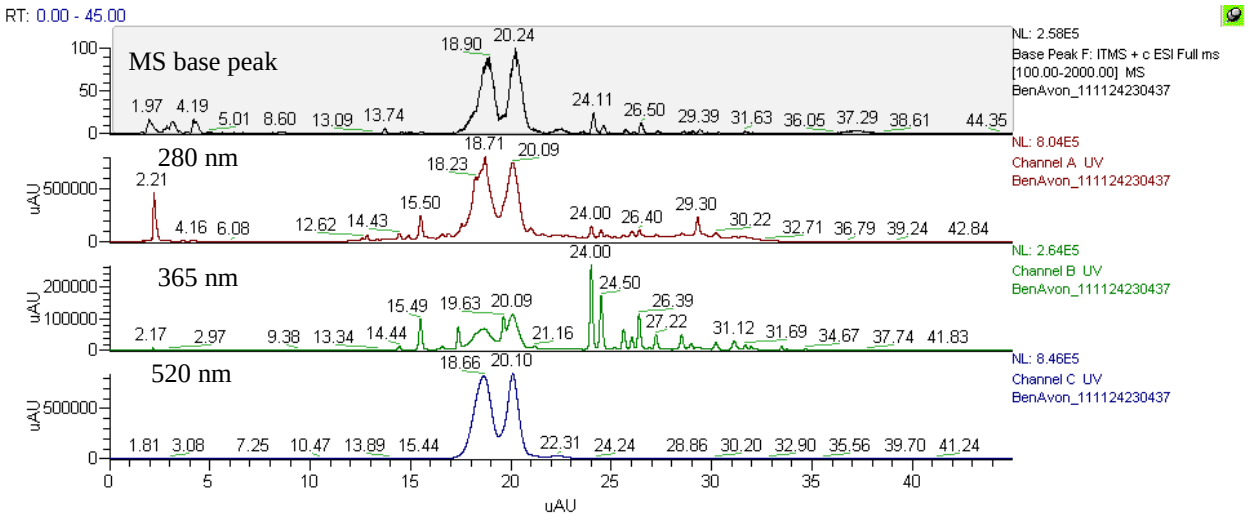
Black currant contains anthocyanins (analysed in positive ion mode) as major components together with significant levels of flavonols (negative and sometimes positive ion modes).

The following table gives a list of polyphenols found in black currant and expected masses.

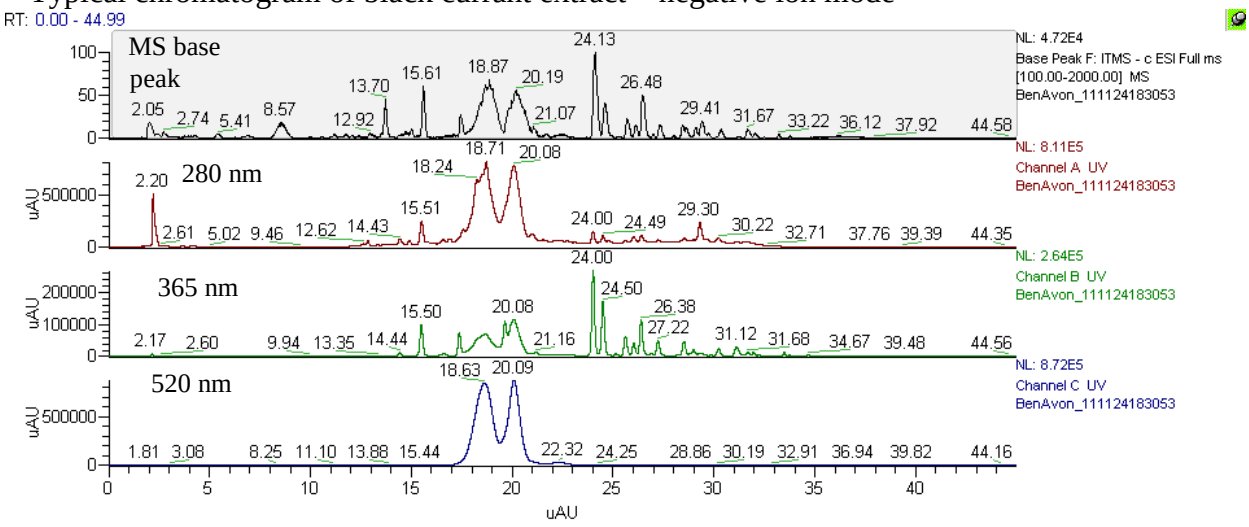
Compound	**MS**	**MS2**
Anthocyanins	**$[M+H]^+$**	
Delphinidin 3-galactoside	465	303
Delphinidin 3-glucoside	465	303
Delphinidin 3-rutinoside	611	465, 303
Cyanidin 3-glucoside	449	287
Cyanidin 3-rutinoside	595	449, 287
Petunidin 3-rutinoside	625	479, 317
Peonidin 3-galactoside	463	301
Malvidin 3-galactoside	493	331
Peonidin 3-glucoside	463	301
Peonidin 3-rutinoside	609	463, 301
Delphinidin 3-(6’’-coumaroyl)glucoside	611	303
Flavonols	**$[M-H]^-$**	
Myricetin 3-rutinoside	625	317
Myricetin 3-glucuronide	493	317
Myricetin 3-glucoside	479	317
Myricetin 3-(6’’-malonyl)glucoside	565	317
Aureusidin glucoside	447	285
Quercetin 3-rutinoside	609	463, 301
Quercetin 3-glucoside	463	301
Quercetin 3-(6’’-malonyl)glucoside	549	301
Kaempferol 3-rutinoside	593	285
Kaempferol 3-galactoside	447	285
Kaempferol 3-glucoside	447	285
Isorhamnetin 3-rutinoside	623	315

The following chromatograms show typical black currant (Ben Avon) profiles and identification of some major components.

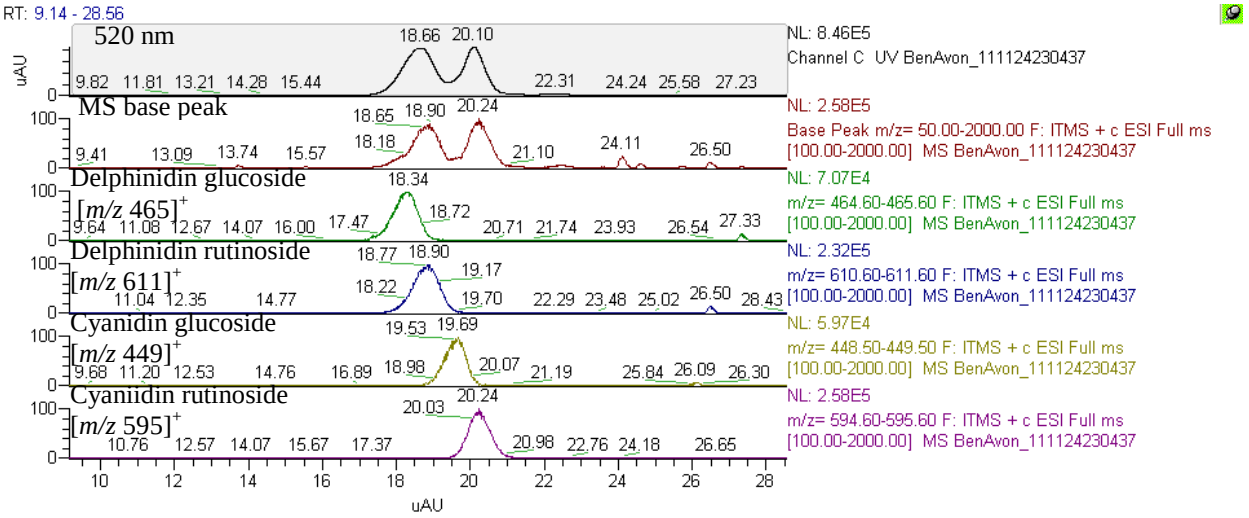
Typical chromatogram of black currant extract – positive ion mode



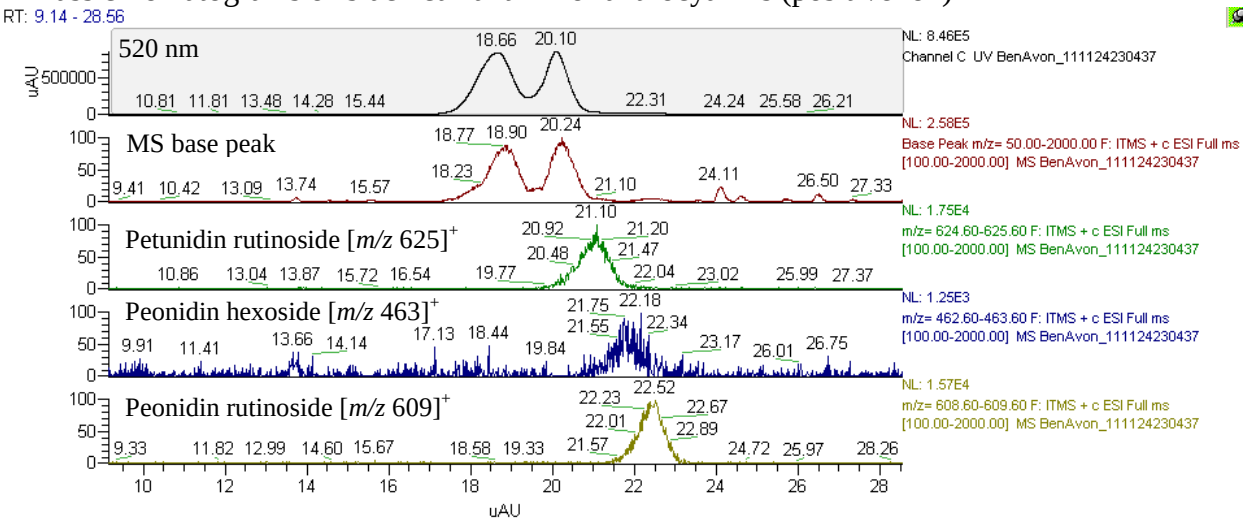
Typical chromatogram of black currant extract – negative ion mode



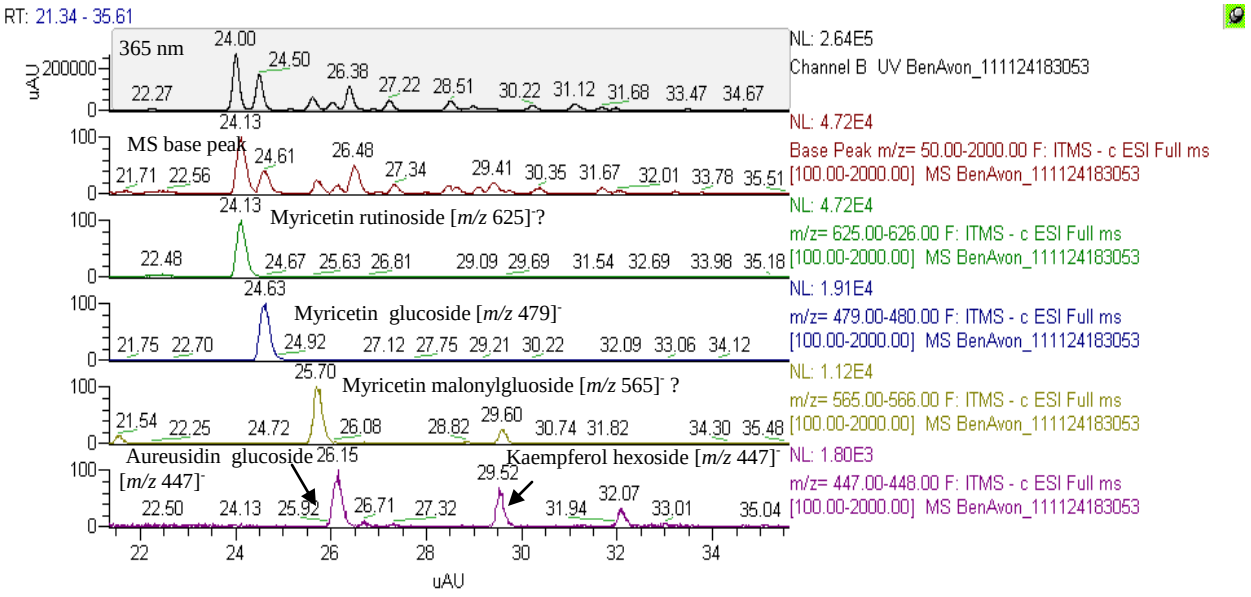
Mass chromatograms of black currant major anthocyanins (positive ion)

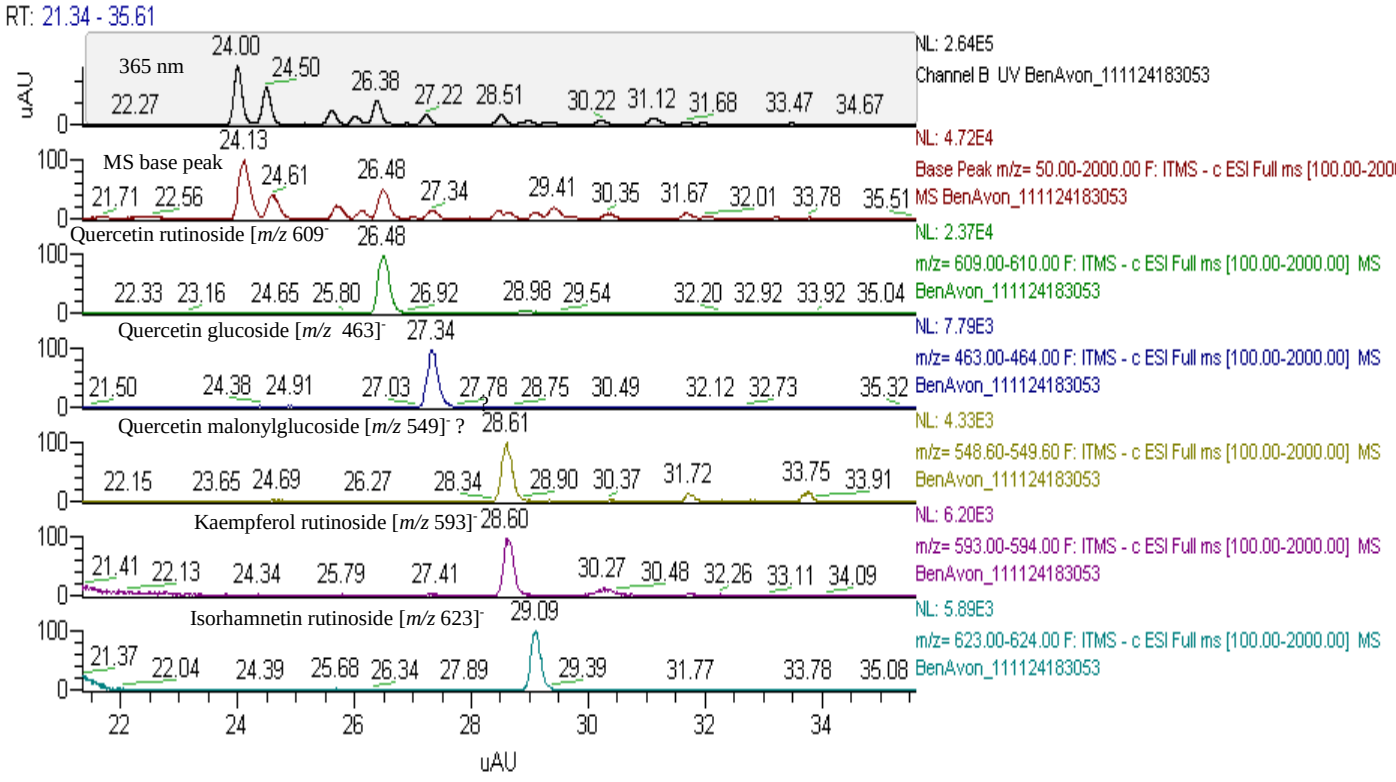


Mass chromatograms of black currant minor anthocyanins (positive ion)



Mass chromatograms of black currant flavonols (negative ion)





2.3.1.4 Blueberry polyphenols

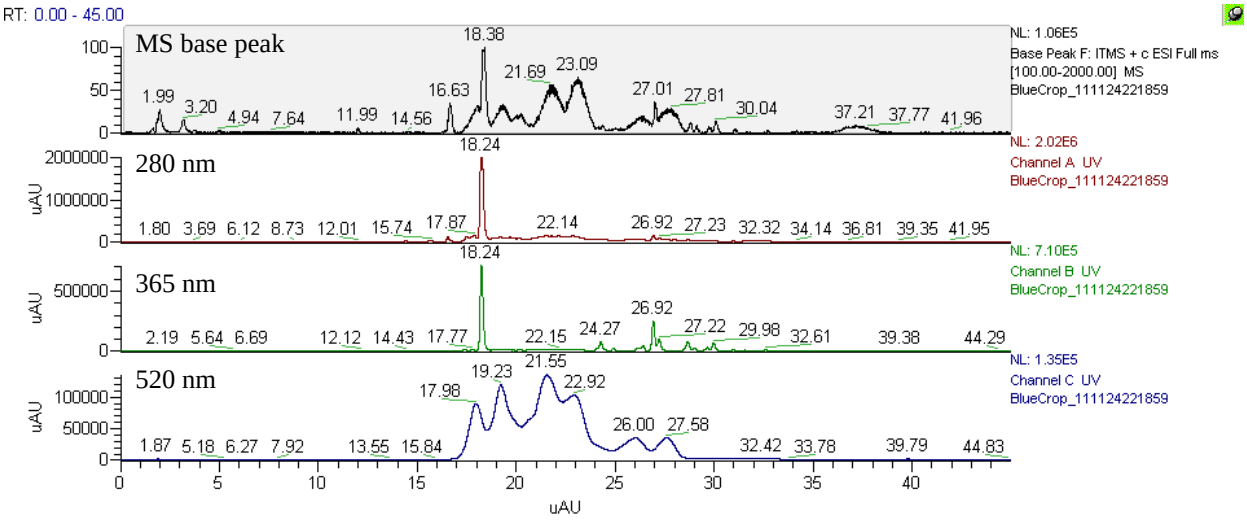
Blueberry contains a complex mixture of anthocyanins (analysed in positive ion mode) and less of flavonols (negative and sometimes positive ion modes).
The following table gives a list of polyphenols found in blueberry and expected masses.

Compound	**MS**	**MS2**
Anthocyanins	**$[M+H]^+$**	
Delphinidin 3-galactoside	465	303
Delphinidin 3-glucoside	465	303
Cyanidin 3-galactoside	449	287
Delphinidin 3-arabinoside	435	303
Cyanidin 3-glucoside	449	287
Petunidin 3-galactoside	479	317
Cyanidin 3-arabinoside	419	287
Petunidin 3-glucoside	479	317
Peonidin 3-galactoside	463	301
Petunidin 3-arabinoside	449	317
Peonidin 3-glucoside	463	301
Malvidin 3-galactoside	493	331
Malvidin 3-glucoside	493	331
Peonidin 3-arabinoside	433	301
Malvidin 3-arabinoside	463	331
Cyanidin 3-(malonyl)glucoside	535	287
Cyanidin 3-(6'-acetyl)galactoside	491	287
Petunidin pentoside	449	317
Delphinidin 3-(malonyl)glucoside	551	303
Malvidin 3-(malonyl)glucoside	579	331
Delphinidin 3-(6'-acetyl)glucoside	507	303
Peonidin 3-(6'-acetyl)galactoside	505	301
Cyanidin 3-(6'-acetyl)glucoside	491	287
Malvidin 3-(6'-acetyl)galactoside	535	331
Petunidin 3-(6'-acetyl)glucoside	521	317
Peonidin 3-(6'-acetyl)glucoside	505	301
Malvidin 3-(6'-acetyl)glucoside	535	331
Flavonols	**$[M-H]^-$**	
Myricetin 3-galactoside	479	317
Quercetin diglucoside	625	301
Quercetin 3-rutinoside	609	463, 301
Quercetin 3-galactoside	463	301
Quercetin 3-methoxyhexoside	493	463, 301
Quercetin 3-glucoside	463	301
Quercetin 3-arabinoside	433	301
Quercetin glucuronide	477	301
Quercetin 3-glucosylpentoside	595	433, 301
Quercetin 3-caffeoylgalactoside	625	463, 301
Quercetin 3-caffeoylglucoside	625	463, 301
Quercetin 3-oxalylpentoside	505	433, 301
Quercetin 3-rhamnoside	447	301
Quercetin 3-dimethoxyrhamnoside	507	477, 447, 301
Quercetin 3-(6'-acetyl)galactoside	505	463, 301
Quercetin 3-(6'-acetyl)glucoside	505	463, 301

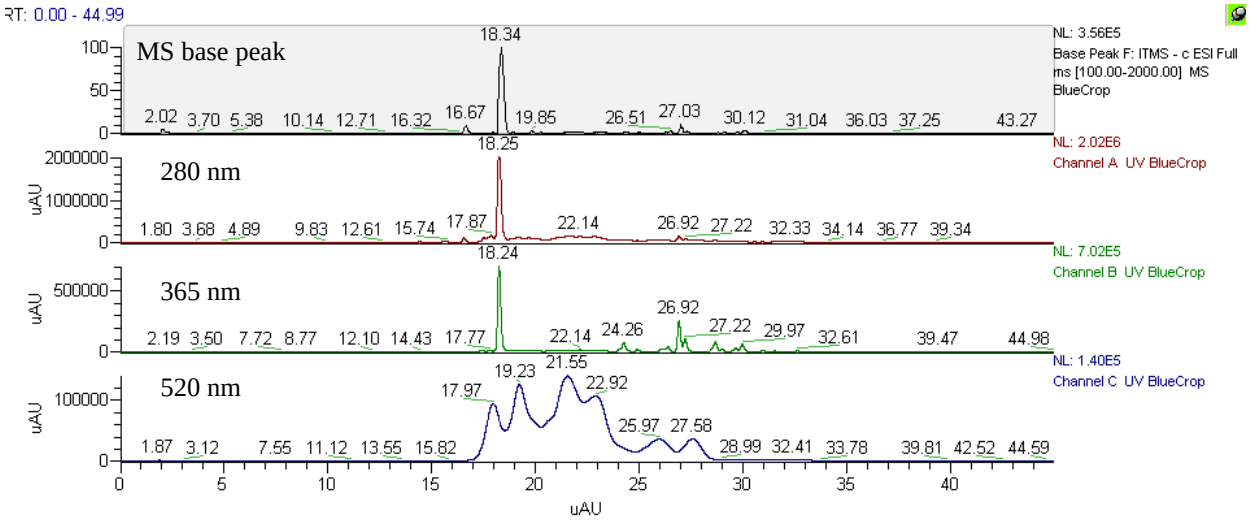
Quercetin	301	

The following chromatograms show typical blueberry (Blue Crop) profiles and identification of some major components.

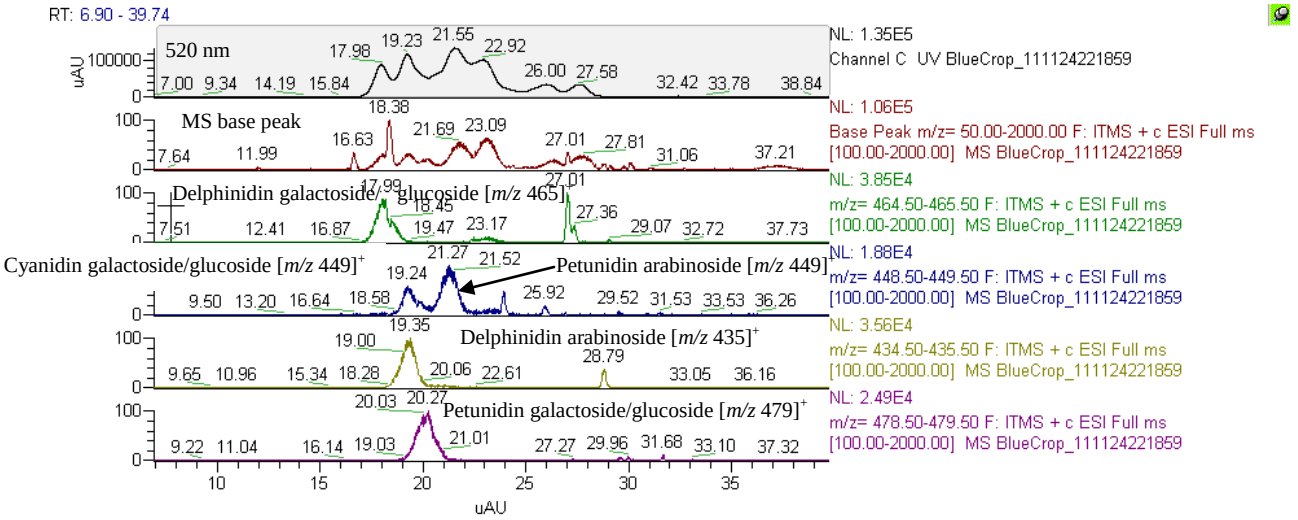
Typical chromatogram of blueberry extract – positive ion mode

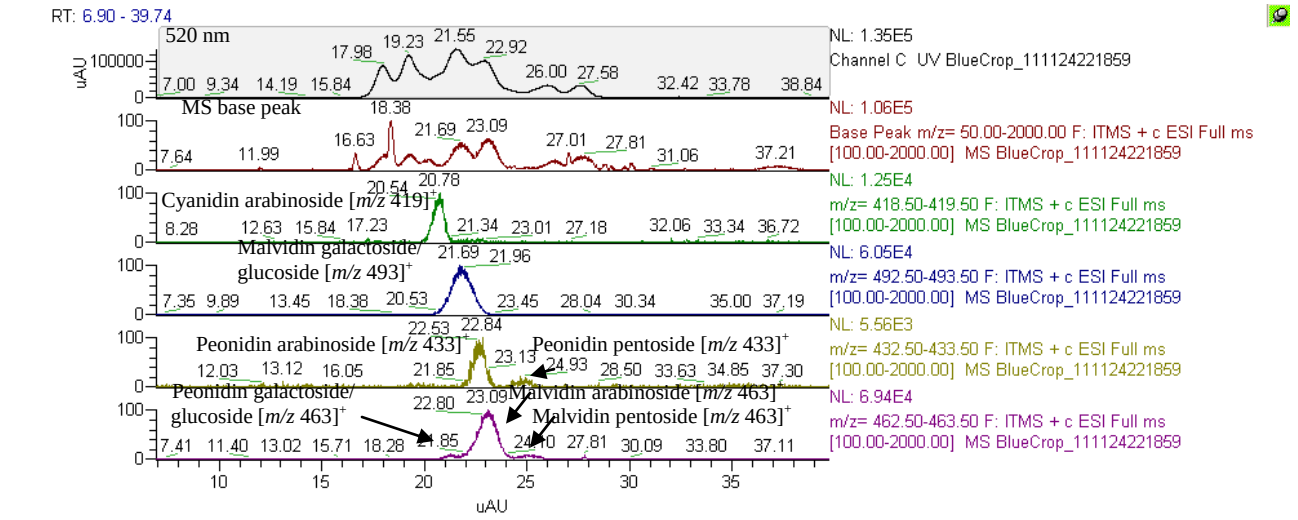


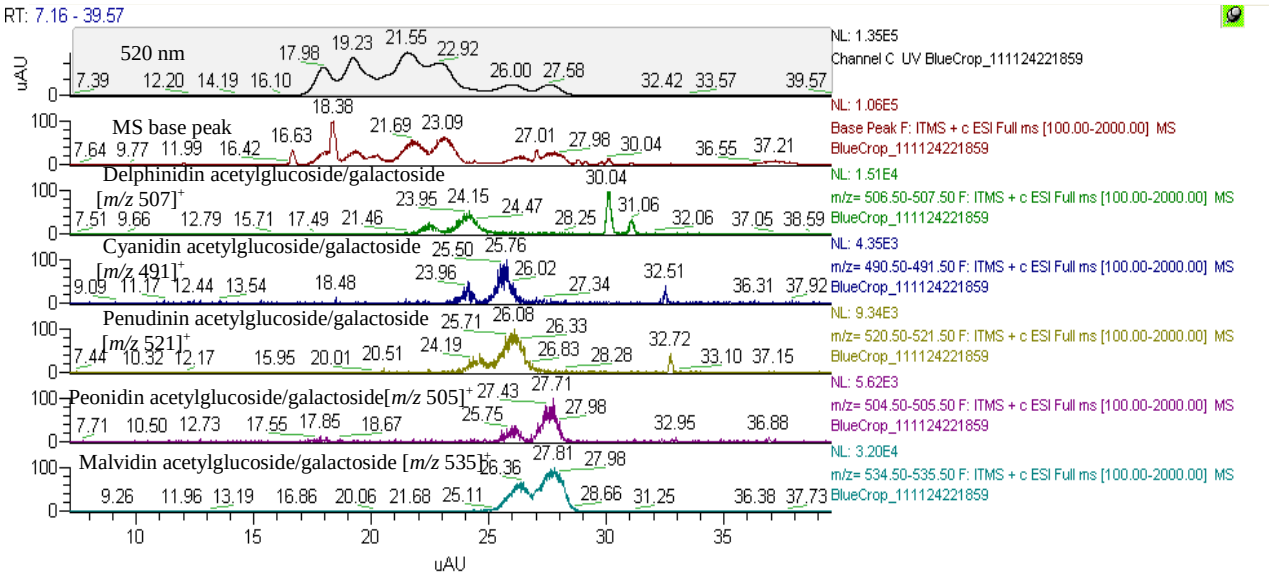
Typical chromatogram of blueberry extract – negative ion mode



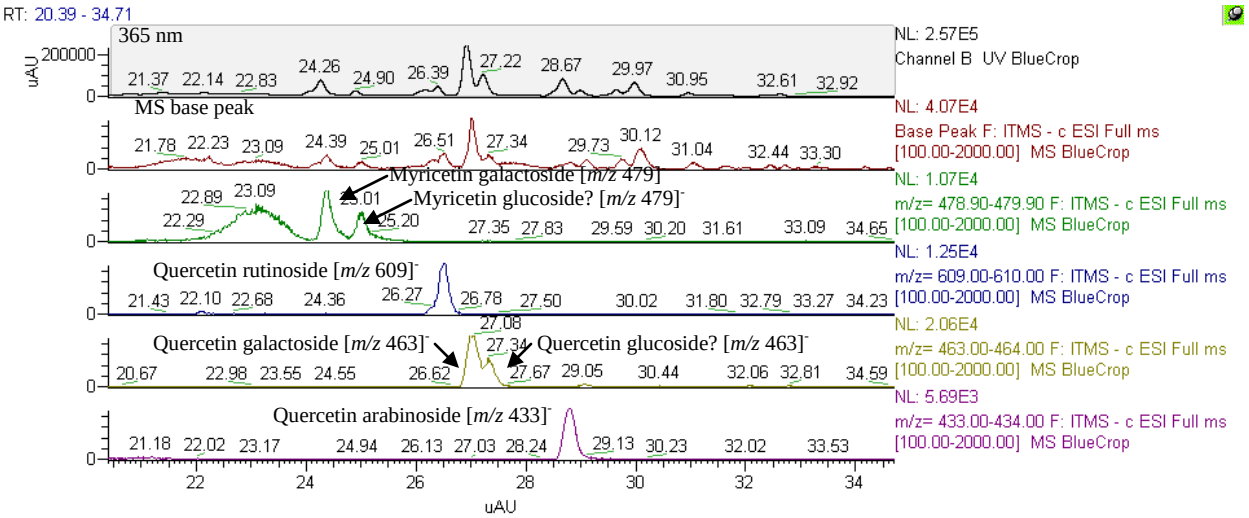
Mass chromatograms of blueberry anthocyanins (positive ion)







Mass chromatograms of blueberry flavonols (negative ion)



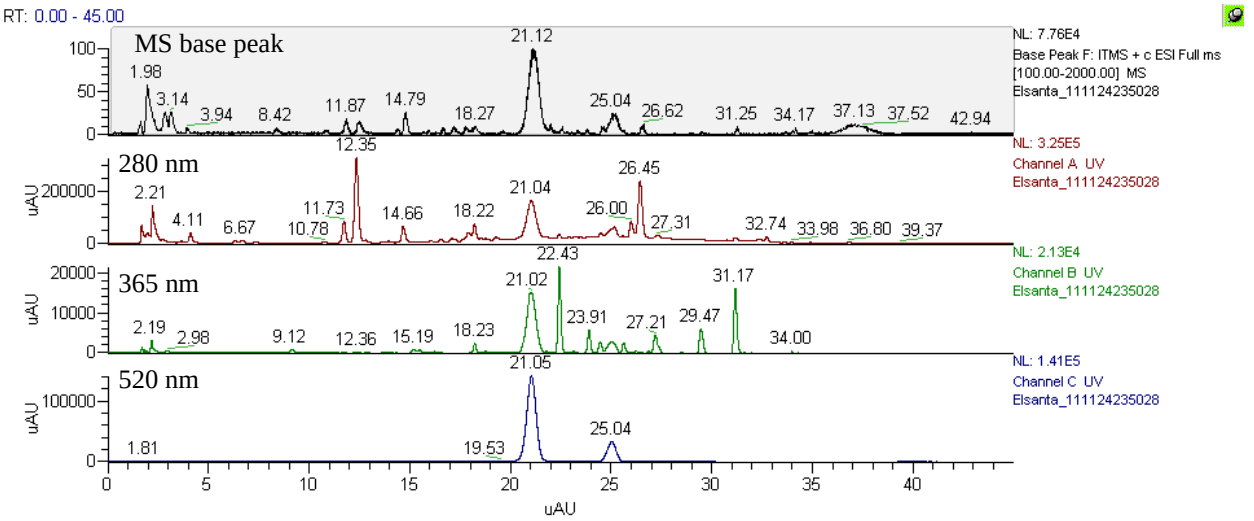
2.3.1.5 Strawberry polyphenols

Strawberry contains large amounts of proanthocyanidins and less anthocyanins (analysed in positive ion mode) and ellagitannins (negative ion mode). Flavonols (negative and sometimes positive ion modes) are minor components.
The following table gives a list of polyphenols found in strawberry and expected masses.

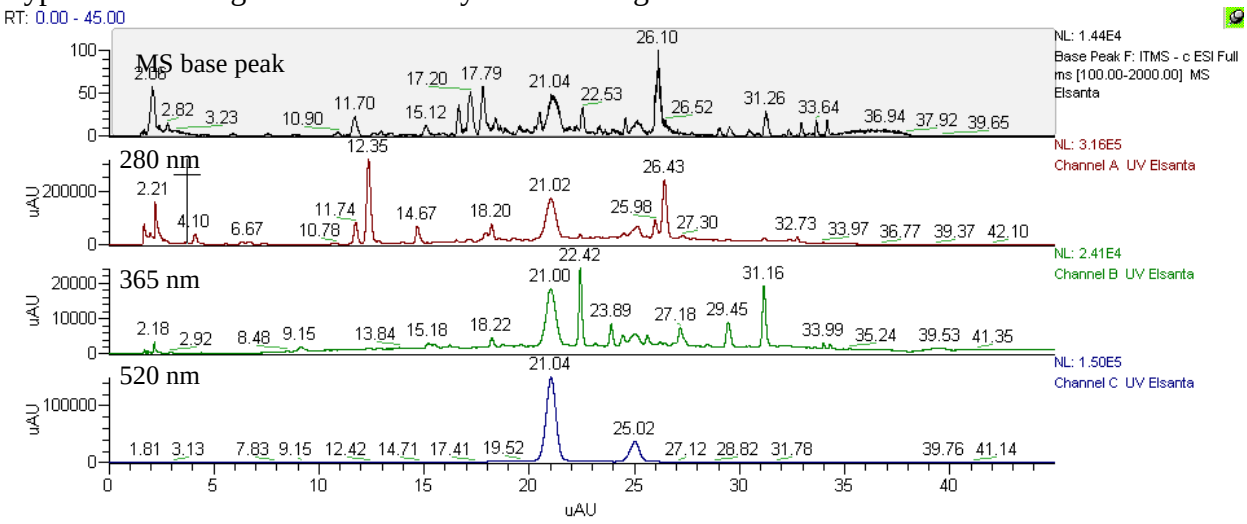
Compound	**MS**	**MS2**
Anthocyanins	**$[M+H]^+$**	
Cyanidin 3-sophoroside	611	287
Cyanidin 3-glucoside	449	287
Cyanidin 3-rutinoside	595	449, 287
Pelargonidin 3-glucoside	433	271
Petunidin 3-glucoside	479	317
Pelargonidin 3-rutinoside	579	433, 271
Pelargonidin 3-(malonyl)glucoside	519	433, 271
Pelargonidin 3-(6'-acetyl)glucoside	475	271
Flavonol	**$[M-H]^-$**	
Quercetin 3-glucuronide	477	301
Kaempferol 3-coumaroylglucoside	593	447, 285
Kaempferol 3-glucoside	447	285
Kaempferol 3-acetylglucoside	489	285
Kaempferol 3-glucuronide	461	285
Ellagitannins/ellagic acid derivatives	$[M-H]^-$	
Bis-HDDP-glucoside	783	301
Galloyl bis-HHDP-glucoside 1	935	633, 301
Galloyl bis-HHDP-glucoside 2	935	633, 301
Sanguiin-H6	$[1869]^{-1}$ $[935]^{-2}$	1697, 1567, 1407, 897, 783, 633, 301
Lambertianin C	$[1401]^{-2}$	2019, 1869, 1567, 1402, 935, 897, 633, 301
Ellagic rhamnoside 1	447	301
Ellagic rhamnoside 2	447	301
Flavan-3-ols/proanthocyanidins	**$[M-H]^-$**	
Catechin	289	
Dimer (Cat-Cat)	577	
Dimer (Cat-Afz)	561	
Trimer (Cat-Cat-Cat)	865	
Trimer (Cat-Cat-Afz)	849	
Trimer Cat-Afz-Afz)	833	
Tetramer (Cat-Cat-Cat-Cat)	1153	
Tetramer (Cat-Cat-Cat-Afz)	1137	

The following chromatograms show typical strawberry (Elsanta) profiles and identification of some major components.

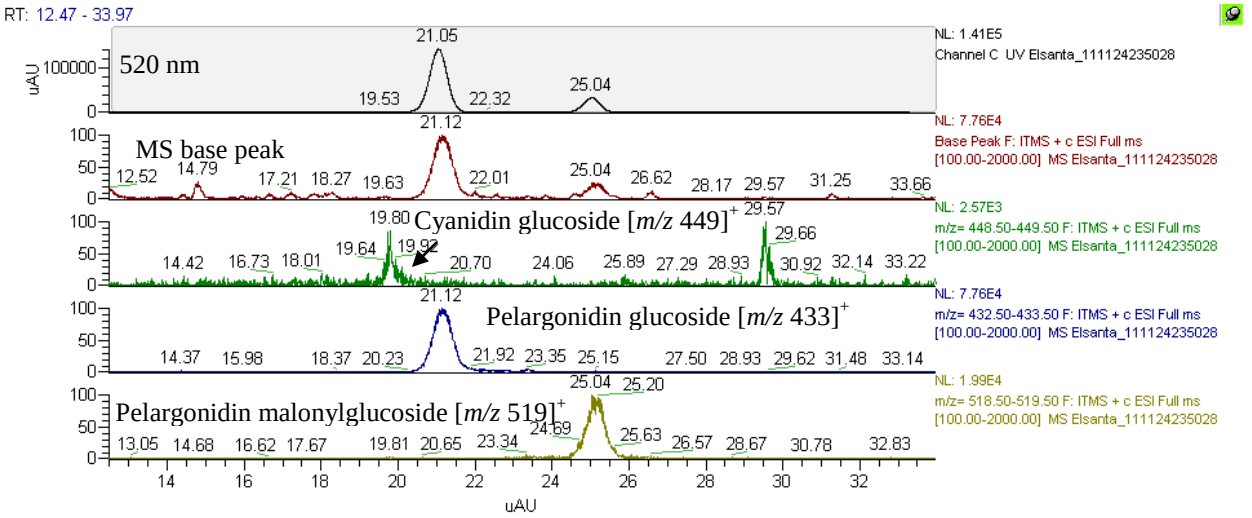
Typical chromatogram of strawberry extract – positive ion mode



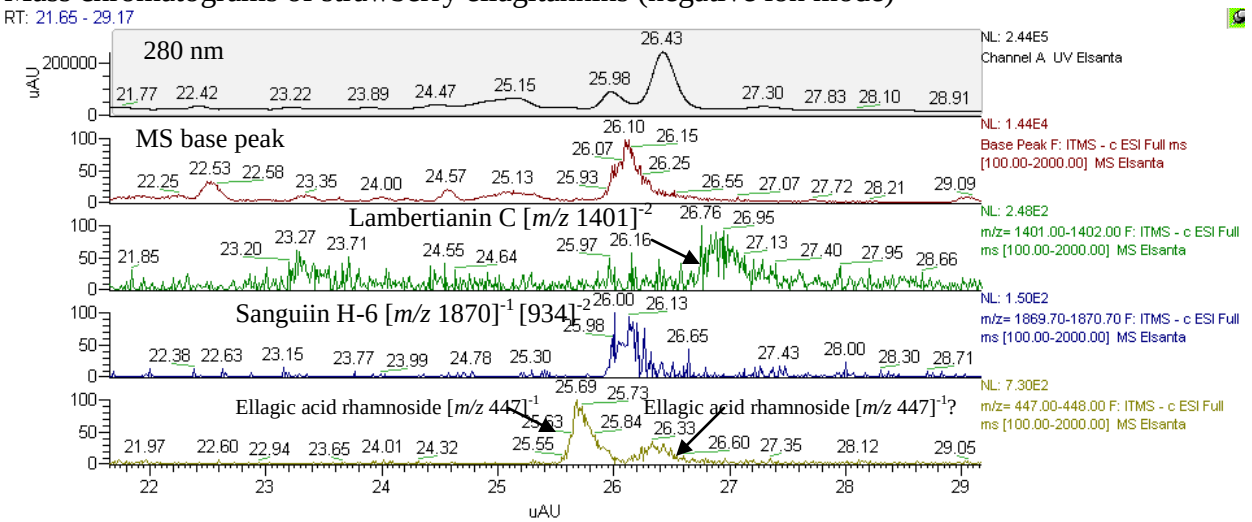
Typical chromatogram of strawberry extract – negative ion mode



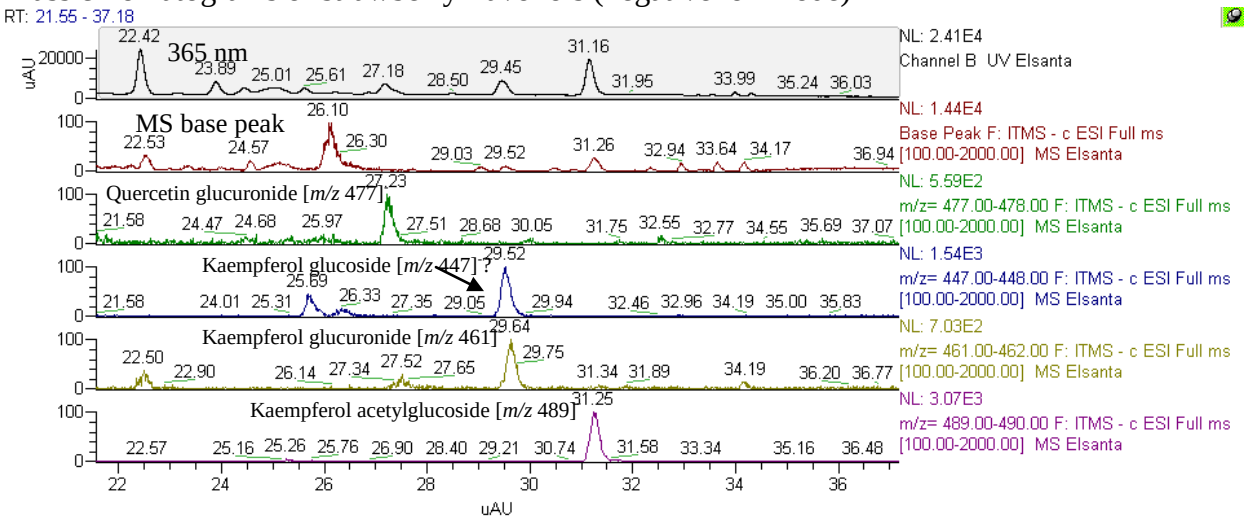
Mass chromatograms of strawberry anthocyanins (positive ion)



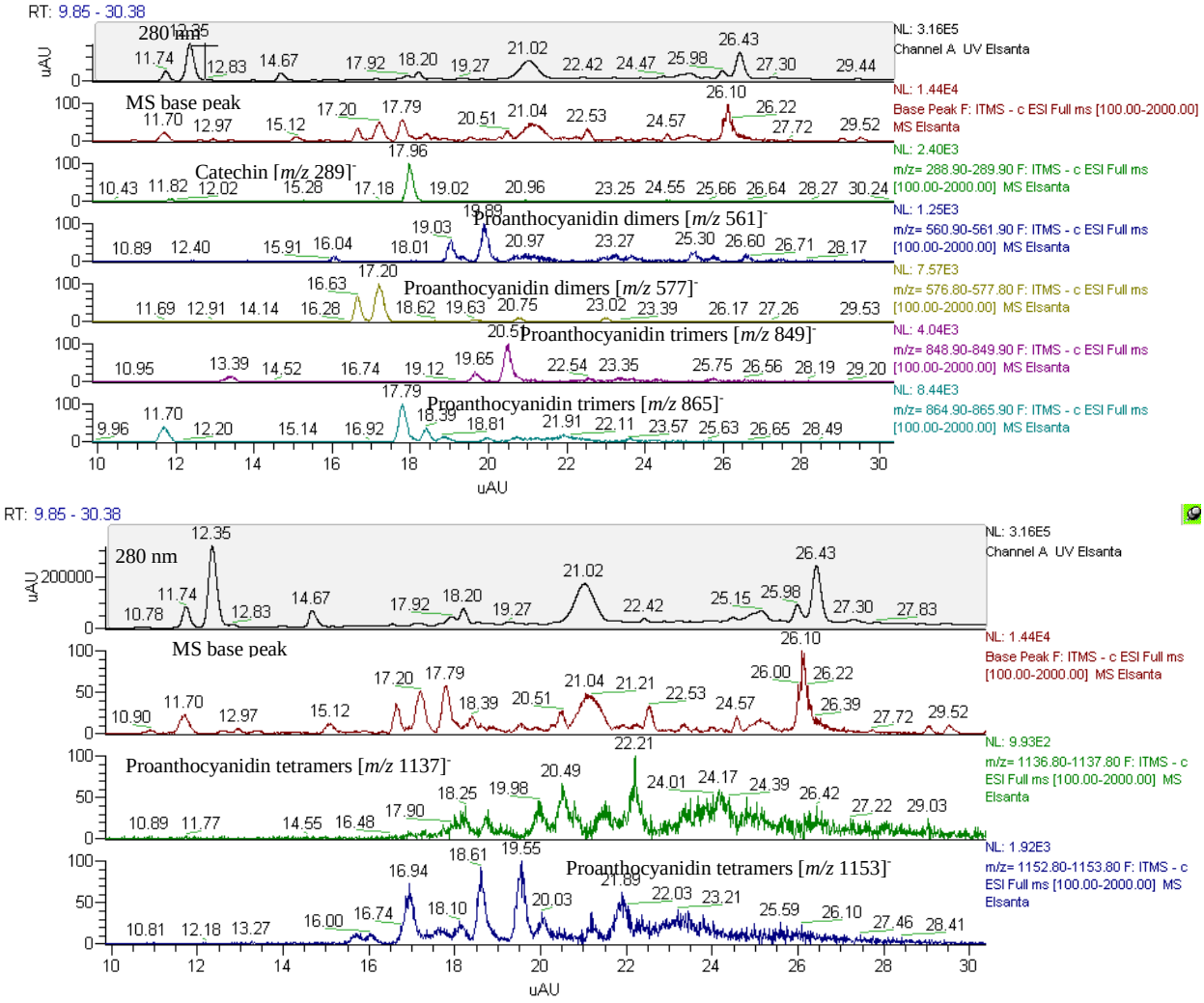
Mass chromatograms of strawberry ellagitannins (negative ion mode)



Mass chromatograms of strawberry flavonols (negative ion mode)



Mass chromatograms of strawberry flavan-3-ols (negative ion mode)



2.3.2 Quantification

For quantification of compounds, morin is added to the juice sample at a concentration of 0.1 mg mL^{-1} (add 100 µl of 1 mg mL^{-1} morin to 900 µl juice). Suitable external standards similar to the compounds of interest are prepared to cover the concentrations in the juice. Morin is added to each standard at the same concentration (0.1 mg mL^{-1}) as in the juice and the standards are analysed together with samples. Samples and standards are processed so that the ratio of the area of a specific ion of the compound of interest to that for morin internal standard (*m*/*z* 301 and 303 for negative and positive mode, respectively) is calculated. In Excel, calibration curves of response ratios against concentration are plotted for the standards and best-fit equations are used to calculate the concentrations in the juices.

Analysis of Polyphenolics in Raspberry, Blackberry, Blueberry, Black Currant and Strawberry by Ultra High Pressure Liquid Chromatography-Photodiode Array-Mass Spectrometry (UPLC-PDA-MS)

Introduction

1.1 Purpose

To describe the procedure for aanlysing the polyphenolic compounds from raspberry, blackberry, blueberry, black currant or strawberry by ultra high pressure liquid chromatography-mass spectrometry (UPLC-PDA-MS). This SOP is to be used for training of new and substitute staff, for auditing purposes and for general reference.

1.2 Scope

The method can be used for fresh, frozen or freeze-dried raspberry, blackberry, blueberry, black currant or strawberry.

1.3 Overview

The method is for analysing the polyphenolics from raspberry, blackberry, blueberry, black currant or strawberry by UPLC-PDA-MS. Following extraction the solvent is removed by Speed Vac and then freeze-dried to remove any remaining water. The sample is re-dissolved in 0.5% formic acid in 5% aqueous acetonitrile and is analysed by reversed phase UPLC-PDA-MS using a mobile phase of 0.1% formic acid in water with increasing proportions of acetonitrile.

2. Methods

2.1 Inputs to process

2.1.1 Apparatus and reagents required

Apparatus
Balance (3 place or better)
Microcentrifuge (Eppendorf 5415D or equivalent)
Borosilicate glass mortars (homogenisers), 5 mL capacity
1.5ml and 2ml microfuge tubes
8ml glass vials
96 well microfuge racks
Scalpel
Freeze-drier
Speed vac SPD131DA (Savant) or equivalent, with refridgerated vapour trap and pump
Eppendorf P1000 pipette or equivalent

Handystep electronic pipette (Brand)
Single StEP^{TM} filter vial 0.45 μm PTFE (Thomson)
Thermo LCQ fleet ion trap mass spectrometer with Accela 600 pump, autosampler and PDA detector or equivalent
Hypersil Gold (50 mm x 2.1 mm; 1.9 □m reversed-phase UPLC column (Thermo)

Chemicals
Acetonitrile
Formic acid
Double distilled water

2.2 Procedures

2.2.1 Sample preparation

2.2.1.1 Berries are extracted with 0.5% formic acid in acetonitrile to give a "50% juice" as described in SOP Extraction of Polphenolics from Berries.
2.2.1.2 Store samples in -20°C freezer until ready to be taken to dryness.
2.2.1.3 Thaw samples in cold room (4°C) and leave on Speed Vac at 45°C for 3 h. Freeze samples in -20°C freezer.
2.2.1.4 Freeze-dry samples overnight using side arm of freeze-drier and keep in -20°C freezer.

2.2.2 Analysis of polyphenolics by UPLC-PDA-MS

2.2.2.1 Prior to use, dissolve sample in 0.5% formic acid in water/acetonitrile (95:5, vol/vol) in same volume (usually 500 ul) as prior to freeze-drying, vortex to disrupt pellet and centrifuge at 13200 rpm for 3 min. Transfer 0.4 mL sample to filter vial and push filter into vial.
2.2.2.2 The sample is injected via an autosampler on to a UPLC column linked to a PDA system (Thermo Accela) and then an electrospray ionization ion trap mass spectrometer (Thermo LCQ Fleet). The important parameters for the autosampler, mobile phase, PDA and mass spectrometer are as follows:-

Injection volume: 8 μl.

Column type: Hypersil Gold (50 mm x 2.1 mm; 1.9 □m) (Thermo)

Autosampler temperature: 6°C

Column temperature: 30°C.

Mobile phase: Solvent A – 0.1% (vol/vol) aqueous formic acid
Solvent B – 0.1% (vol/vol) formic acid in acetonitrile/water (50:50, vol/vol).

Flow rate: 450 μl min^{-1}

Gradient :

Time (min)	A%	B%
0	97	3
3	85	15
7	75	25
10	50	50
13	50	50
14	100	0
16	100	0

PDA range: 200-600 nm.

PDA channels: A. 280 nm (general polyphenols).
B. 365 nm (flavonols).
C 520 nm (anthocyanins).

Mass range: *m/z* 100-2000.

Source parameters: Capillary temperature 300°C, sheath gas and auxillary gas 35 and 5 units and spray voltage 3.8 kV

Scan events: Alternative full scan MS and MS/MS (data-dependant scan).

The sample is run twice – in positive ion and negative ion modes.

2.3 Polyphenol composition

2.3.1 Identification

Identification of polyphenols is based on PDA characteristics and mass spectra including MS and MS/MS data.

2.3.1.1 Raspberry polyphenols

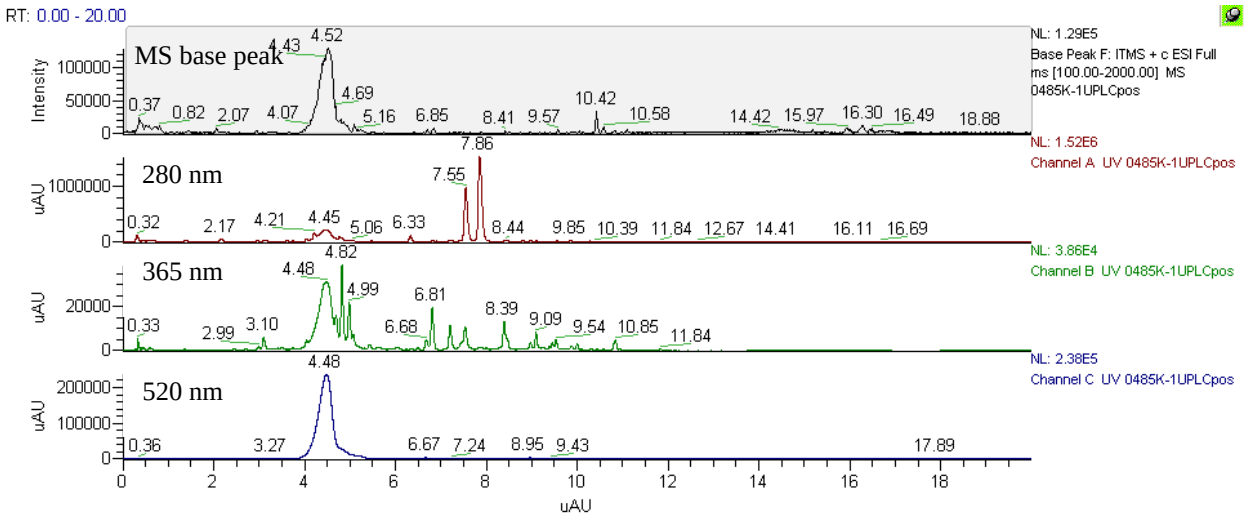
Raspberry contains mainly anthocyanins (analysed in positive ion mode) and ellagitannins (negative ion mode) with flavonols (negative and sometimes positive ion modes) as minor compounds.
The following table gives a list of polyphenols found in raspberry and expected masses.

Compound	**MS**	**MS2**
Anthocyanins	**$[M+H]^+$**	
Cyanidin 3-sophoroside	611	287
Cyanidin 3,5-diglucoside	611	449, 287
Cyanidin 3-(2'-glucosyl)rutinoside	757	287

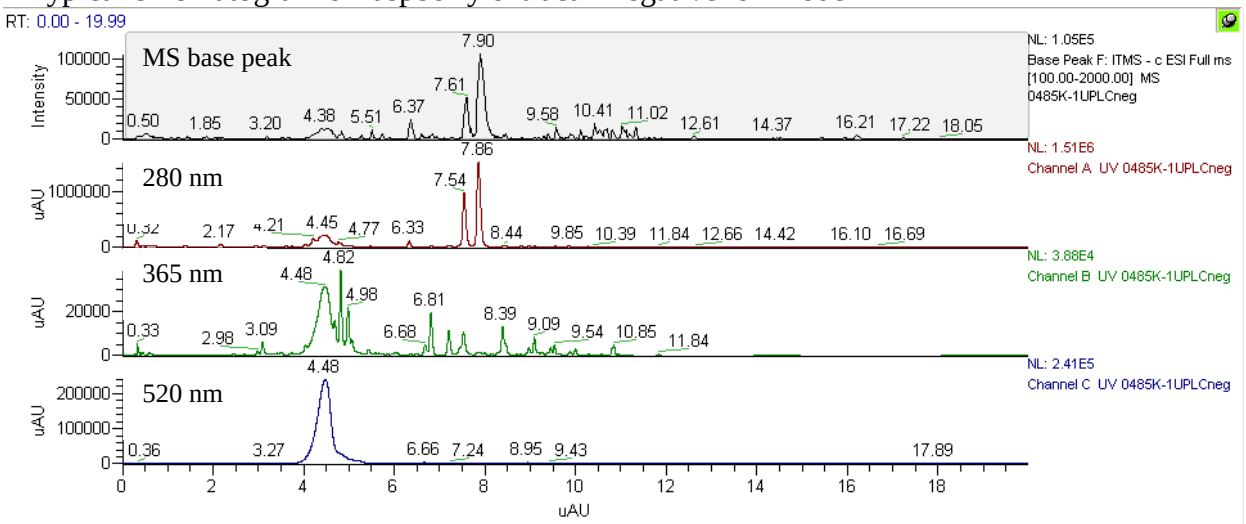
Cyanidin 3-sophoroside-5-rhamnoside	757	611, 433, 287
Cyanidin 3-sambubioside	581	287
Cyanidin 3-(2'-xylosyl)rutinoside	727	287
Cyanidin 3-glucoside	449	287
Cyanidin 3-sambubioside-5-rhamnoside	727	581, 433, 287
Cyanidin 3-rutinoside	595	449, 287
Pelargonidin 3-sophoroside	595	271
Pelargonidin 3-(2'-glucosyl)rutinoside	741	271
Pelargonidin 3-sambubioside	565	271
Pelargonidin 3-glucoside	433	271
Pelargonidin 3-rutinoside	579	433, 271
Flavonols	**$[M-H]^-$**	
Quercetin galactosylrhamnoside/rutinoside	609	301
Quercetin (2'-glucosyl)rutinoside	771	301
Quercetin galactoside	463	301
Quercetin glucoside	463	301
Quercetin glucuronide	477	301
Quercetin diglucoside	625	301
Kaempferol glucuronide	461	285
Methylquercetin glucuronide	491	315
Ellagitannins/ellagic acid derivatives	**$[M-H]^-$**	
Sanguiin H-2	$[1103]^{-1}$ $[551]^{-2}$	301
Sanguiin-H6	$[1869]^{-1}$ $[935]^{-2}$	1697, 1567, 1407,897, 783, 633, 301
Sanguiin-H10 isomers	$[1567]^{-1}$ $[783]^{-2}$	935, 633, 301
Lambertianin C	$[1401]^{-2}$	2019, 1869, 1567, 1402, 935, 897, 633, 301
Ellagic acid pentoside 1	433	301
Ellagic acid pentoside 2	433	301
Ellagic acid acetyl arabinoside	475	301
Ellagic acid 4-acetyl xyloside	475	301
Ellagic acid	301	
Methyl ellagic acid pentoside	447	315

The following chromatograms show typical raspberry (cultivar 0485K-1) profiles and identification of some major components.

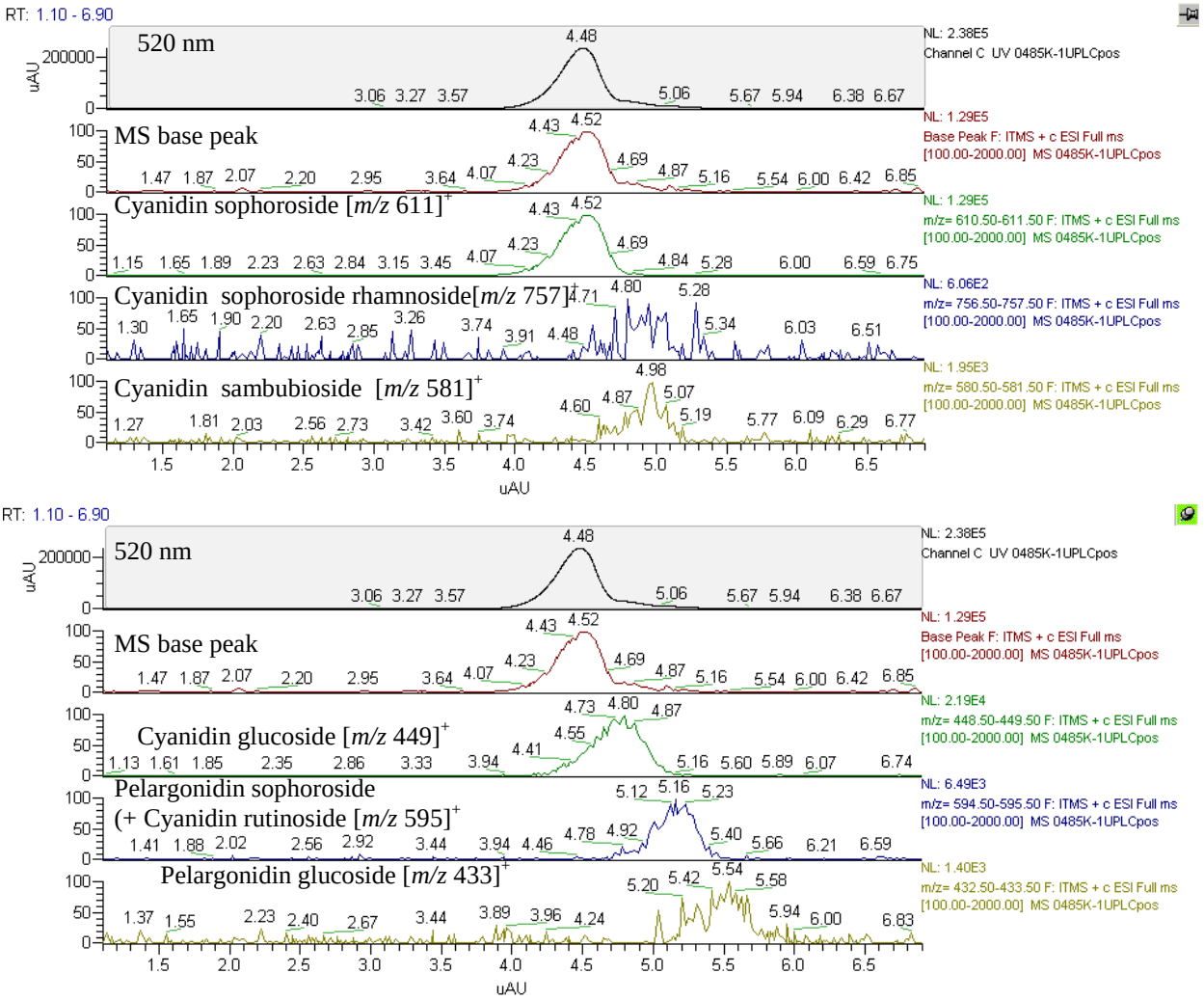
Typical chromatogram of raspberry extract – positive ion mode



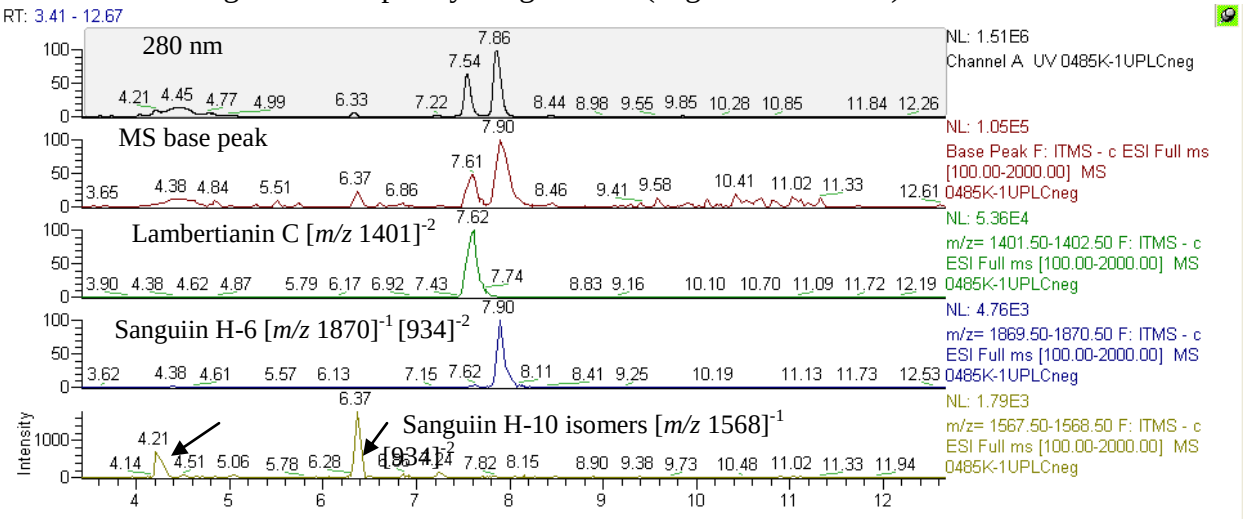
Typical chromatogram of raspberry extract – negative ion mode



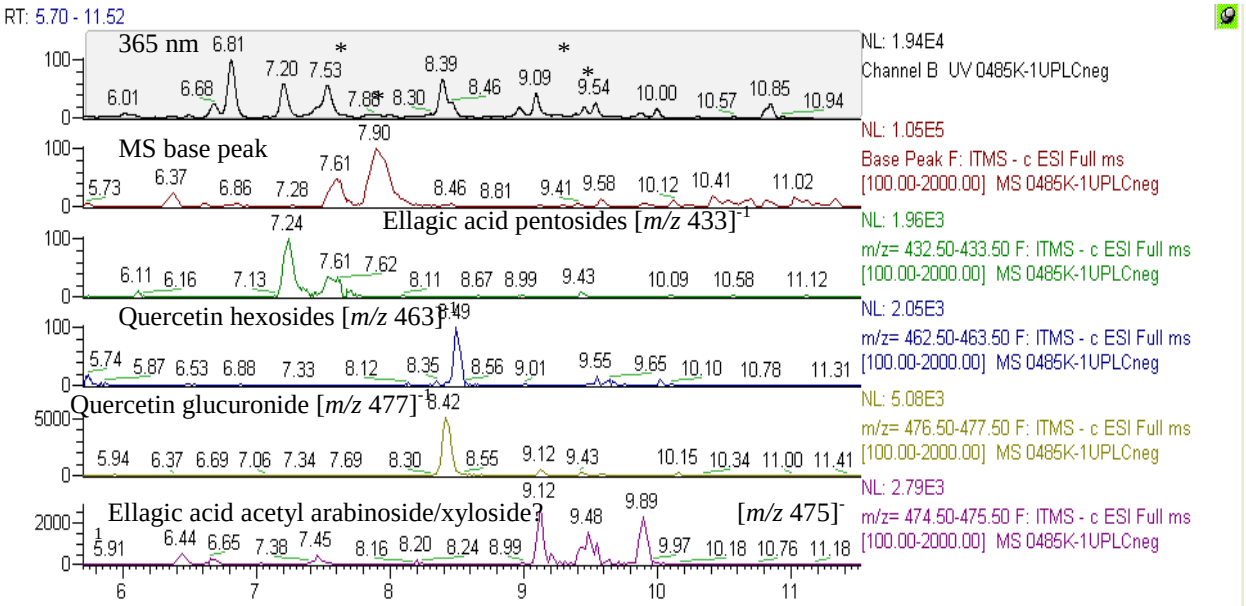
Mass chromatograms of raspberry anthocyanins (positive ion mode)



Mass chromatograms of raspberry ellagitannins (negative ion mode)



Mass chromatograms of raspberry flavonols/ellagic acid derivatives (negative ion mode)



2.3.1.2 Blackberry polyphenols

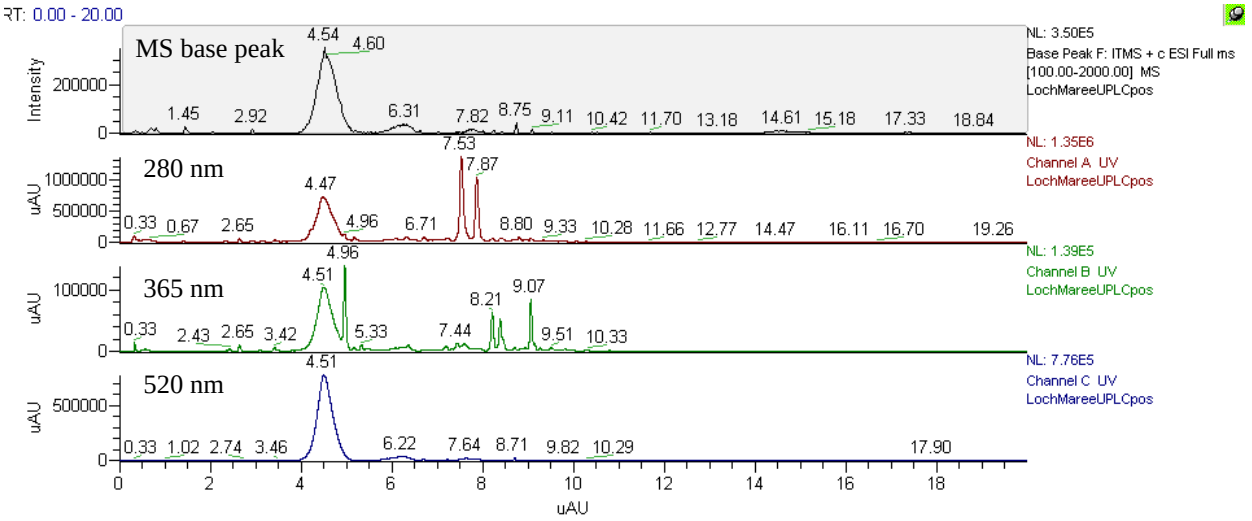
Blackberry contains mainly anthocyanins (analysed in positive ion mode) and ellagitannins (negative ion mode) with flavonols (negative and sometimes positive ion modes) as minor compounds.

The following table gives a list of polyphenols found in blackberry and expected masses.

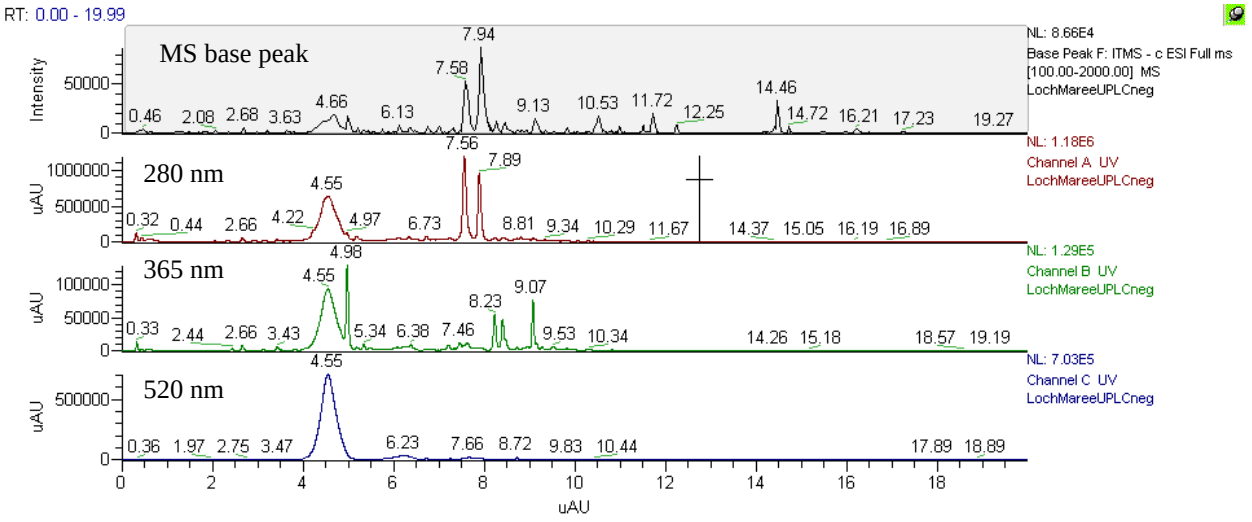
Compound	**MS**	**MS2**
Anthocyanins	**$[M+H]^+$**	
Cyanidin 3-glucoside	449	287
Cyanidin 3-arabinoside	419	287
Cyanidin 3-rutinoside	595	449, 287
Pelargonidin 3-glucoside	433	271
Cyanidin 3-(3'-malonyl)glucoside	535	287
Peonidin 3-glucoside	463	301
Cyanidin 3-xyloside	419	287
Cyanidin 3-(6'-malonyl)glucoside	535	449, 287
Cyanidin 3-dioxalylglucoside	593	287
Cyanidin 3-[6''-(3-hydroxy-3-methylglutaroyl) galactoside	593	287
Flavonols	**$[M-H]^-$**	
Quercetin 3-rutinoside	609	463, 301
Quercetin 3-galactoside	463	301
Quercetin 3-methoxyhexoside	493	463, 301
Quercetin 3-glucoside	463	301
Quercetin 3-pentoside 1	433	301
Quercetin 3-pentoside 2	433	301
Quercetin glucuronide	477	301
Quercetin 3-[6''-(3-hydroxy-3-methylglutaroyl) galactoside	607	463, 301
Quercetin acetylhexoside	505	301
Quercetin 3-glucosylpentoside	595	433, 301
Quercetin 3-oxalylpentoside	505	433, 301
Quercetin	301	
Kaempferol hexoside	447	285
Kaempferol 3-[6''-(3-hydroxy-3-methylglutaroyl) galactoside	591	285, 447
Ellagitannins/ellagic acid derivatives	**$[M-H]^-$**	
Sanguiin H-2	$[110]^{-1}$ $[551]^{-2}$	301
Sanguiin-H6	$[1869]^{-1}$ $[935]^{-2}$	1697, 1567, 1407,897, 783, 633, 301
Sanguiin-H10 isomers	$[1567]^{-1}$ $[783]^{-2}$	935, 633, 301
Lambertianin C	$[1401]^{-2}$	2019, 1869, 1567, 1402, 935, 897, 633, 301
Ellagic acid	301	
Methyl ellagic acid glucuronide	491	315
Methyl ellagic acid pentoside	447	315

The following chromatograms show typical blackberry (cultivar Loch Maree) profiles and identification of some major components.

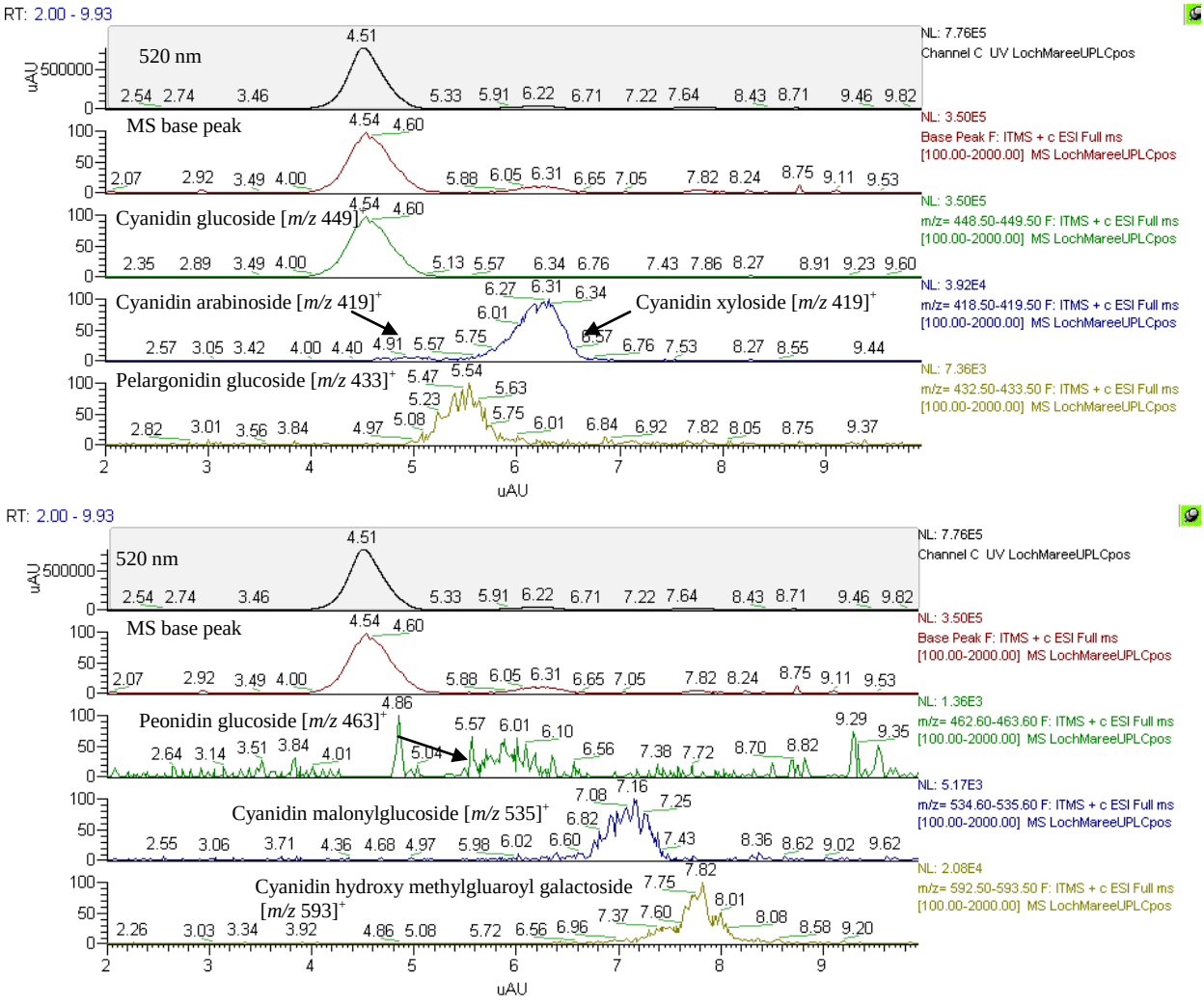
Typical chromatogram of blackberry extract – positive ion mode



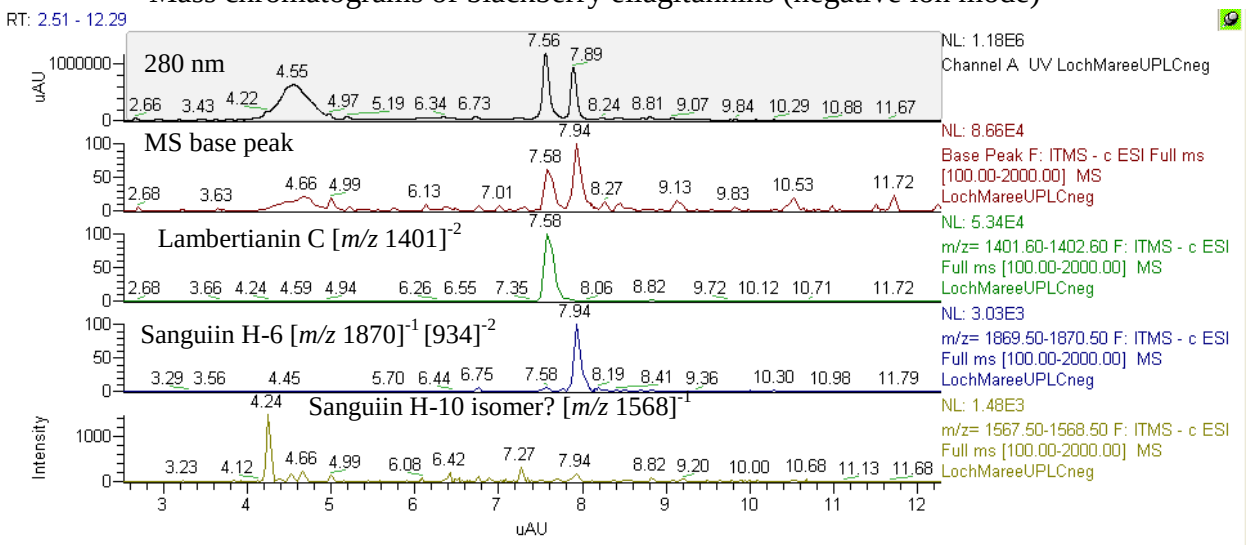
Typical chromatogram of blackberry extract – negative ion mode



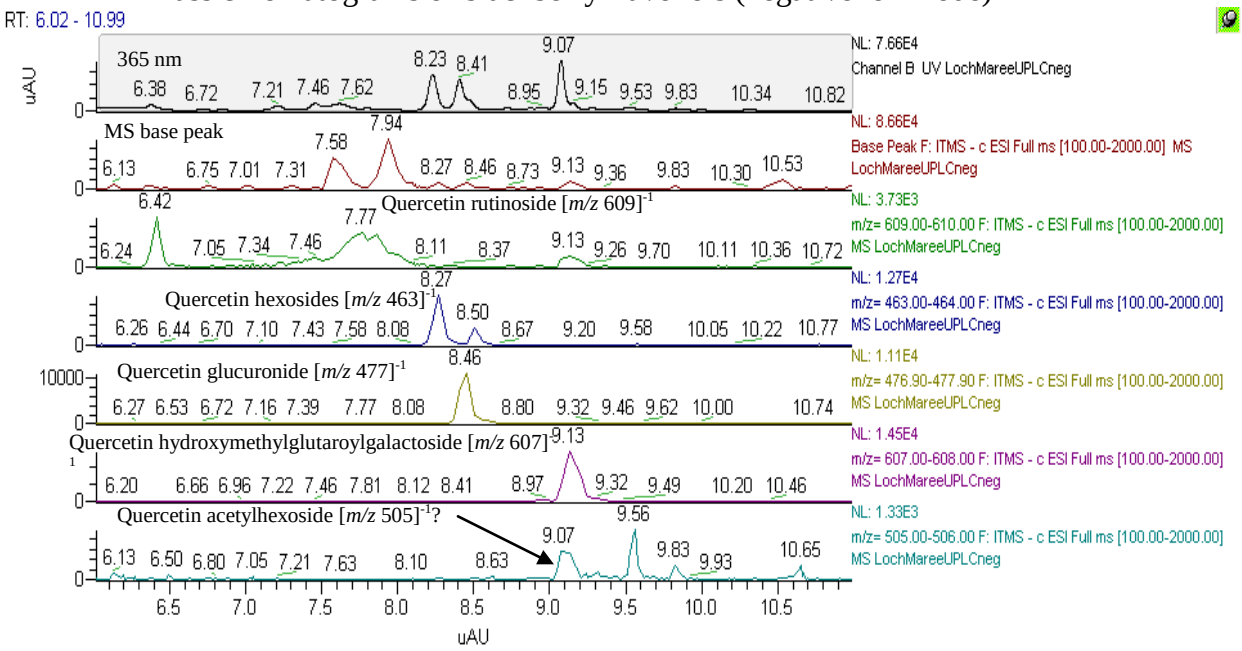
Mass chromatograms of blackberry anthocyanins (positive ion)



Mass chromatograms of blackberry ellagitannins (negative ion mode)



Mass chromatograms of blackberry flavonols (negative ion mode)



2.3.1.3 Black currant polyphenols

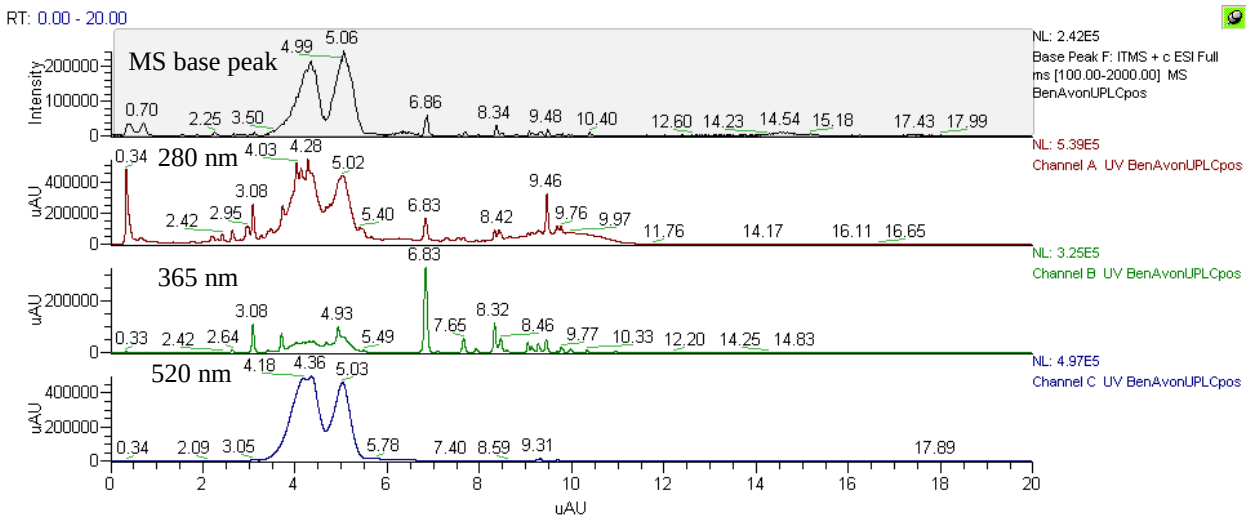
Black currant contains anthocyanins (analysed in positive ion mode) as major components together with significant levels of flavonols (negative and sometimes positive ion modes).

The following table gives a list of polyphenols found in black currant and expected masses.

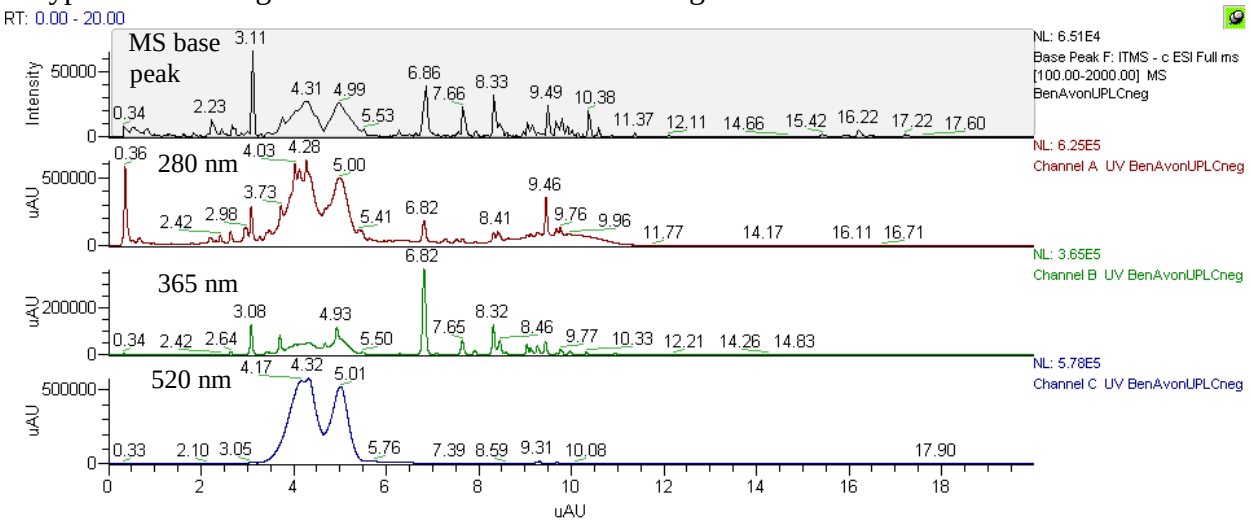
Compound	**MS**	**MS2**
Anthocyanins	**$[M+H]^+$**	
Delphinidin 3-galactoside	465	303
Delphinidin 3-glucoside	465	303
Delphinidin 3-rutinoside	611	465, 303
Cyanidin 3-glucoside	449	287
Cyanidin 3-rutinoside	595	449, 287
Petunidin 3-rutinoside	625	479, 317
Peonidin 3-galactoside	463	301
Malvidin 3-galactoside	493	331
Peonidin 3-glucoside	463	301
Peonidin 3-rutinoside	609	463, 301
Delphinidin 3-(6’’-coumaroyl)glucoside	611	303
Flavonols	**$[M-H]^-$**	
Myricetin 3-rutinoside	625	317
Myricetin 3-glucuronide	493	317
Myricetin 3-glucoside	479	317
Myricetin 3-(6’’-malonyl)glucoside	565	317
Aureusidin glucoside	447	285
Quercetin 3-rutinoside	609	463, 301
Quercetin 3-glucoside	463	301
Quercetin 3-(6’’-malonyl)glucoside	549	301
Kaempferol 3-rutinoside	593	285
Kaempferol 3-galactoside	447	285
Kaempferol 3-glucoside	447	285
Isohamnetin 3-rutinoside	623	315

The following chromatograms show typical black currant (Ben Avon) profiles and identification of some major components.

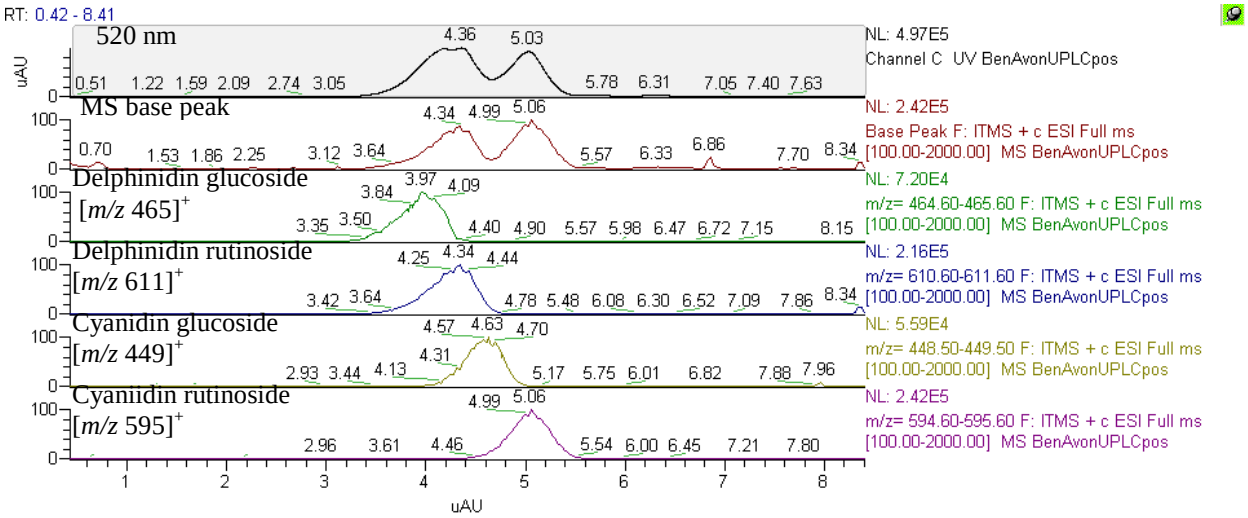
Typical chromatogram of black currant extract – positive ion mode



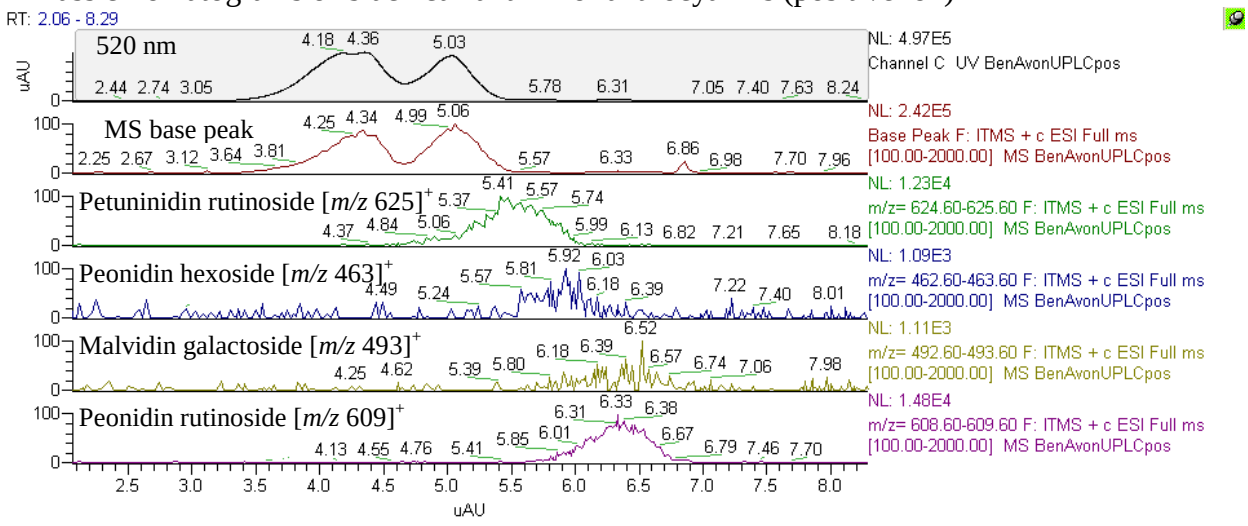
Typical chromatogram of black currant extract – negative ion mode



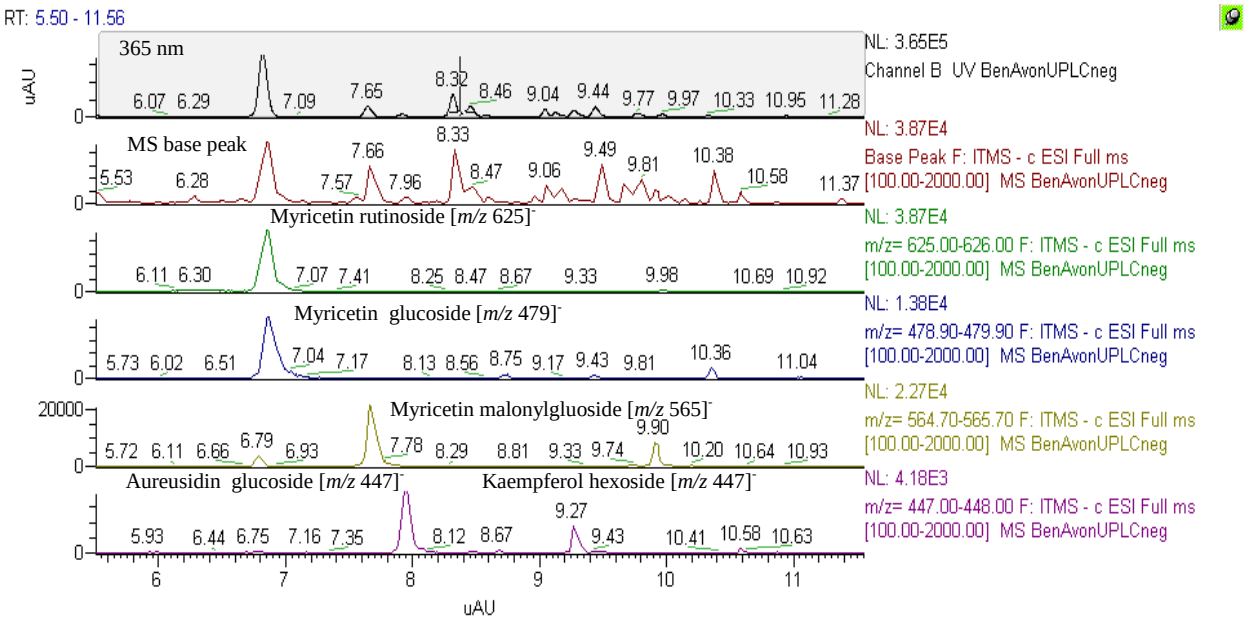
Mass chromatograms of black currant major anthocyanins (positive ion)

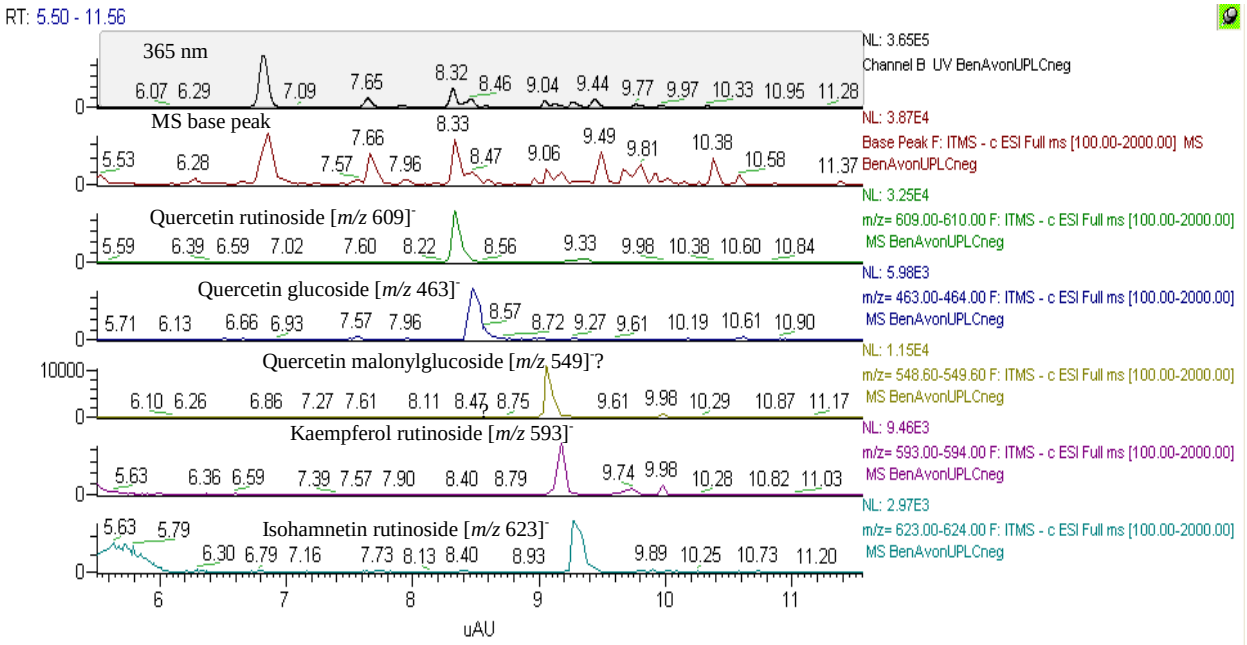


Mass chromatograms of black currant minor anthocyanins (positive ion)



Mass chromatograms of black currant flavonols (negative ion)





2.3.1.4 Blueberry polyphenols

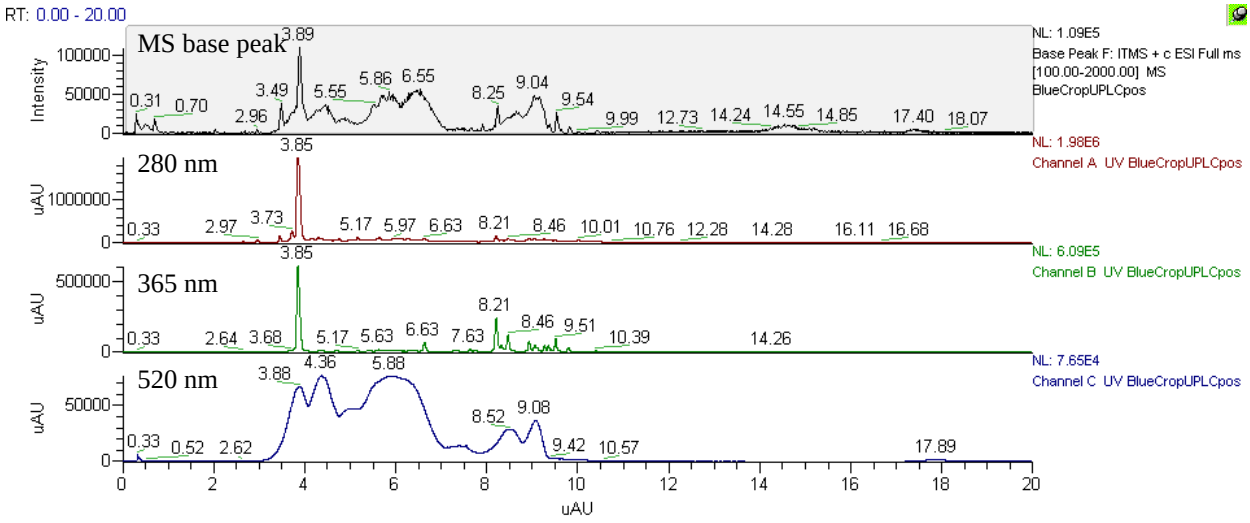
Blueberry contains a complex mixture of anthocyanins (analysed in positive ion mode) and less of flavonols (negative and sometimes positive ion modes).
The following table gives a list of polyphenols found in blueberry and expected masses.

Compound	**MS**	**MS2**
Anthocyanins	**$[M+H]^+$**	
Delphinidin 3-galactoside	465	303
Delphinidin 3-glucoside	465	303
Cyanidin 3-galactoside	449	287
Delphinidin 3-arabinoside	435	303
Cyanidin 3-glucoside	449	287
Petunidin 3-galactoside	479	317
Cyanidin 3-arabinoside	419	287
Petunidin 3-glucoside	479	317
Peonidin 3-galactoside	463	301
Petunidin 3-arabinoside	449	317
Peonidin 3-glucoside	463	301
Malvidin 3-galactoside	493	331
Malvidin 3-glucoside	493	331
Peonidin 3-arabinoside	433	301
Malvidin 3-arabinoside	463	331
Cyanidin 3-(malonyl)glucoside	535	287
Cyanidin 3-(6'-acetyl)galactoside	491	287
Petunidin pentoside	449	317
Delphinidin 3-(malonyl)glucoside	551	303
Malvidin 3-(malonyl)glucoside	579	331
Delphinidin 3-(6'-acetyl)glucoside	507	303
Peonidin 3-(6'-acetyl)galactoside	505	301
Cyanidin 3-(6'-acetyl)glucoside	491	287
Malvidin 3-(6'-acetyl)galactoside	535	331
Petunidin 3-(6'-acetyl)glucoside	521	317
Peonidin 3-(6'-acetyl)glucoside	505	301
Malvidin 3-(6'-acetyl)glucoside	535	331
Flavonols	**$[M-H]^-$**	
Myricetin 3-galactoside	479	317
Quercetin diglucoside	625	301
Quercetin 3-rutinoside	609	463, 301
Quercetin 3-galactoside	463	301
Quercetin 3-methoxyhexoside	493	463, 301
Quercetin 3-glucoside	463	301
Quercetin 3-arabinoside	433	301
Quercetin glucuronide	477	301
Quercetin 3-glucosylpentoside	595	433, 301
Quercetin 3-caffeoylgalactoside	625	463, 301
Quercetin 3-caffeoylglucoside	625	463, 301
Quercetin 3-oxalylpentoside	505	433, 301
Quercetin 3-rhamnoside	447	301
Quercetin 3-dimethoxyrhamnoside	507	477, 447, 301
Quercetin 3-(6'-acetyl)galactoside	505	463, 301
Quercetin 3-(6'-acetyl)glucoside	505	463, 301

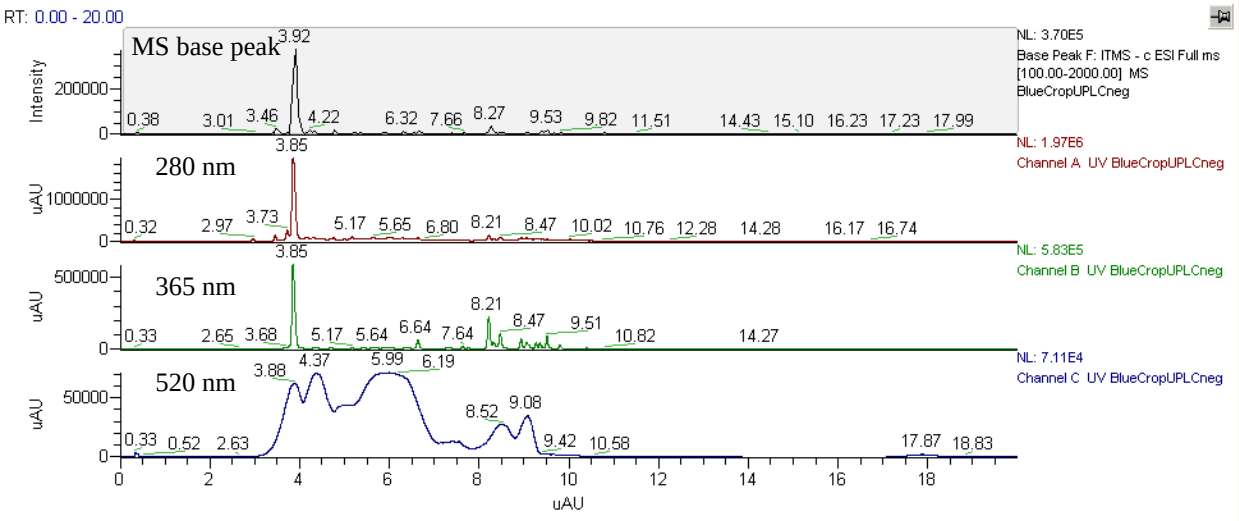
Quercetin	301	

The following chromatograms show typical blueberry (Blue Crop) profiles and identification of some major components.

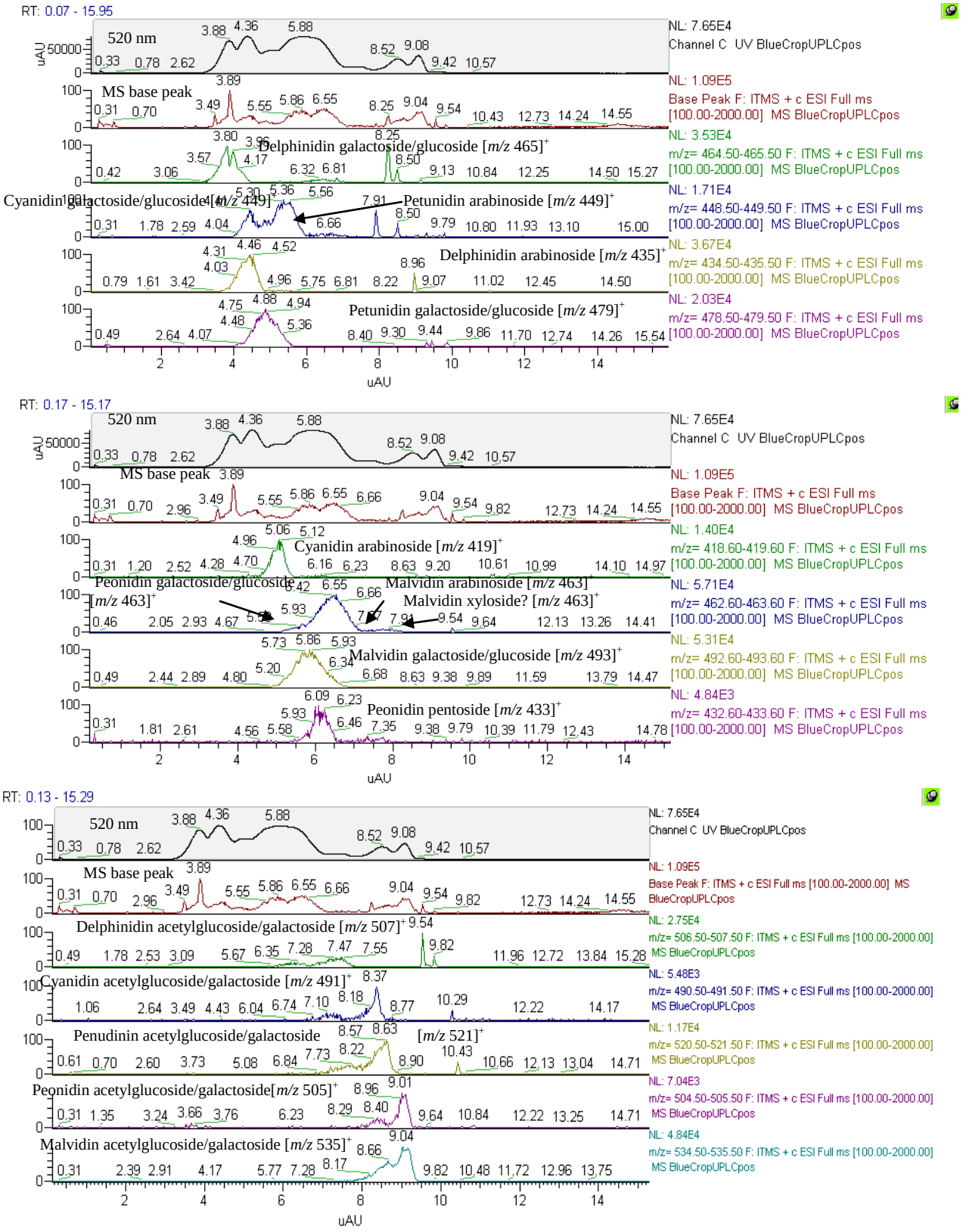
Typical chromatogram of blueberry extract – positive ion mode



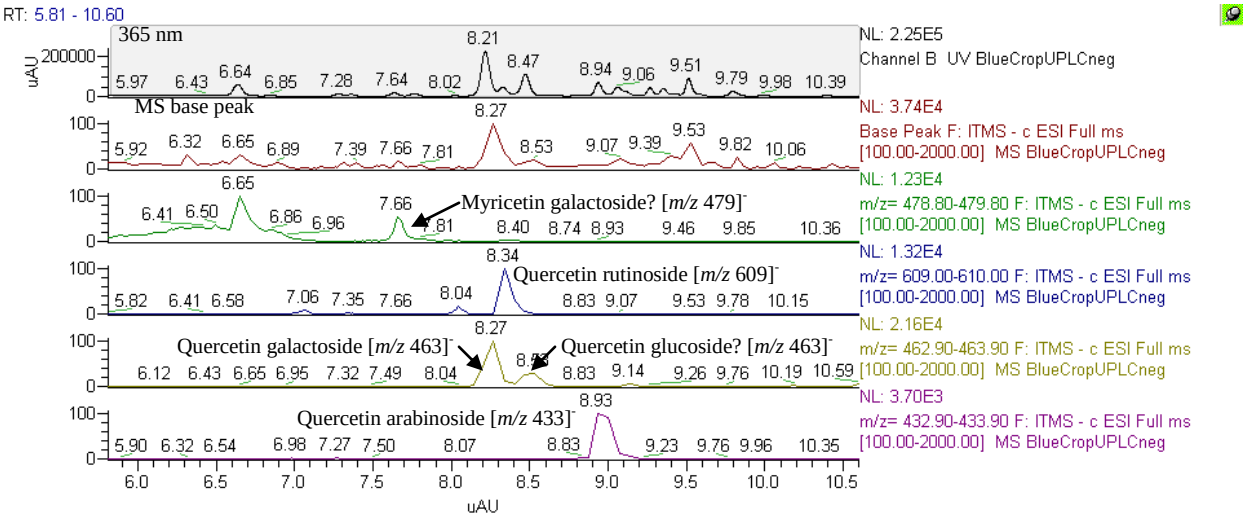
Typical chromatogram of blueberry extract – negative ion mode



Mass chromatograms of blueberry anthocyanins (positive ion)



Mass chromatograms of blueberry flavonols (negative ion)



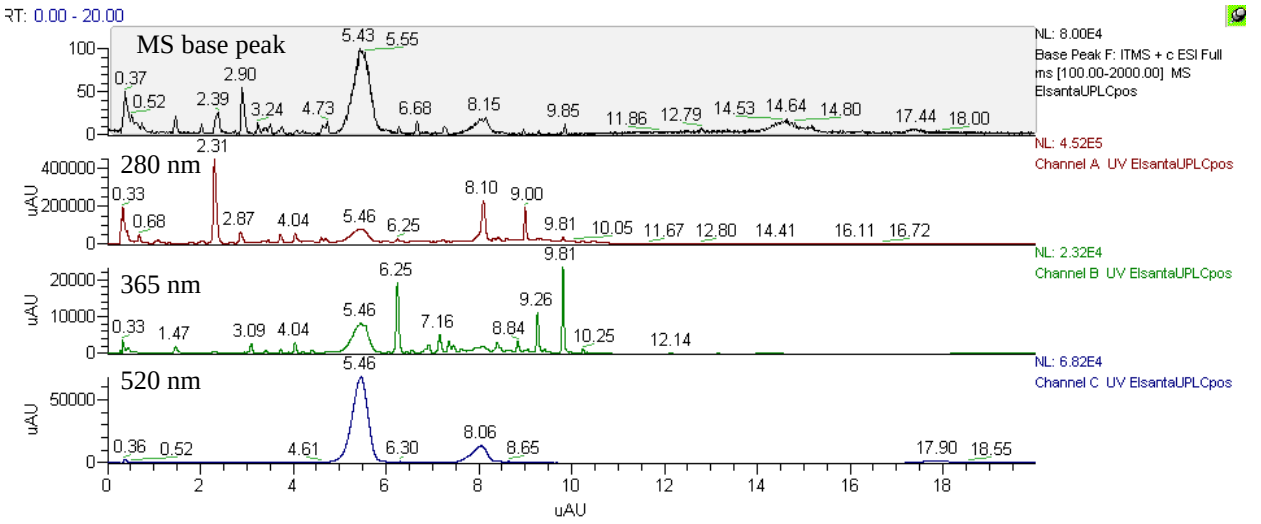
2.3.1.5 Strawberry polyphenols

Strawberry contains large amounts of proanthocyanidins and less anthocyanins (analysed in positive ion mode) and ellagitannins (negative ion mode). Flavonols (negative and sometimes positive ion modes) are minor components.
The following table gives a list of polyphenols found in strawberry and expected masses.

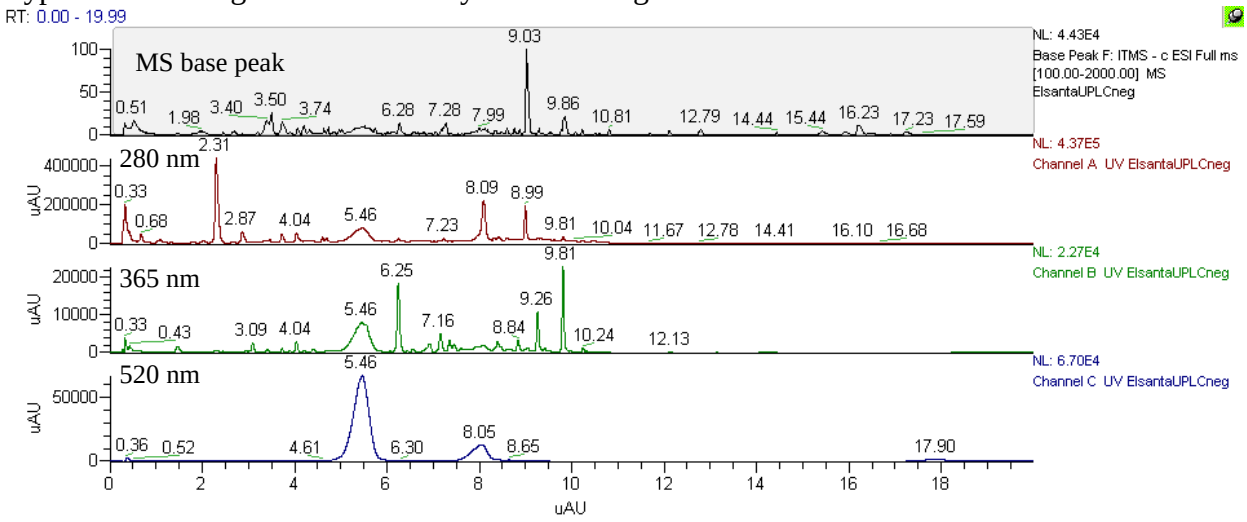
Compound	**MS**	**MS2**
Anthocyanins	**$[M+H]^+$**	
Cyanidin 3-sophoroside	611	287
Cyanidin 3-glucoside	449	287
Cyanidin 3-rutinoside	595	449, 287
Pelargonidin 3-glucoside	433	271
Petunidin 3-glucoside	479	317
Pelargonidin 3-rutinoside	579	433, 271
Pelargonidin 3-(malonyl)glucoside	519	433, 271
Pelargonidin 3-(6'-acetyl)glucoside	475	271
Flavonol	**$[M-H]^-$**	
Quercetin 3-glucuronide	477	301
Kaempferol 3-coumaroylglucoside	593	447, 285
Kaempferol 3-glucoside	447	285
Kaempferol 3-acetylglucoside	489	285
Kaempferol 3-glucuronide	461	285
Ellagitannins/ellagic acid derivatives	**$[M-H]^-$**	
Bis-HDDP-glucoside	783	301
Galloyl bis-HHDP-glucoside 1	935	633, 301
Galloyl bis-HHDP-glucoside 2	935	633, 301
Sanguiin-H6	$[1869]^{-1}$ $[935]^{-2}$	1697, 1567, 1407,897, 783, 633, 301
Lambertianin C	$[1401]^{-2}$	2019, 1869, 1567, 1402, 935, 897, 633, 301
Ellagic rhamnoside 1	447	301
Ellagic rhamnoside 2	447	301
Flavan-3-ols/proanthocyanidins	**$[M-H]^-$**	
Catechin	289	
Dimer (Cat-Cat)	577	
Dimer (Cat-Afz)	561	
Trimer (Cat-Cat-Cat)	865	
Trimer (Cat-Cat-Afz)	849	
Trimer Cat-Afz-Afz)	833	
Tetramer (Cat-Cat-Cat-Cat)	1153	
Tetramer (Cat-Cat-Cat-Afz)	1137	

The following chromatograms show typical strawberry (Elsanta) profiles and identification of some major components.

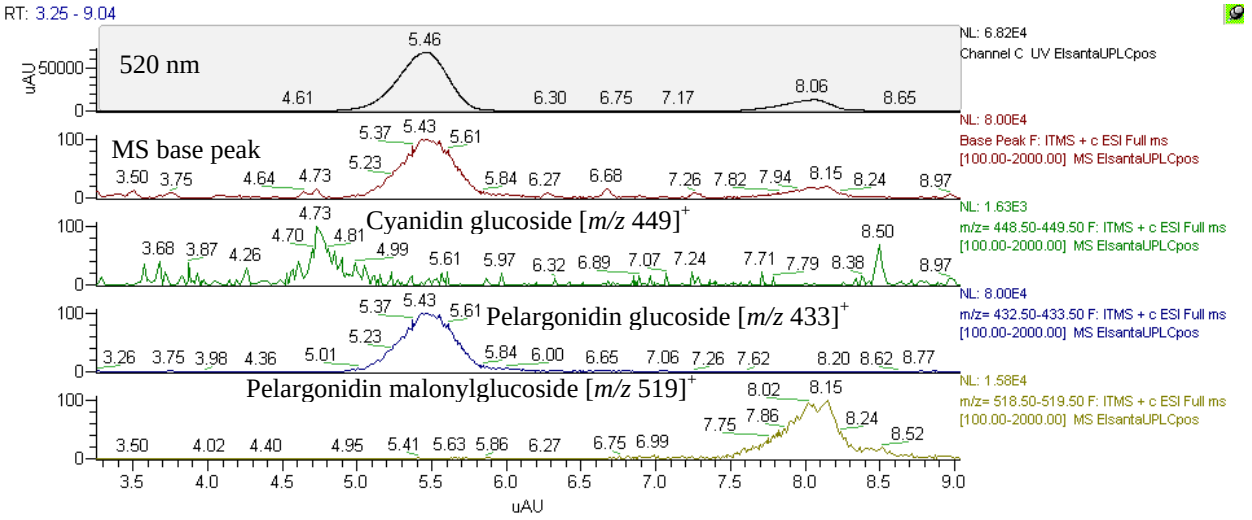
Typical chromatogram of strawberry extract – positive ion mode



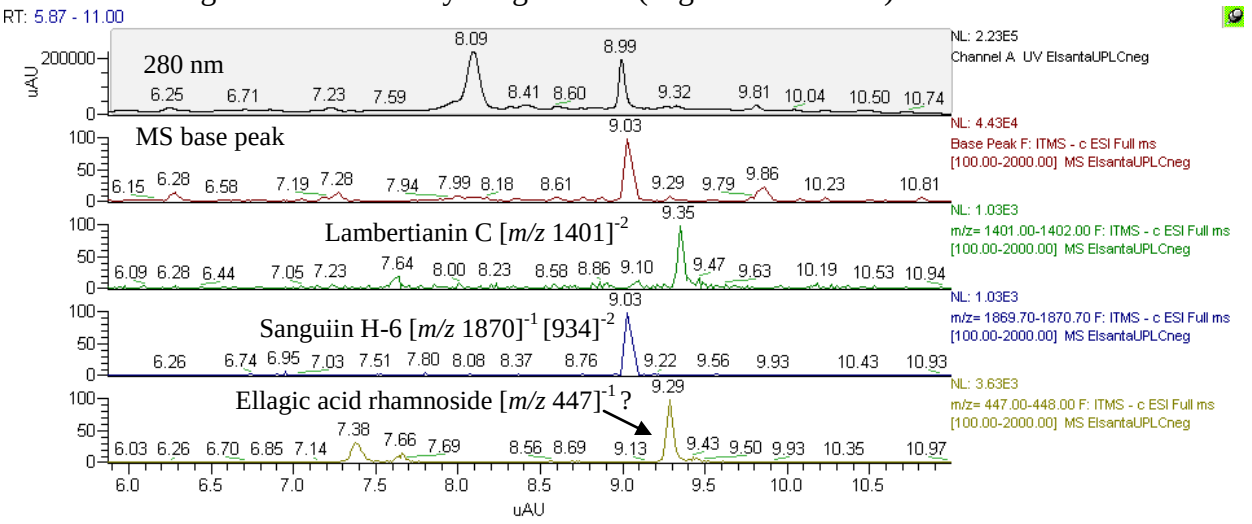
Typical chromatogram of strawberry extract – negative ion mode



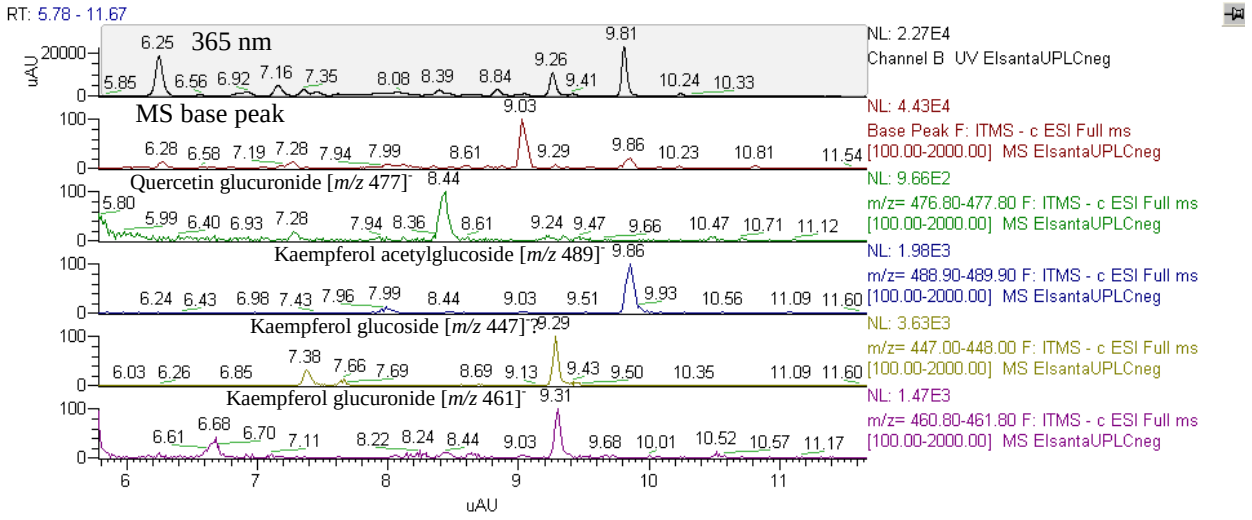
Mass chromatograms of strawberry anthocyanins (positive ion)



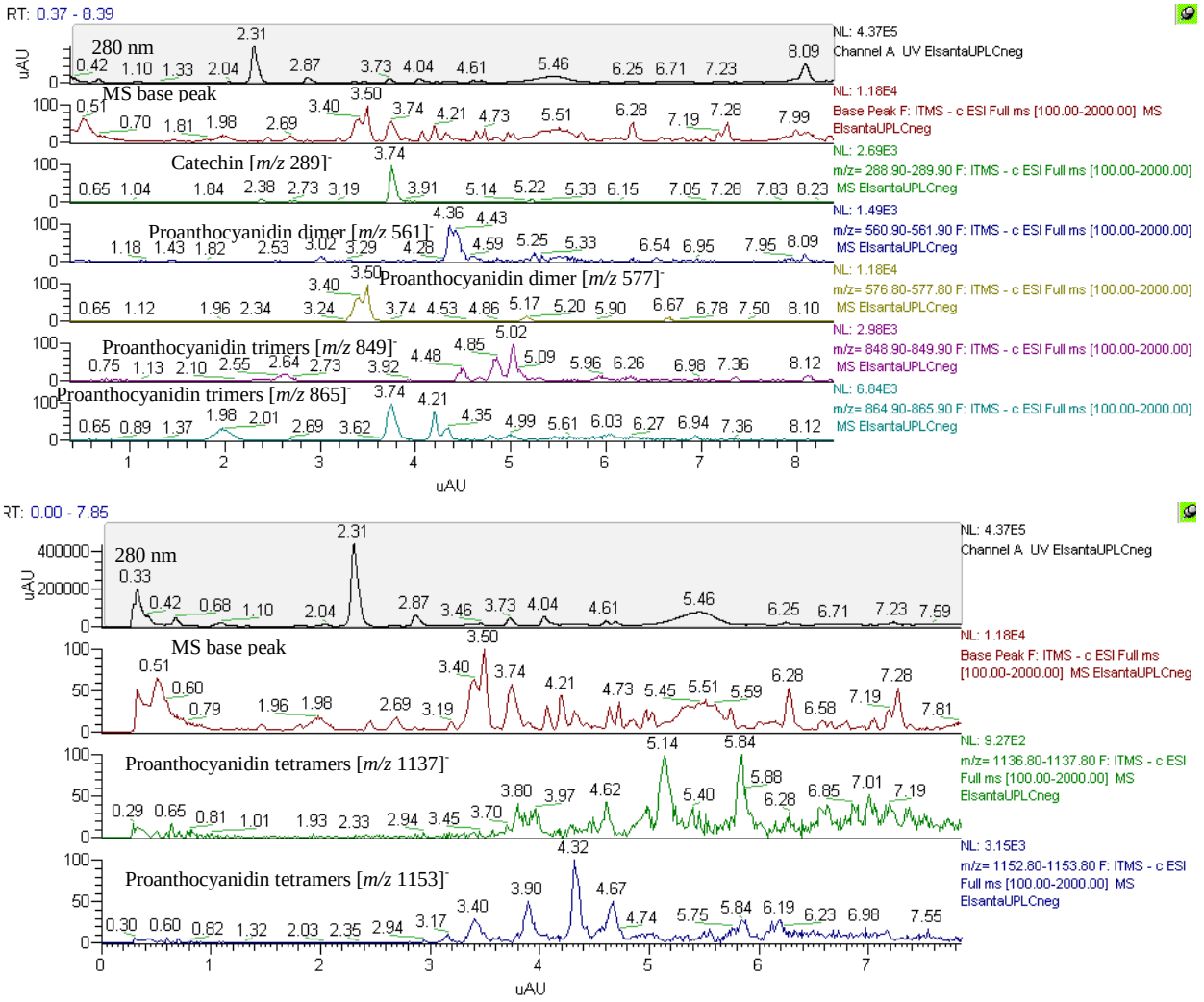
Mass chromatograms of strawberry ellagitannins (negative ion mode)



Mass chromatograms of strawberry flavonols (negative ion mode)



Mass chromatograms of strawberry flavan-3-ols (negative ion mode)



2.3.2 Quantification

For quantification of compounds, morin is added to the juice sample at a concentration of 0.1 mg mL^{-1} (add 100 µl of 1 mg mL^{-1} morin to 900 µl juice). Suitable external standards similar to the compounds of interest are prepared to cover the concentrations in the juice. Morin is added to each standard at the same concentration (0.1 mg mL^{-1}) as in the juice and the standards are analysed together with samples. Samples and standards are processed so that the ratio of the area of a specific ion of the compound of interest to that for morin internal standard (*m*/*z* 301 and 303 for negative and positive mode, respectively) is calculated. In Excel, calibration curves of response ratios against concentration are plotted for the standards and best-fit equations are used to calculate the concentrations in the juices.

Analysis of Polyphenolics in Raspberry, Blackberry, Blueberry, Black Currant and Strawberry by High Pressure Liquid Chromatography-Photodiode Array-High Resolution-Mass Spectrometry (HPLC-PDA-HR-MS)

1. Introduction

1.1 Purpose

To describe the procedure for analysing the polyphenolic compounds from raspberry, blackberry, blueberry, black currant or strawberry by high pressure liquid chromatography-high resolution (accurate mass)-mass spectrometry (HPLC-PDA-HR-MS). The use of accurate mass allows differentiation between isobaric compounds and therefore improves accuracy of compound identification.This SOP is to be used for training of new and substitute staff, for auditing purposes and for general reference.

1.2 Scope

The method can be used for fresh, frozen or freeze-dried raspberry, blackberry, blueberry, black currant or strawberry.

1.3 Overview

Following extraction, the solvent is removed by Speed Vac and then freeze-dried to remove any remaining water. The sample is re-dissolved in 0.5% formic acid in 5% aqueous acetonitrile and is analysed by reversed phase HPLC-PDA-HR-MS using a mobile phase of 0.1% formic acid in water with increasing proportions of acetonitrile.

2. Methods

2.1 Inputs to process

2.1.1 Apparatus and reagents required

Apparatus
Balance (3 place or better)
Microcentrifuge (Eppendorf 5415D or equivalent)
Borosilicate glass mortars (homogenisers), 5 mL capacity
1.5ml and 2ml microfuge tubes
8ml glass vials
96 well microfuge racks
Scalpel
Freeze-drier
Speed vac SPD131DA (Savant) or equivalent, with refridgerated vapour trap and pump
Eppendorf P1000 pipette or equivalent

Handystep electronic pipette (Brand)
Single StEP^{TM} filter vial 0.45 μm PTFE (Thomson)
Thermo LTQ Orbitrap XL mass spectrometer with Accela 600 pump, autosampler and PDA detector or equivalent
Synergi 4 Hydro-RP 80A (150 mm x 2.0 mm; 4 μm reversed-phase HPLC column (Phenomenex)

Chemicals
Acetonitrile
Formic acid
Double distilled water

2.2 Procedures

2.2.1 Sample preparation

2.2.1.1 Berries are extracted with 0.5% formic acid in acetonitrile to give a "50% juice" as described in SOP Extraction of Polphenolics from Berries.
2.2.1.2 Store samples in -20°C freezer until ready to be taken to dryness.
2.2.1.3 Thaw samples in cold room (4°C) and leave on Speed Vac at 45°C for 3 h. Freeze samples in -20°C freezer.
2.2.1.4 Freeze-dry samples overnight using side arm of freeze-drier and keep in -20°C freezer.

2.2.2 Analysis of polyphenolics by HPLC-PDA-HR-MS

2.2.2.1 Prior to use, dissolve sample in 0.5% formic acid in water/acetonitrile (95:5, vol/vol) in same volume (usually 500 ul) as prior to freeze-drying, vortex to disrupt pellet and centrifuge at 13200 rpm for 3 min. Transfer 0.4 mL sample to filter vial and push filter into vial.
2.2.2.2 The sample is injected via an autosampler on to a HPLC column linked to a PDA system (Thermo Accela) and then an electrospray ionization ion trap mass spectrometer (Thermo LTQ Orbitrap XL). The important parameters for the autosampler, mobile phase, PDA and mass spectrometer are as follows:-

Injection volume: 8 μl.

Column type: Synergi 4μ Hydro-RP 80A (150 mm x 2.0 mm; 4 m reversed-phase HPLC column (Phenomenex)

Autosampler temperature: 6°C

Column temperature: 30°C.

Mobile phase: Solvent A – 0.1% (vol/vol) aqueous formic acid
Solvent B – 0.1% (vol/vol) formic acid in acetonitrile/water (50:50, vol/vol).

Flow rate: 200 μl min^{-1}

Gradient :

Time (min)	A%	B%
0	95	5
4	95	5
28	50	50
32	0	100
34	0	100
36	95	5
45	95	5

PDA range: 200-600 nm.

Mass range: *m/z* 100-2000 at a resolution of 30000.

Source parameters: Capillary temperature 275°C, sheath gas and auxillary gas 40 and 5 units and spray voltage 4.0 kV

Scan events: Alternative full scan MS and MS/MS (data-dependant scan).
The sample is run twice – in positive ion and negative ion modes.

2.3 Polyphenol composition

2.3.1 Identification

Identification of polyphenols is based on PDA characteristics and mass spectra including MS and MS/MS data.

2.3.1.1 Raspberry polyphenols

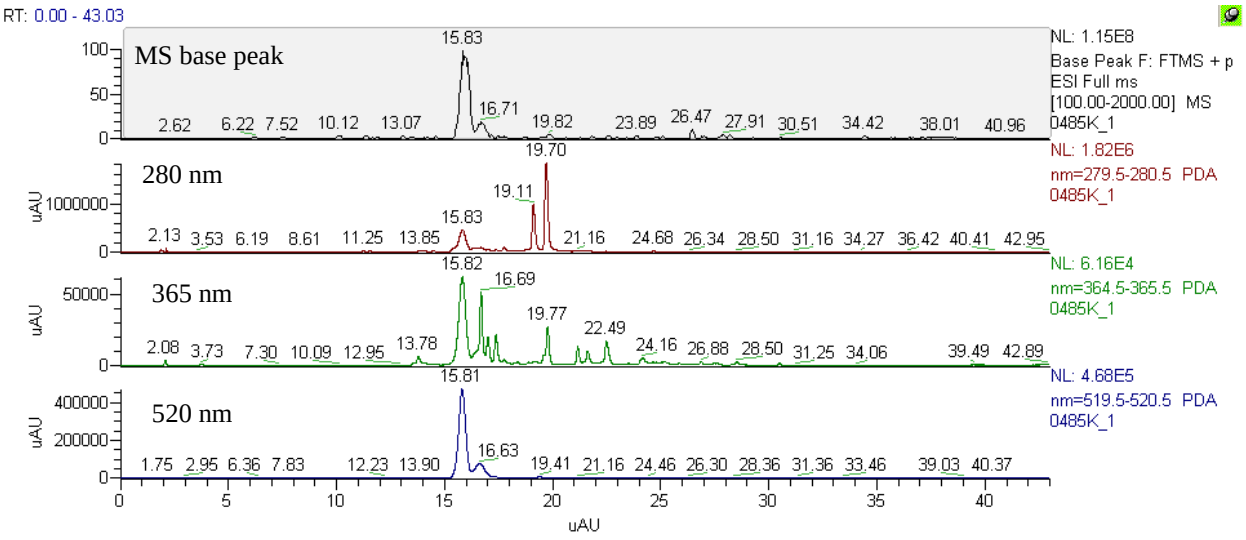
Raspberry contains mainly anthocyanins (analysed in positive ion mode) and ellagitannins (negative ion mode) with flavonols (negative and sometimes positive ion modes) as minor compounds.
The following table gives a list of polyphenols found in raspberry and expected masses.

Compound	**Formula**	**MS**	**MS2**
Anthocyanins		**$[M+H]^+$**	
Cyanidin 3-sophoroside	$C_{27}H_{30}O_{16}$	611.161	287
Cyanidin 3,5-diglucoside	$C_{27}H_{30}O_{16}$	611.161	449, 287

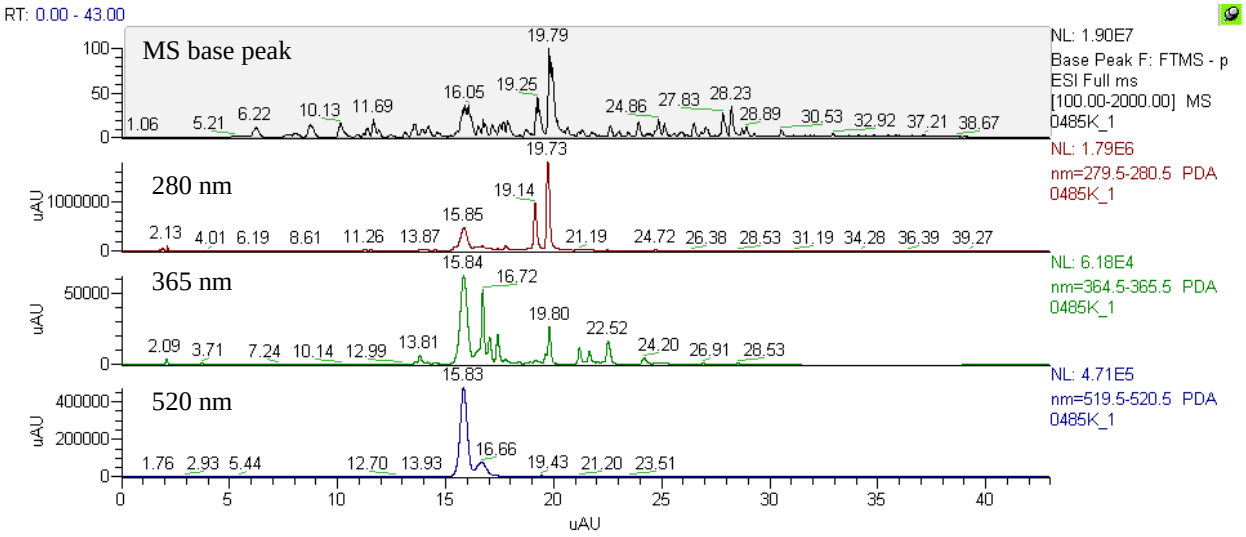
Cyanidin 3-(2'-glucosyl)rutinoside	$C_{33}H_{40}O_{20}$	757.219	287
Cyanidin 3-sophoroside-5-rhamnoside	$C_{33}H_{40}O_{20}$	757.219	611, 433, 287
Cyanidin 3-sambubioside	$C_{26}H_{28}O_{15}$	581.151	287
Cyanidin 3-(2'-xylosyl)rutinoside	$C_{32}H_{38}O_{19}$	727.209	287
Cyanidin 3-glucoside	$C_{21}H_{20}O_{11}$	449.108	287
Cyanidin 3-sambubioside-5-rhamnoside	$C_{32}H_{38}O_{19}$	727.209	581, 433, 287
Cyanidin 3-rutinoside	$C_{27}H_{30}O_{15}$	595.166	449, 287
Pelargonidin 3-sophoroside	$C_{27}H_{30}O_{15}$	595.166	271
Pelargonidin 3-(2'-glucosyl)rutinoside	$C_{33}H_{40}O_{19}$	741.223	271
Pelargonidin 3-sambubioside	$C_{26}H_{28}O_{14}$	565.155	271
Pelargonidin 3-glucoside	$C_{21}H_{20}O_{10}$	433.113	271
Pelargonidin 3-rutinoside	$C_{27}H_{30}O_{14}$	579.150	433, 271
Flavonols		**$[M-H]^-$**	
Quercetin galactosylrhamnoside/rutinoside	$C_{27}H_{30}O_{16}$	609.146	463, 301
Quercetin (2'-glucosyl)rutinoside	$C_{33}H_{40}O_{21}$	771.198	301
Quercetin galactoside	$C_{21}H_{20}O_{12}$	463.088	301
Quercetin glucoside	$C_{21}H_{20}O_{12}$	463.088	301
Quercetin glucuronide	$C_{21}H_{18}O_{13}$	477.067	301
Quercetin diglucoside	$C_{27}H_{30}O_{17}$	625.141	301
Kaempferol glucuronide	$C_{21}H_{18}O_{12}$	461.072	285
Methylquercetin glucuronide	$C_{22}H_{20}O_{13}$	491.083	315
Ellagitannins/ellagic acid derivatives		**$[M-H]^-$**	
Sanguiin H-2	$C_{48}H_{32}O_{31}$	$[1103.085]^{-1}$ $[551.038]^{-2}$	301
Sanguiin-H6	$C_{82}H_{54}O_{52}$	$[1869.150]^{-1}$ $[934.071]^{-2}$	1697, 1567, 1407, 897, 783, 633, 301
Sanguiin-H10 isomers	$C_{68}H_{48}O_{44}$	$[1567.144]^{-1}$ $[783.068]^{-2}$	935, 633, 301
Lambertianin C	$C_{123}H_{80}O_{78}$	$[1401.107]^{-2}$	2019, 1869, 1567, 1402, 935, 897, 633, 301
Ellagic acid pentoside 1	$C_{19}H_{14}O_{12}$	433.041	301
Ellagic acid pentoside 2	$C_{19}H_{14}O_{12}$	433.041	301
Ellagic acid acetyl arabinoside	$C_{21}H_{16}O_{13}$	475.051	301
Ellagic acid 4-acetyl xyloside	$C_{21}H_{16}O_{13}$	475.051	301
Ellagic acid	$C_{14}H_6O_8$	300.998	
Methyl ellagic acid pentoside	$C_{20}H_{16}O_{12}$	447.056	315

The following chromatograms show typical raspberry (cultivar 0485K-1) profiles and identification of some major components.

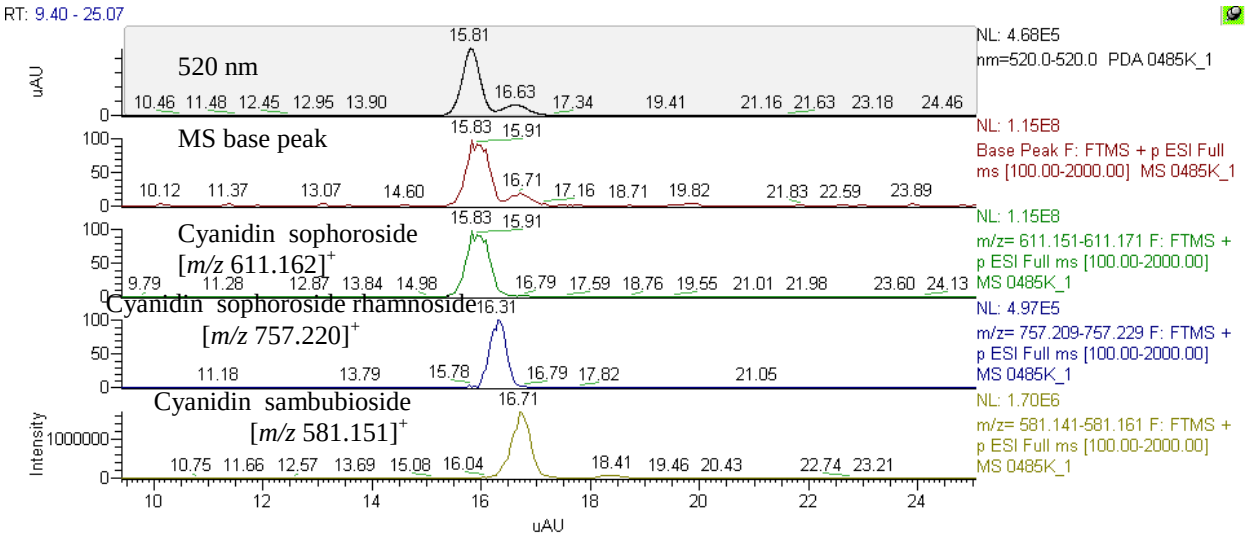
Typical chromatogram of raspberry extract – positive ion mode

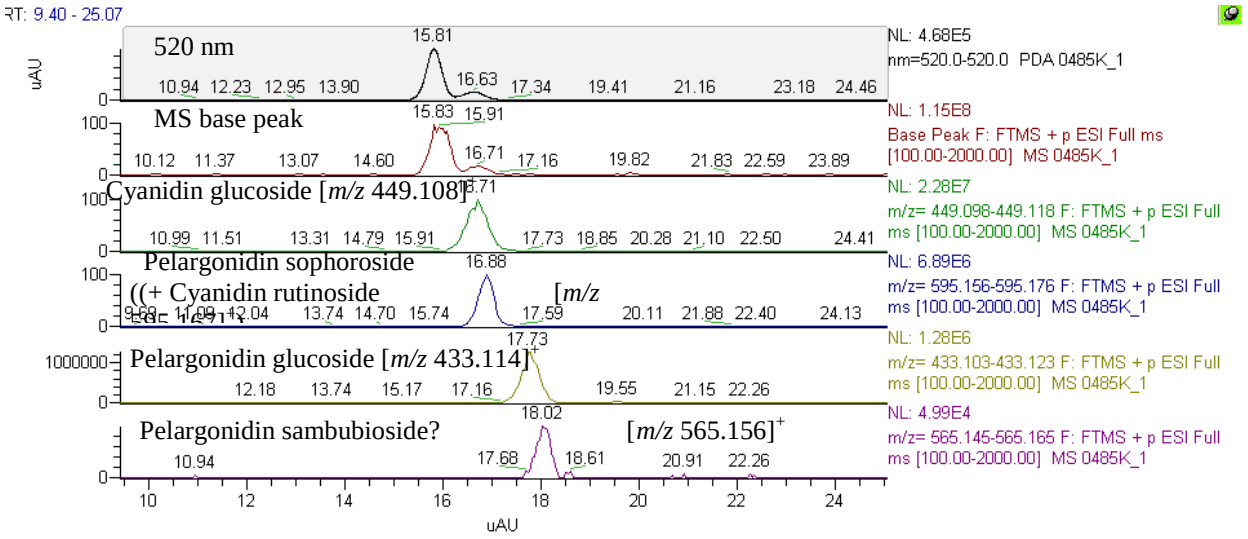


Typical chromatogram of raspberry extract – negative ion mode

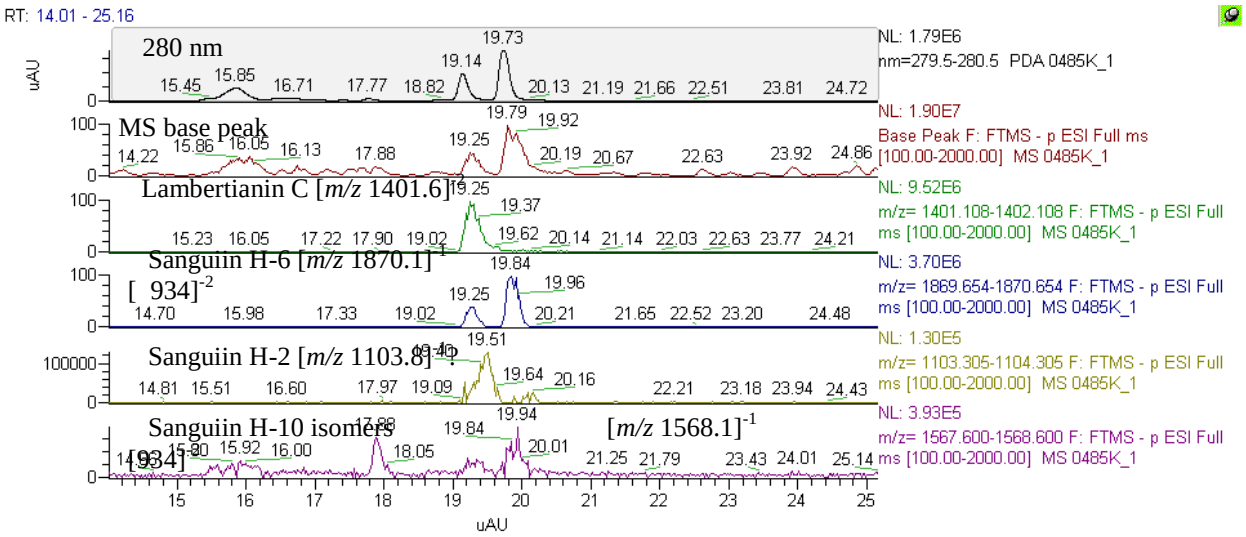


Mass chromatograms of raspberry anthocyanins (positive ion mode)

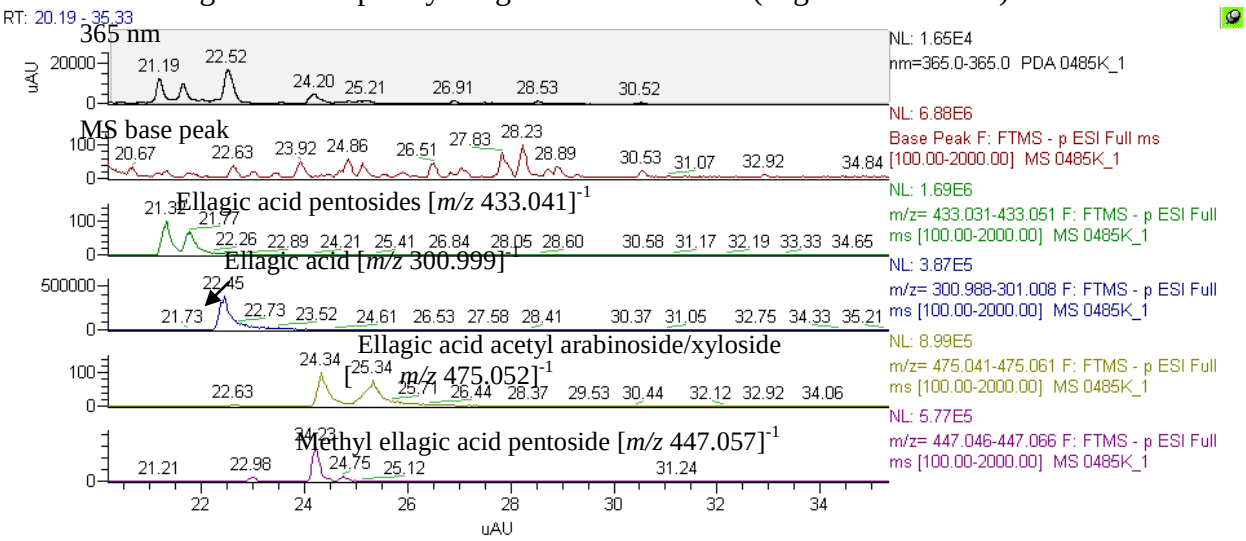




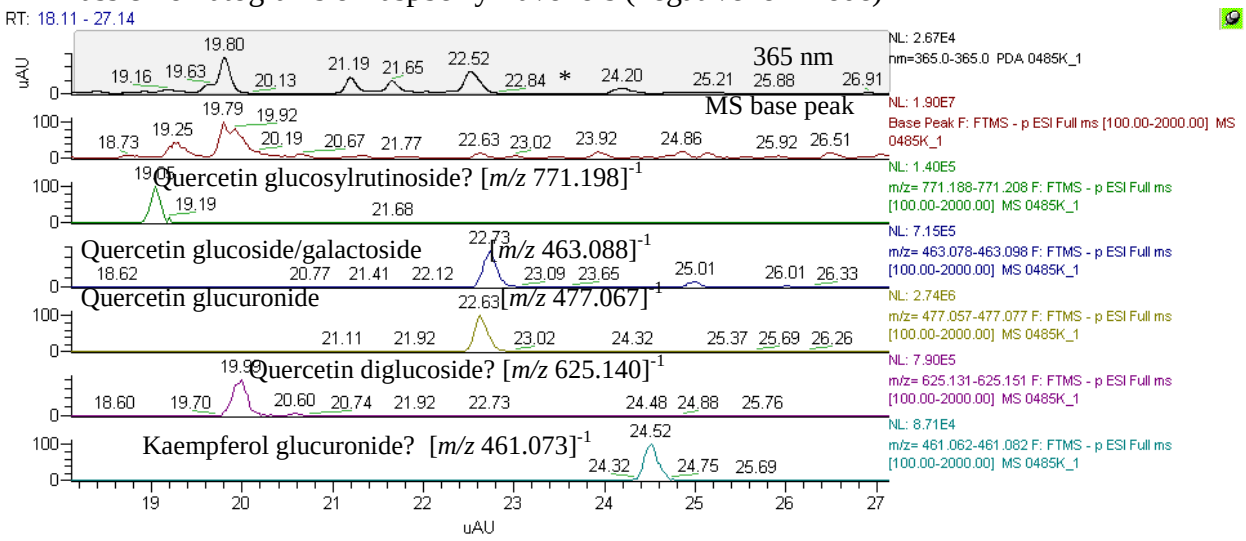
Mass chromatograms of raspberry ellagitannins (negative ion mode)



Mass chromatograms of raspberry ellagic acid derivatives (negative ion mode)



Mass chromatograms of raspberry flavonols (negative ion mode)



2.3.1.2 Blackberry polyphenols

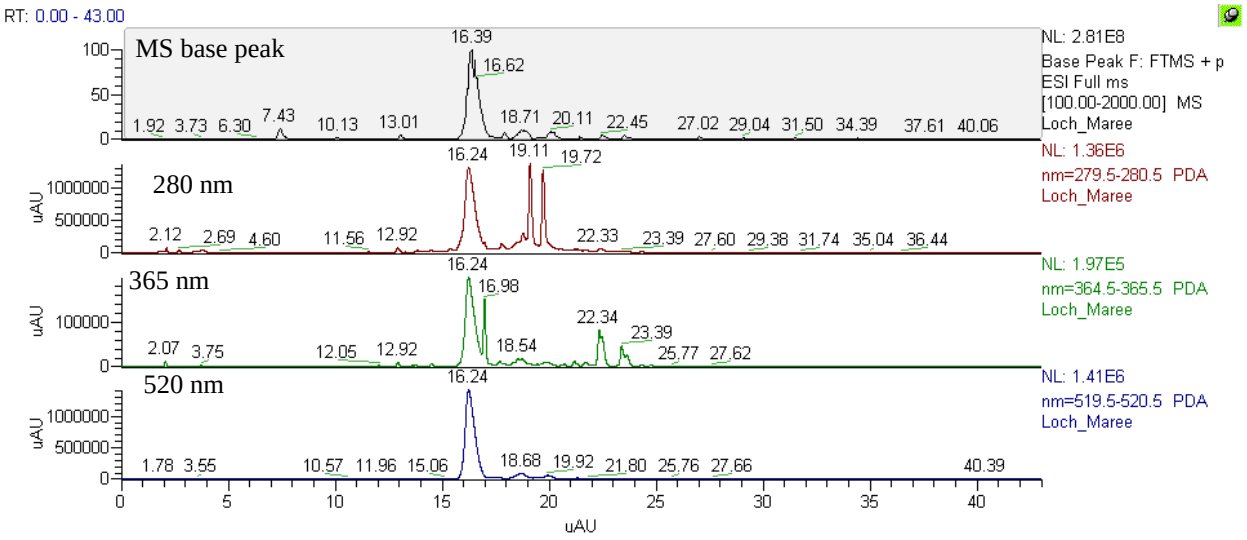
Blackberry contains mainly anthocyanins (analysed in positive ion mode) and ellagitannins (negative ion mode) with flavonols (negative and sometimes positive ion modes) as minor compounds.

The following table gives a list of polyphenols found in blackberry and expected masses.

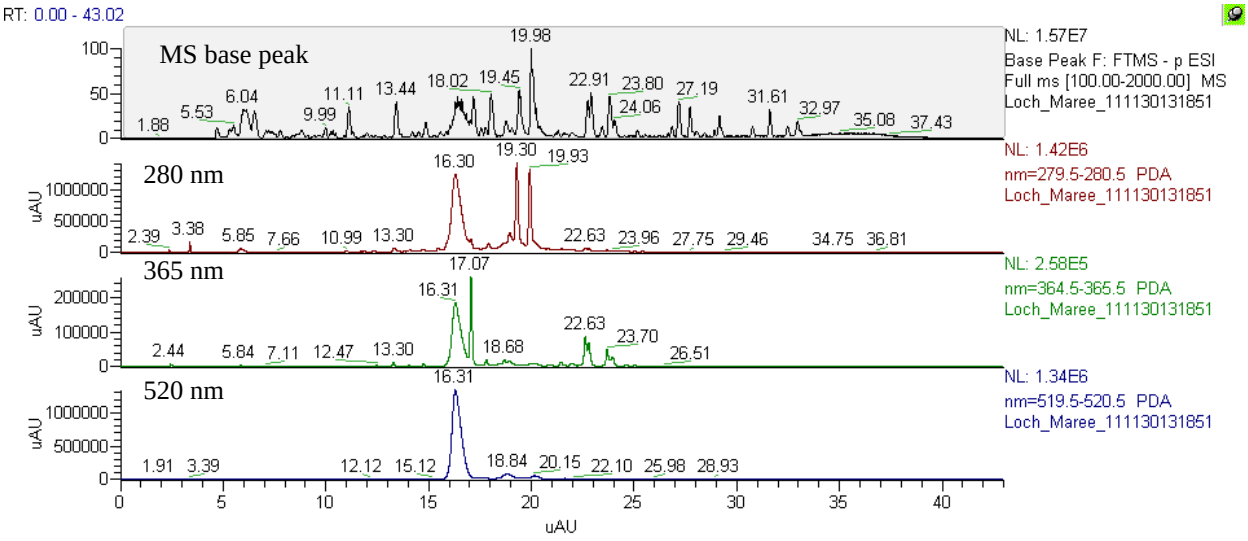
Compound	**Formula**	**MS**	**MS2**
Anthocyanins		**$[M+H]^+$**	
Cyanidin 3-glucoside	$C_{21}H_{20}O_{11}$	449.108	287
Cyanidin 3-arabinoside	$C_{20}H_{18}O_{10}$	419.097	287
Cyanidin 3-rutinoside	$C_{27}H_{30}O_{15}$	595.166	449, 287
Pelargonidin 3-glucoside	$C_{21}H_{20}O_{10}$	433.113	271
Cyanidin 3-(3'-malonyl)glucoside	$C_{24}H_{22}O_{14}$	535.109	287
Peonidin 3-glucoside	$C_{22}H_{22}O_{11}$	463.124	301
Cyanidin 3-xyloside	$C_{20}H_{18}O_{10}$	419.097	287
Cyanidin 3-(6'-malonyl)glucoside	$C_{24}H_{22}O_{14}$	535.109	449, 287
Cyanidin 3-dioxalylglucoside	$C_{25}H_{20}O_{17}$	593.078	287
Cyanidin 3-[6''-(3-hydroxy-3-methylglutaroyl) galactoside	$C_{27}H_{28}O_{15}$	593.151	287
Flavonols		**$[M-H]^-$**	
Quercetin 3-rutinoside	$C_{27}H_{30}O_{16}$	609.146	463, 301
Quercetin 3-galactoside	$C_{21}H_{20}O_{12}$	463.088	301
Quercetin 3-methoxyhexoside	$C_{22}H_{22}O_{13}$	493.099	463, 301
Quercetin 3-glucoside	$C_{21}H_{20}O_{12}$	463.088	301
Quercetin 3-pentoside 1	$C_{20}H_{18}O_{11}$	433.077	301
Quercetin 3-pentoside 2	$C_{20}H_{18}O_{11}$	433.077	301
Quercetin glucuronide	$C_{21}H_{18}O_{13}$	477.067	301
Quercetin 3-[6''-(3-hydroxy-3-methylglutaroyl) galactoside	$C_{27}H_{28}O_{16}$	607.130	463, 301
Quercetin acetylhexoside	$C_{23}H_{22}O_{13}$	505.099	301
Quercetin 3-glucosylpentoside	$C_{26}H_{28}O_{16}$	595.130	433, 301
Quercetin 3-oxalylpentoside	$C_{22}H_{18}O_{14}$	505.062	433, 301
Quercetin	$C_{15}H_{10}O_6$	301.035	
Kaempferol hexoside	$C_{21}H_{20}O_{11}$	447.093	285
Kaempferol 3-[6''-(3-hydroxy-3-methylglutaroyl) galactoside	$C_{27}H_{28}O_{15}$	591.135	285, 447
Ellagitannins/ellagic acid derivatives		**$[M-H]^-$**	
Sanguiin H-2	$C_{48}H_{32}O_{31}$	$[1103.085]^{-1}$ $[551.038]^{-2}$	301
Sanguiin-H6	$C_{82}H_{54}O_{52}$	$[1869.150]^{-1}$ $[934.071]^{-2}$	1697, 1567, 1407, 897, 783, 633, 301
Sanguiin-H10 isomers	$C_{68}H_{48}O_{44}$	$[1567.144]^{-1}$ $[783.068]^{-2}$	935, 633, 301
Lambertianin C	$C_{123}H_{80}O_{78}$	$[1401.107]^{-2}$	2019, 1869, 1567, 1402, 935, 897, 633, 301
Ellagic acid	$C_{14}H_6O_8$	300.998	
Methyl ellagic acid glucuronide	$C_{21}H_{16}O_{14}$	491.046	315
Methyl ellagic acid pentoside	$C_{20}H_{16}O_{12}$	447.056	315

The following chromatograms show typical blackberry (cultivar Loch Maree) profiles and identification of some major components.

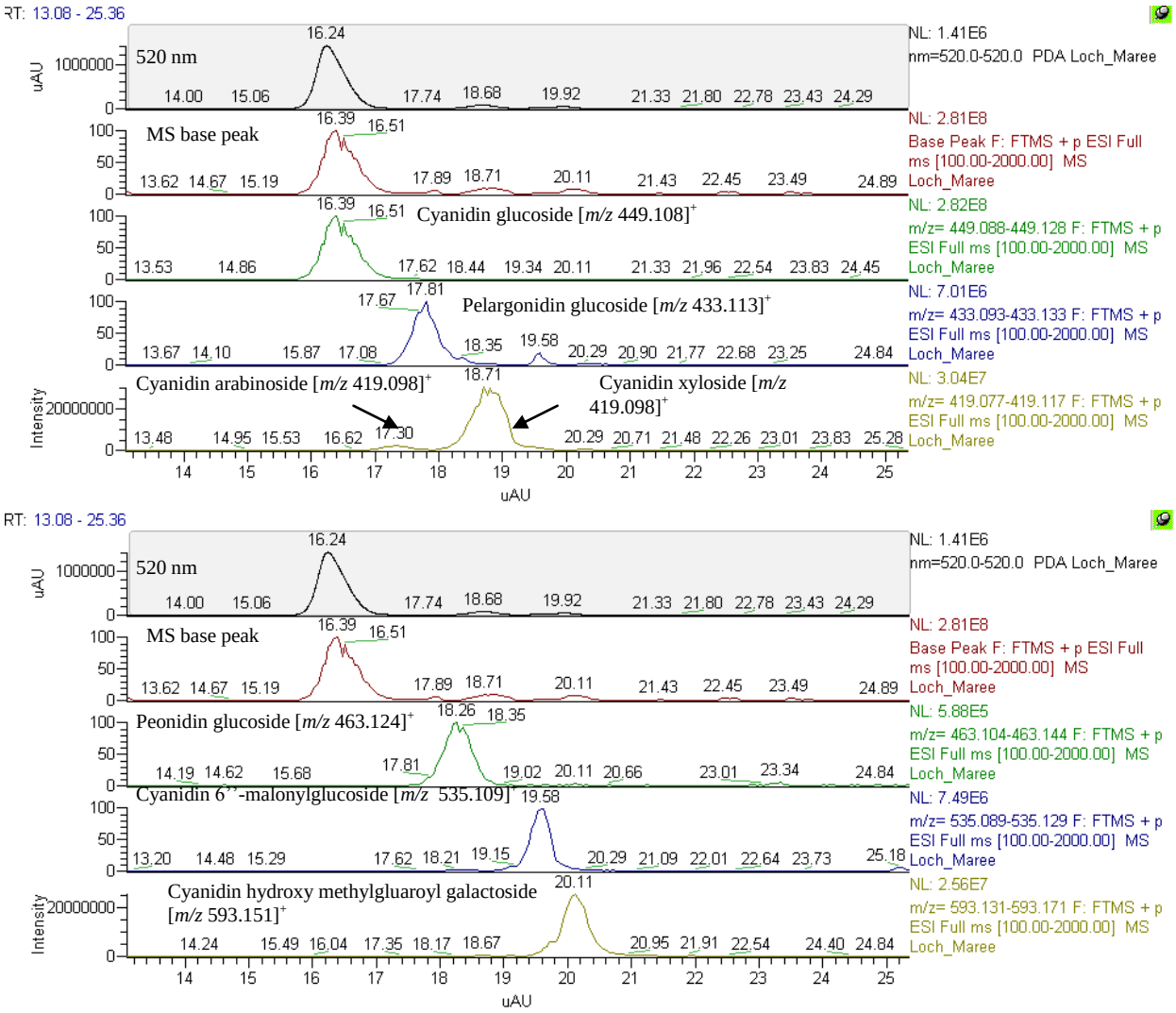
Typical chromatogram of blackberry extract – positive ion mode



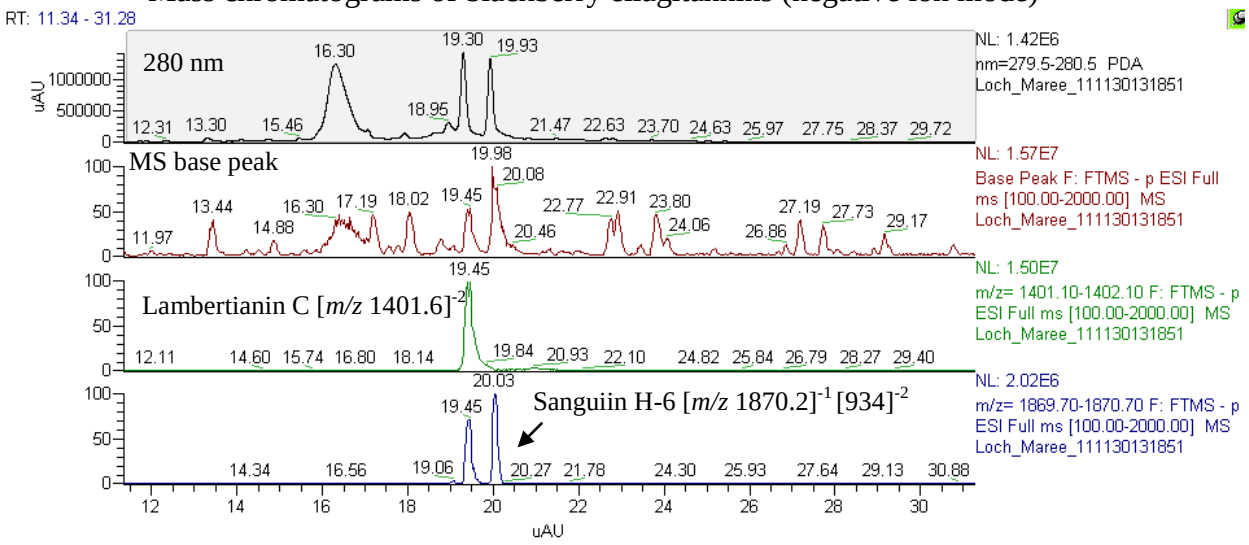
Typical chromatogram of blackberry extract – negative ion mode



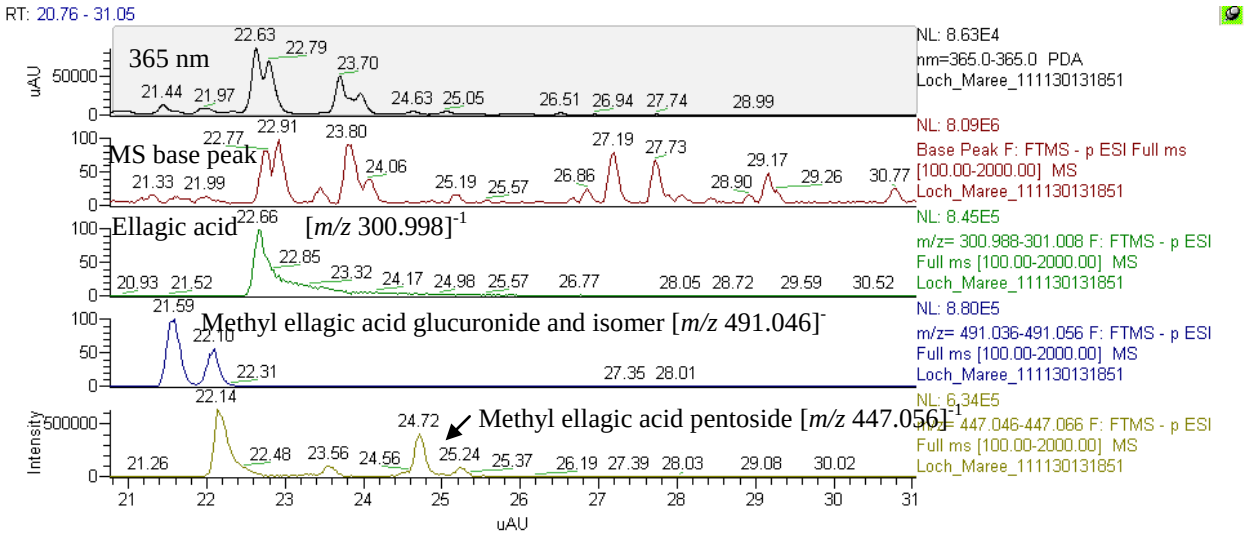
Mass chromatograms of blackberry anthocyanins (positive ion)



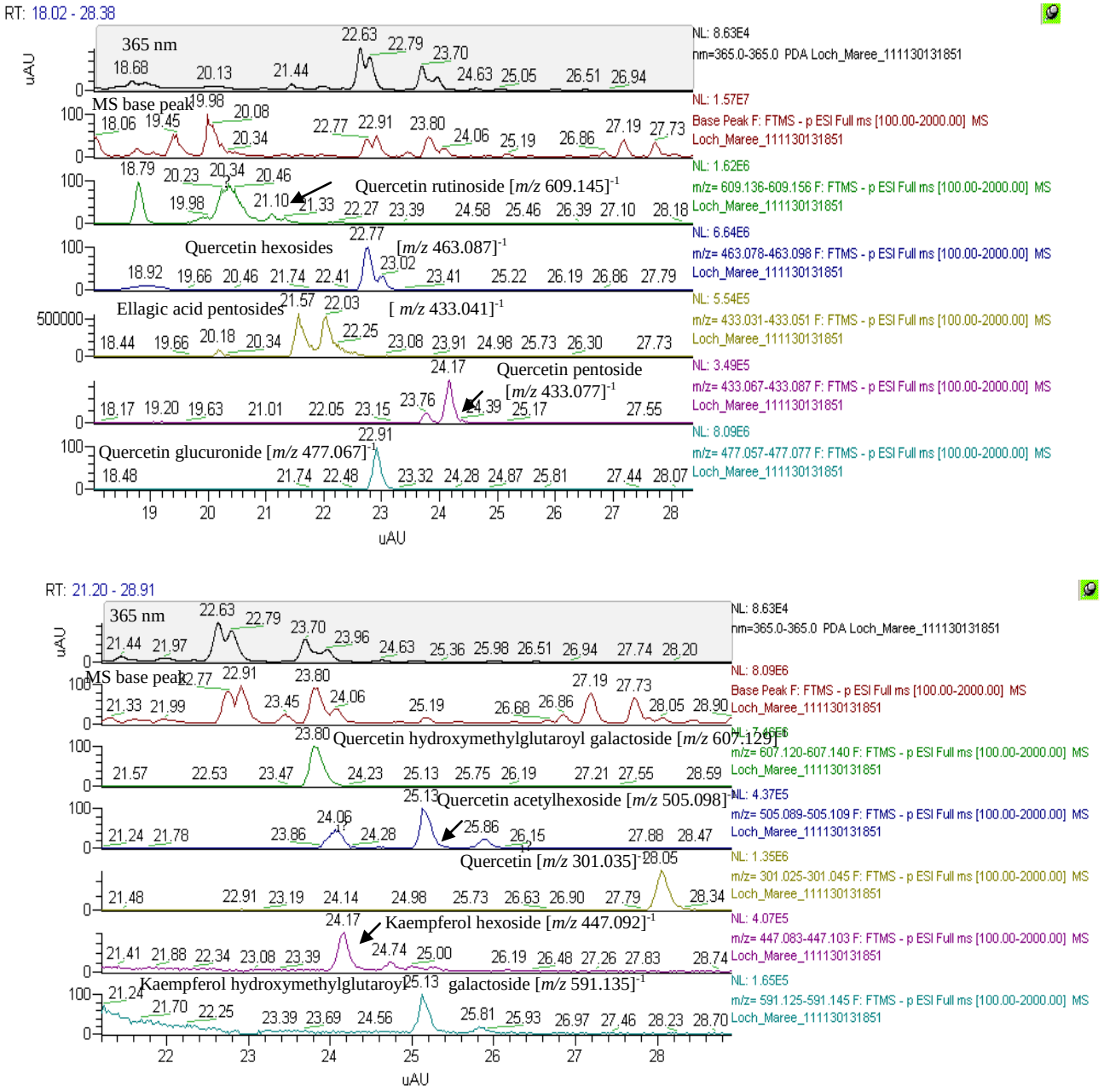
Mass chromatograms of blackberry ellagitannins (negative ion mode)



Mass chromatograms of blackberry ellagic acid derivatives (negative ion mode)



Mass chromatograms of blackberry flavonols (negative ion mode)



2.3.1.3 Black currant polyphenols

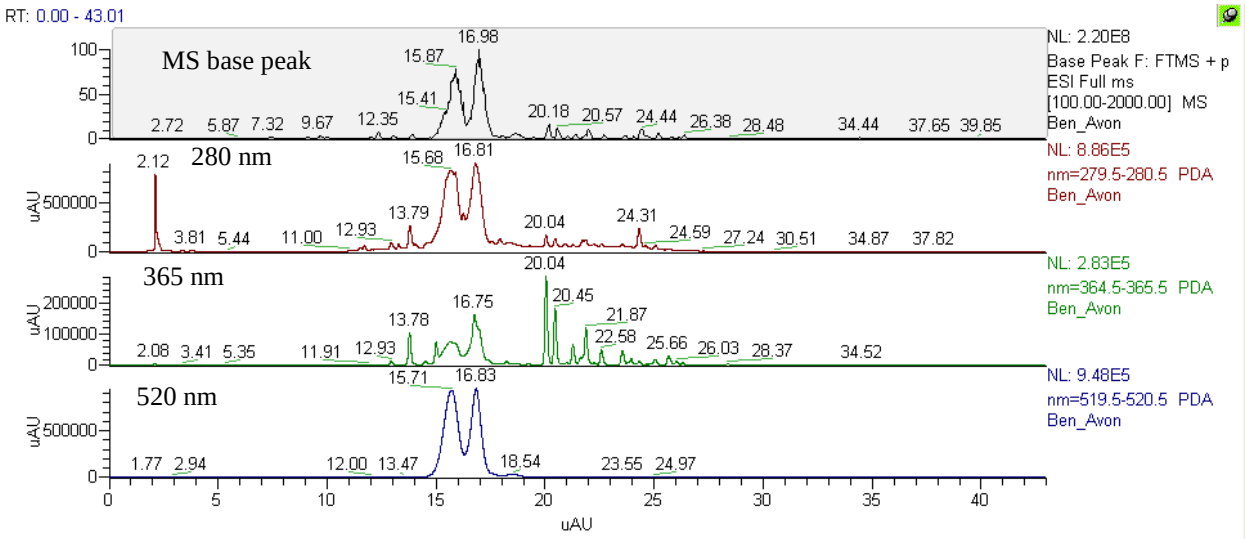
Black currant contains anthocyanins (analysed in positive ion mode) as major components together with significant levels of flavonols (negative and sometimes positive ion modes).

The following table gives a list of polyphenols found in black currant and expected masses.

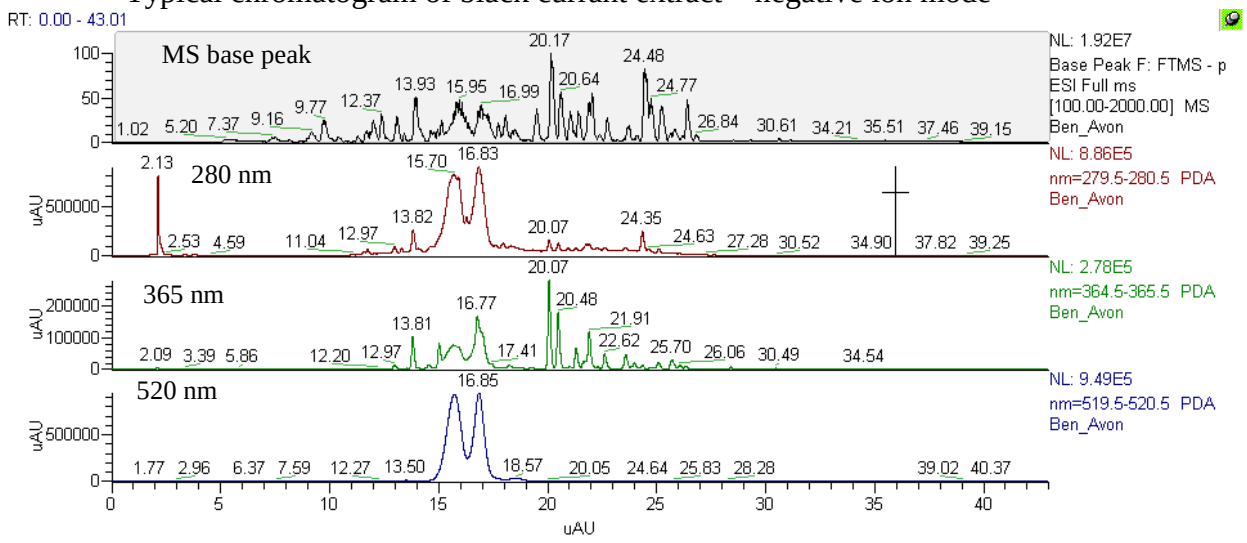
Compound	**Formula**	**MS**	**MS2**
Anthocyanins		**$[M+H]^+$**	
Delphinidin 3-galactoside	$C_{21}H_{20}O_{12}$	465.103	303
Delphinidin 3-glucoside	$C_{21}H_{20}O_{12}$	465.103	303
Delphinidin 3-rutinoside	$C_{27}H_{30}O_{16}$	611.161	465, 303
Cyanidin 3-glucoside	$C_{21}H_{20}O_{11}$	449.108	287
Cyanidin 3-rutinoside	$C_{27}H_{30}O_{15}$	595.166	449, 287
Petunidin 3-rutinoside	$C_{28}H_{32}O_{16}$	625.177	479, 317
Peonidin 3-galactoside	$C_{22}H_{22}O_{11}$	463.124	301
Malvidin 3-galactoside	$C_{23}H_{24}O_{12}$	493.135	331
Peonidin 3-glucoside	$C_{22}H_{22}O_{11}$	463.124	301
Peonidin 3-rutinoside	$C_{28}H_{32}O_{15}$	609.182	463, 301
Delphinidin 3-(6’’-coumaroyl)glucoside	$C_{30}H_{26}O_{14}$	611.140	303
Pelargonidin 3-glucoside	$C_{21}H_{20}O_{10}$	433.113	271
Pelargonidin 3-rutinoside	$C_{27}H_{30}O_{14}$	579.171	433, 271
Petunidin 3-glucoside	$C_{22}H_{22}O_{12}$	479.119	317
Malvidin 3-rutinoside	$C_{28}H_{34}O_{16}$	639.193	493, 331
Cyanidin 3-arabinoside	$C_{20}H_{18}O_{10}$	419.098	287
Cyanidin 3-(6’’-coumaroyl)glucoside	$C_{30}H_{26}O_{13}$	595.145	287
Flavonols		**$[M-H]^-$**	
Myricetin 3-rutinoside	$C_{27}H_{30}O_{17}$	625.140	317
Myricetin 3-glucuronide	$C_{21}H_{18}O_{14}$	493.062	317
Myricetin 3-glucoside	$C_{21}H_{20}O_{13}$	479.083	317
Myricetin 3-(6’’-malonyl)glucoside	$C_{24}H_{22}O_{16}$	565.083	317
Aureusidin glucoside	$C_{21}H_{20}O_{11}$	447.093	285
Quercetin 3-rutinoside	$C_{27}H_{30}O_{16}$	609.146	301
Quercetin 3-glucoside	$C_{21}H_{20}O_{12}$	463.088	301
Quercetin 3-(6’’-malonyl)glucoside	$C_{24}H_{22}O_{15}$	549.088	301
Kaempferol 3-rutinoside	$C_{27}H_{30}O_{15}$	593.151	285
Kaempferol 3-galactoside	$C_{21}H_{20}O_{11}$	447.093	285
Kaempferol 3-glucoside	$C_{21}H_{20}O_{11}$	447.093	285
Isohamnetin 3-rutinoside	$C_{28}H_{32}O_{16}$	623.161	315

The following chromatograms show typical black currant (Ben Avon) profiles and identification of some major components.

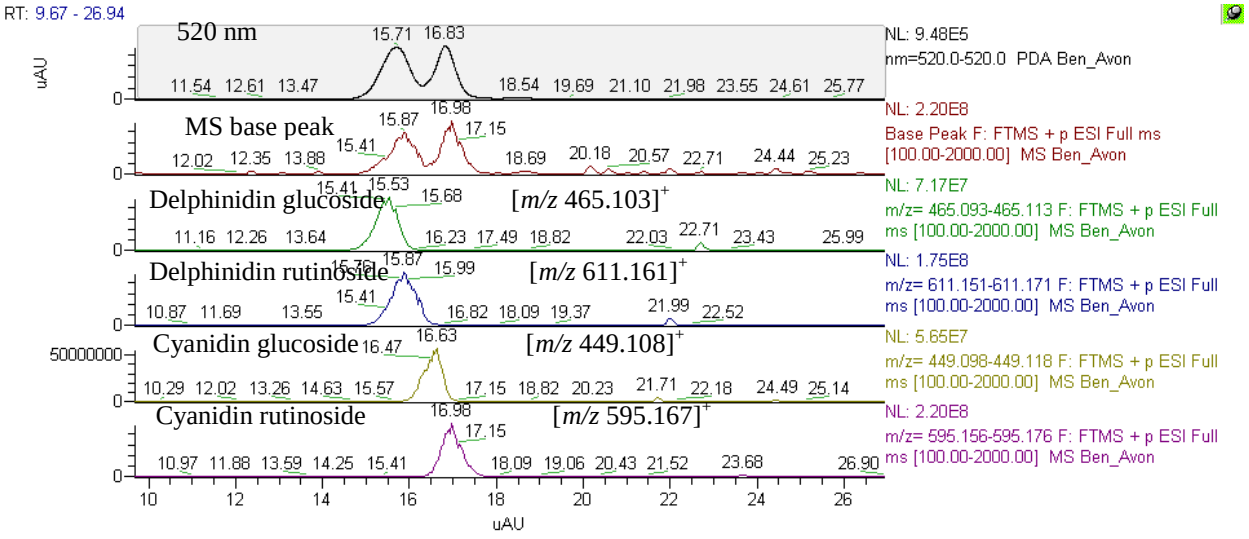
Typical chromatogram of black currant extract – positive ion mode



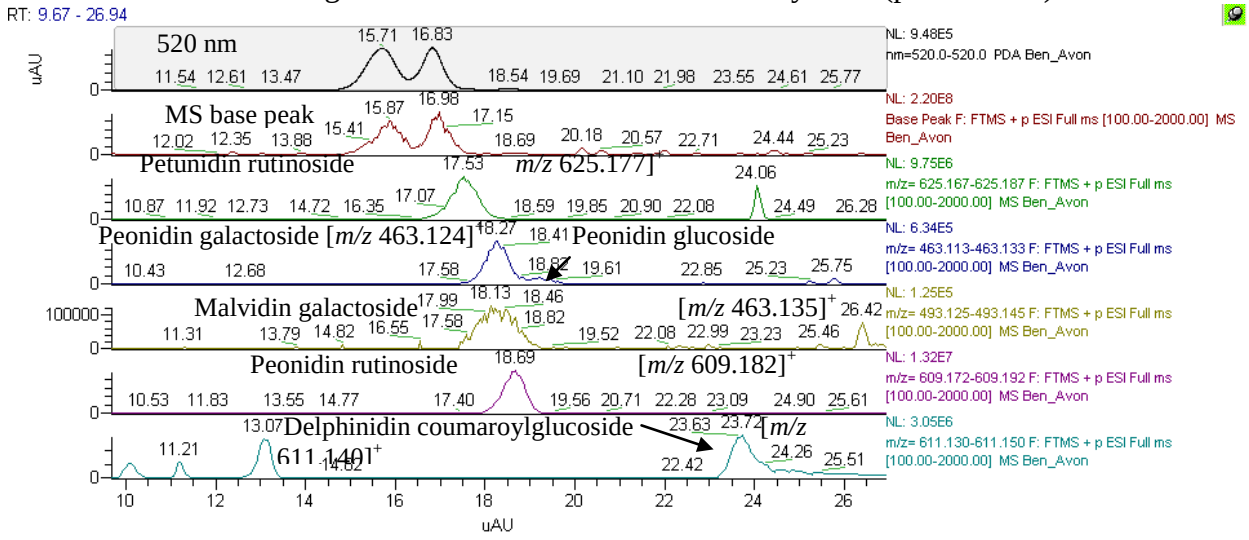
Typical chromatogram of black currant extract – negative ion mode

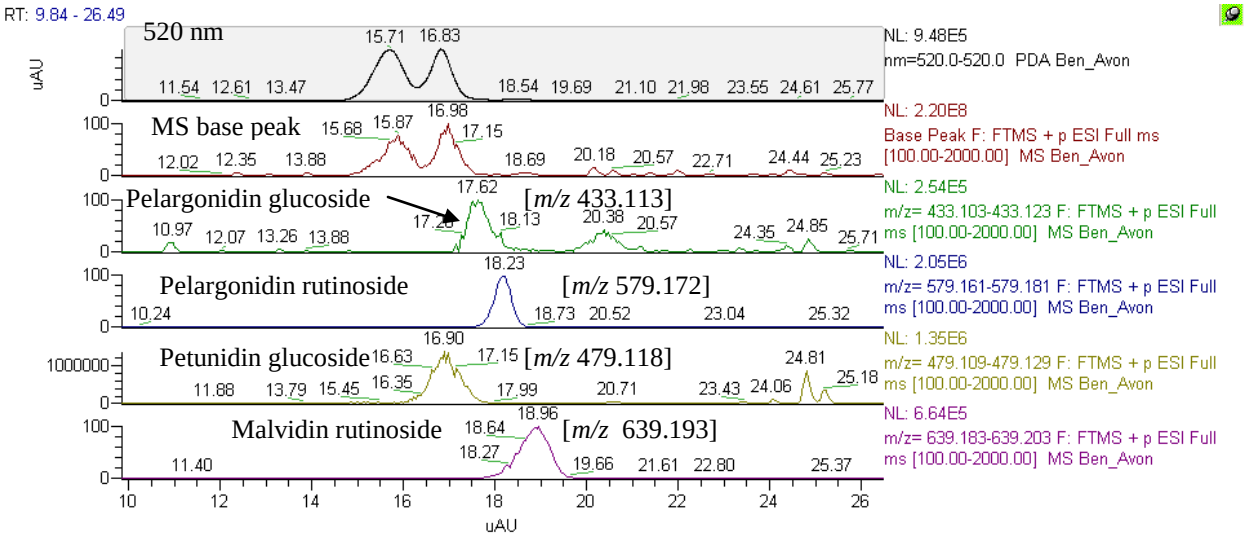


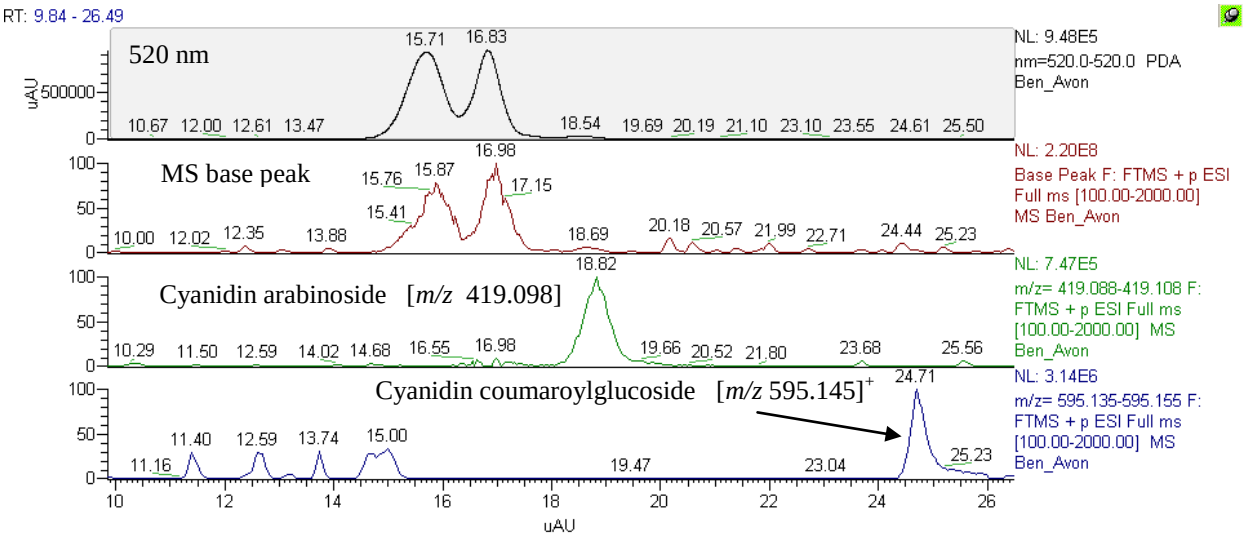
Mass chromatograms of black currant major anthocyanins (positive ion)



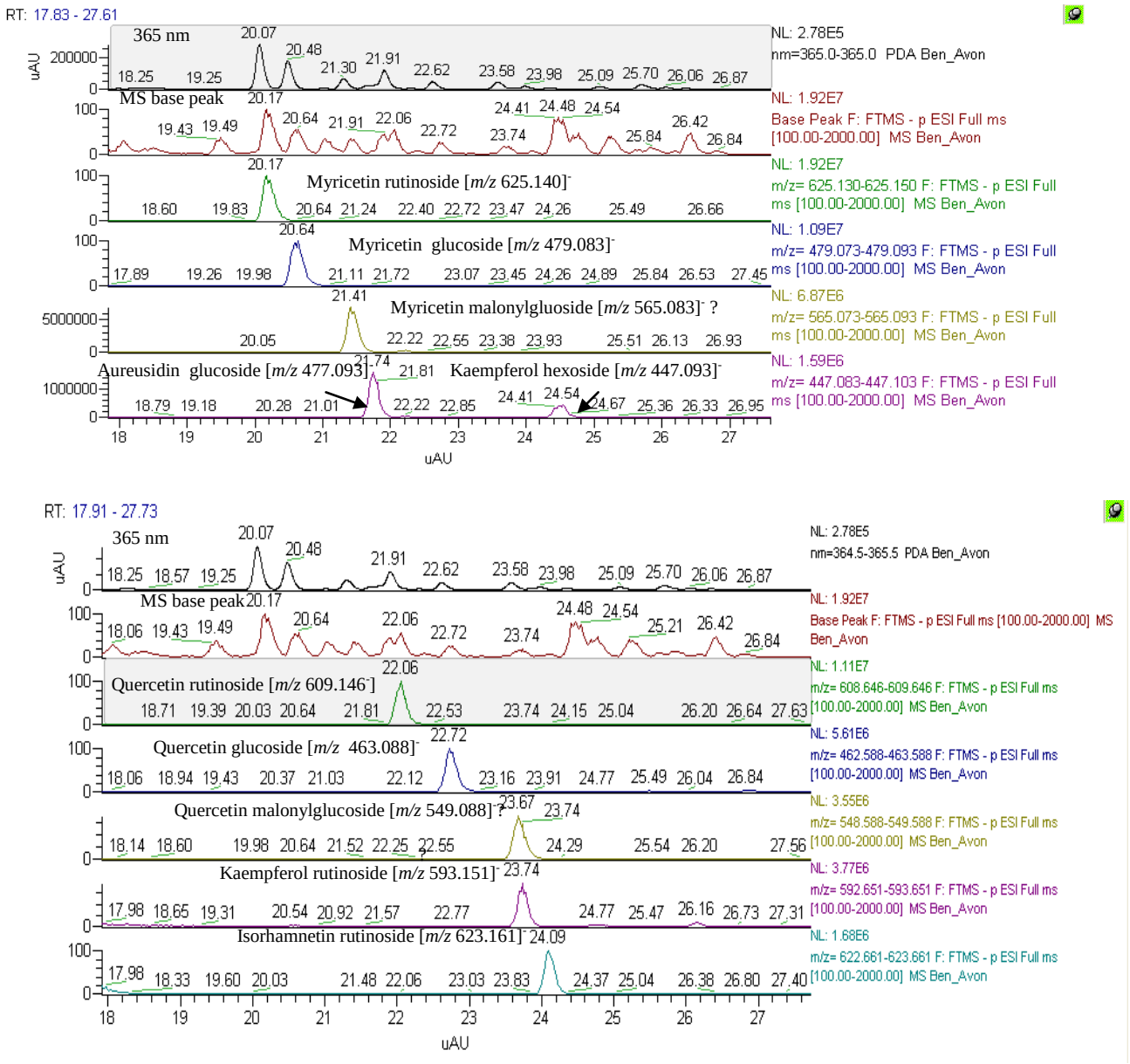
Mass chromatograms of black currant minor anthocyanins (positive ion)




RT: 9.84 - 26.49
520 nm
NL: 9.48E5
nm=520.0-520.0 PDA Ben_Avon
MS base peak
NL: 2.20E8
Base Peak F: FTMS + p ESI Full ms [100.00-2000.00] MS Ben_Avon
Pelargonidin glucoside [m/z 433.113]
NL: 2.54E5
m/z= 433.103-433.123 F: FTMS + p ESI Full ms [100.00-2000.00] MS Ben_Avon
Pelargonidin rutinoside [m/z 579.172]
NL: 2.05E6
m/z= 579.161-579.181 F: FTMS + p ESI Full ms [100.00-2000.00] MS Ben_Avon
Petunidin glucoside [m/z 479.118]
NL: 1.35E6
m/z= 479.109-479.129 F: FTMS + p ESI Full ms [100.00-2000.00] MS Ben_Avon
Malvidin rutinoside [m/z 639.193]
NL: 6.64E5
m/z= 639.183-639.203 F: FTMS + p ESI Full ms [100.00-2000.00] MS Ben_Avon
uAU


RT: 9.84 - 26.49
520 nm
NL: 9.48E5
nm=520.0-520.0 PDA Ben_Avon
MS base peak
NL: 2.20E8
Base Peak F: FTMS + p ESI Full ms [100.00-2000.00] MS Ben_Avon
Cyanidin arabinoside [m/z 419.098]
NL: 7.47E5
m/z= 419.088-419.108 F: FTMS + p ESI Full ms [100.00-2000.00] MS Ben_Avon
Cyanidin coumaroylglucoside [m/z 595.145]+
NL: 3.14E6
m/z= 595.135-595.155 F: FTMS + p ESI Full ms [100.00-2000.00] MS Ben_Avon
uAU

Mass chromatograms of black currant flavonols (negative ion)



2.3.1.4 Blueberry polyphenols

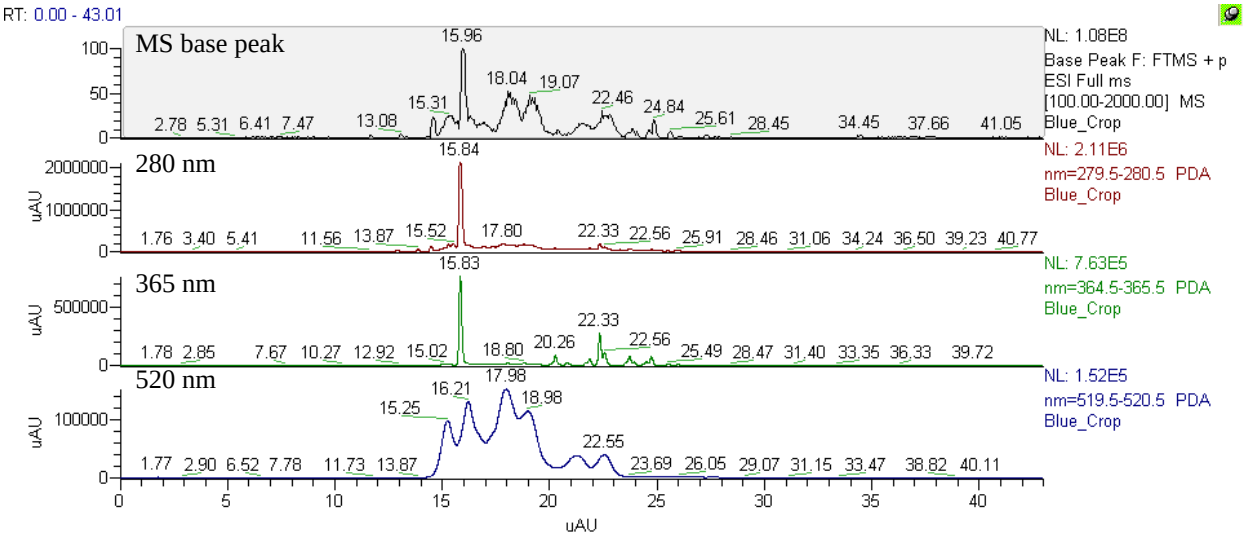
Blueberry contains a complex mixture of anthocyanins (analysed in positive ion mode) and less of flavonols (negative and sometimes positive ion modes).
The following table gives a list of polyphenols found in blueberry and expected masses.

Compound	**Formula**	**MS**	**MS2**
Anthocyanins		**$[M+H]^+$**	
Delphinidin 3-galactoside	$C_{21}H_{20}O_{12}$	465.103	303
Delphinidin 3-glucoside	$C_{21}H_{20}O_{12}$	465.103	303
Cyanidin 3-galactoside	$C_{21}H_{20}O_{11}$	449.108	287
Delphinidin 3-arabinoside	$C_{20}H_{18}O_{11}$	435.093	303
Cyanidin 3-glucoside	$C_{21}H_{20}O_{11}$	449.108	287
Petunidin 3-galactoside	$C_{22}H_{22}O_{12}$	479.119	317
Cyanidin 3-arabinoside	$C_{20}H_{18}O_{10}$	419.098	287
Petunidin 3-glucoside	$C_{22}H_{22}O_{12}$	479.119	317
Peonidin 3-galactoside	$C_{22}H_{22}O_{11}$	463.124	301
Petunidin 3-arabinoside	$C_{21}H_{20}O_{11}$	449.108	317
Peonidin 3-glucoside	$C_{22}H_{22}O_{11}$	463.124	301
Malvidin 3-galactoside	$C_{23}H_{24}O_{12}$	493.135	331
Malvidin 3-glucoside	$C_{23}H_{24}O_{12}$	493.135	331
Peonidin 3-arabinoside	$C_{21}H_{20}O_{10}$	433.113	301
Malvidin 3-arabinoside	$C_{22}H_{22}O_{11}$	463.124	331
Cyanidin 3-(malonyl)glucoside	$C_{24}H_{22}O_{14}$	535.109	287
Cyanidin 3-(6'-acetyl)galactoside	$C_{23}H_{22}O_{12}$	491.119	287
Petunidin pentoside	$C_{25}H_{26}O_{13}$	449.108	317
Delphinidin 3-(malonyl)glucoside	$C_{24}H_{22}O_{15}$	551.104	303
Malvidin 3-(malonyl)glucoside	$C_{26}H_{26}O_{15}$	579.135	331
Delphinidin 3-(6'-acetyl)glucoside	$C_{23}H_{22}O_{13}$	507.114	303
Peonidin 3-(6'-acetyl)galactoside	$C_{24}H_{24}O_{12}$	505.135	301
Cyanidin 3-(6'-acetyl)glucoside	$C_{23}H_{22}O_{12}$	491.119	287
Malvidin 3-(6'-acetyl)galactoside	$C_{25}H_{26}O_{13}$	535.145	331
Petunidin 3-(6'-acetyl)glucoside	$C_{24}H_{24}O_{13}$	521.130	317
Peonidin 3-(6'-acetyl)glucoside	$C_{24}H_{24}O_{12}$	505.135	301
Malvidin 3-(6'-acetyl)glucoside	$C_{25}H_{26}O_{13}$	535.145	331
Flavonols		**$[M-H]^-$**	
Myricetin 3-galactoside	$C_{21}H_{20}O_{13}$	479.083	317
Quercetin diglucoside	$C_{27}H_{30}O_{17}$	625.140	301
Quercetin 3-rutinoside	$C_{27}H_{30}O_{16}$	609.146	463, 301
Quercetin 3-galactoside	$C_{21}H_{20}O_{12}$	463.088	301
Quercetin 3-methoxyhexoside	$C_{22}H_{22}O_{13}$	493.098	463, 301
Quercetin 3-glucoside	$C_{21}H_{20}O_{12}$	463.088	301
Quercetin 3-arabinoside	$C_{20}H_{18}O_{11}$	433.077	301
Quercetin glucuronide	$C_{21}H_{18}O_{13}$	477.067	301
Quercetin 3-glucosylpentoside	$C_{26}H_{28}O_{16}$	595.130	433, 301
Quercetin 3-caffeoylgalactoside	$C_{30}H_{26}O_{15}$	625.119	463, 301
Quercetin 3-caffeoylglucoside	$C_{30}H_{26}O_{15}$	625.119	463, 301
Quercetin 3-oxalylpentoside	$C_{22}H_{18}O_{14}$	505.062	433, 301
Quercetin 3-rhamnoside	$C_{21}H_{20}O_{11}$	447.093	301
Quercetin 3-dimethoxyrhamnoside	$C_{23}H_{24}O_{13}$	507.114	477, 447, 301
Quercetin 3-(6'-acetyl)galactoside	$C_{23}H_{22}O_{13}$	505.098	463, 301
Quercetin 3-(6'-acetyl)glucoside	$C_{23}H_{22}O_{13}$	505.098	463, 301

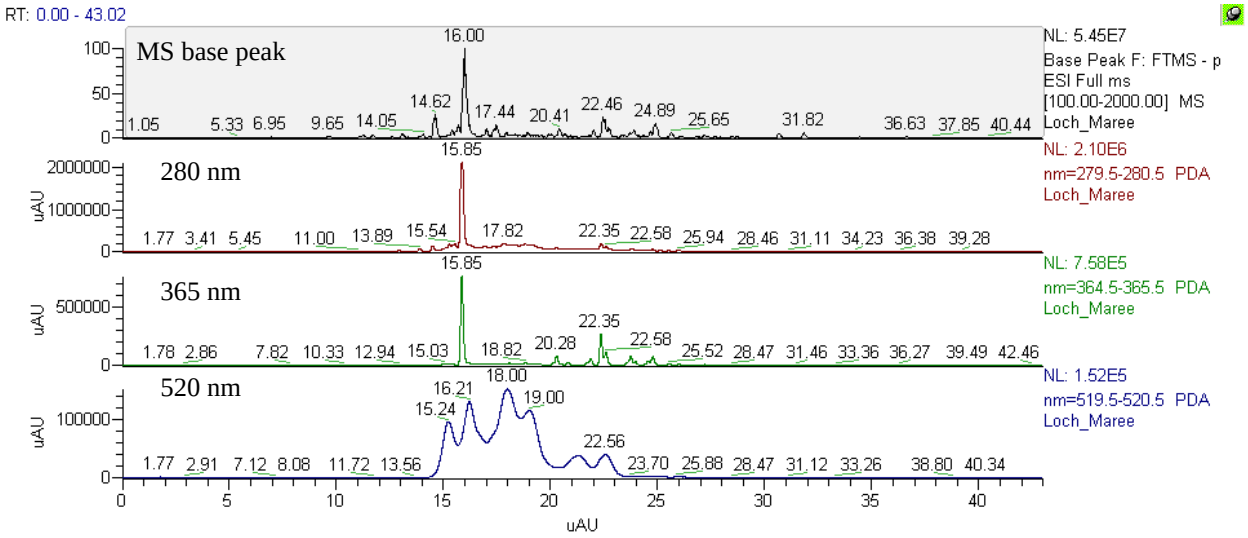
Quercetin	$C_{15}H_{10}O_6$	301.035	

The following chromatograms show typical blueberry (Blue Crop) profiles and identification of some major components.

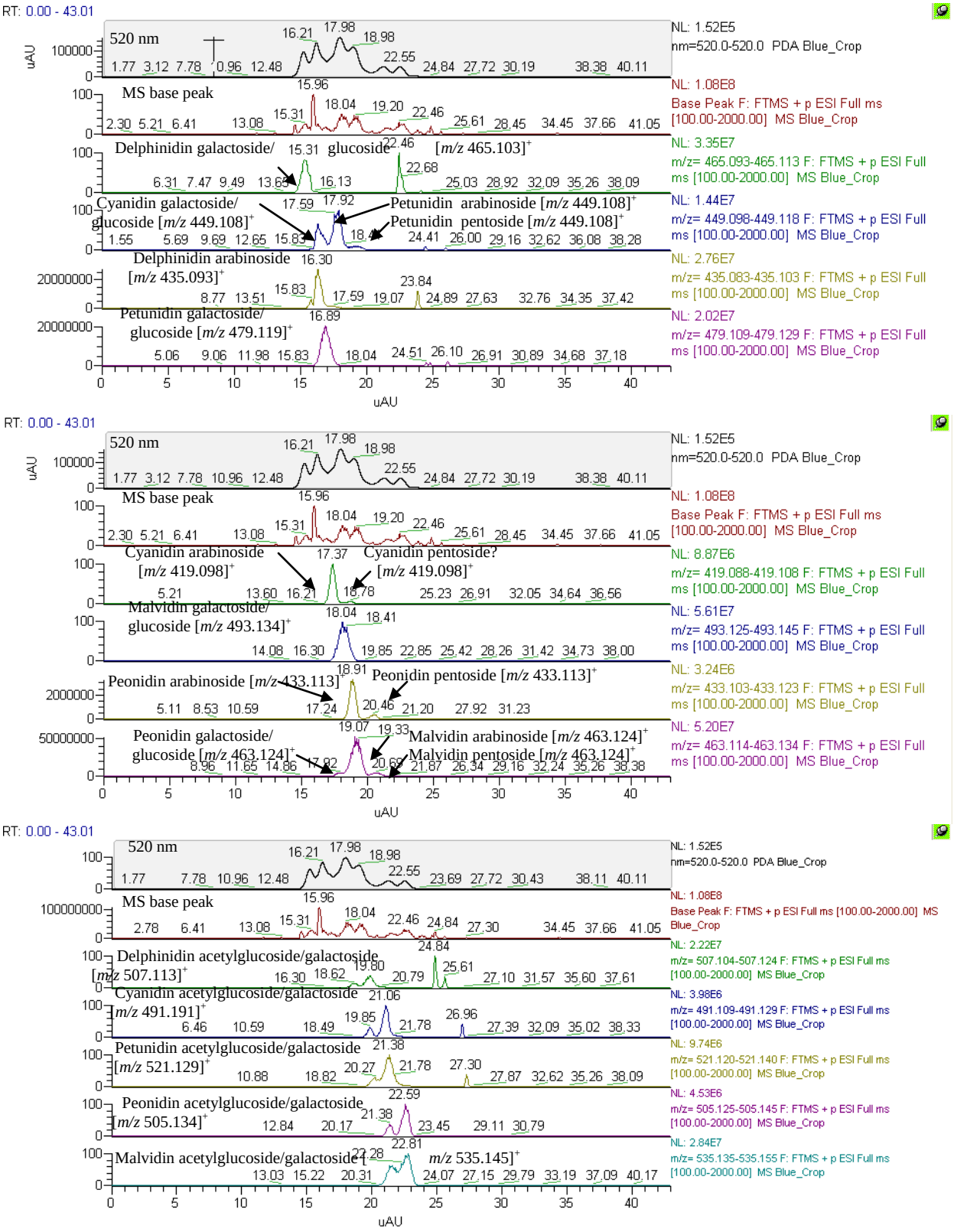
Typical chromatogram of blueberry extract – positive ion mode



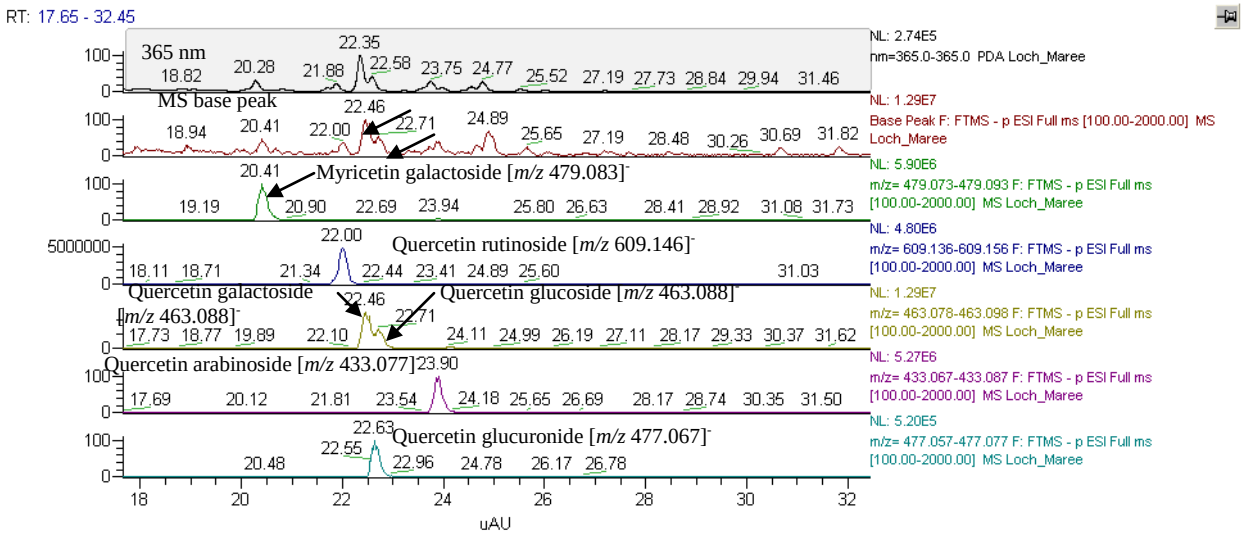
Typical chromatogram of blueberry extract – negative ion mode

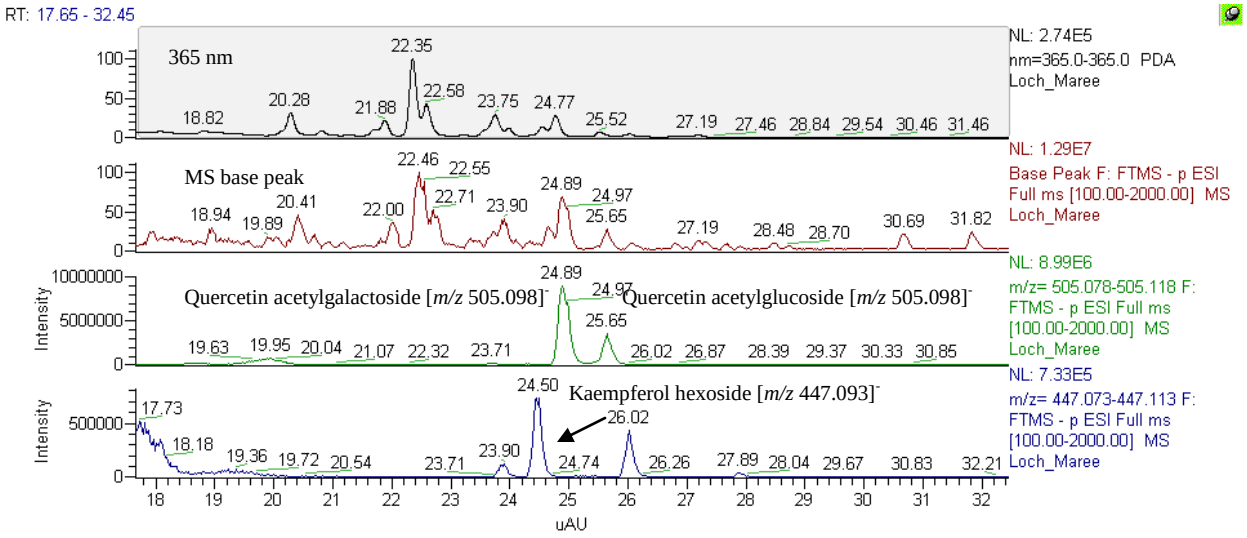


Mass chromatograms of blueberry anthocyanins (positive ion)



Mass chromatograms of blueberry flavonols (negative ion)





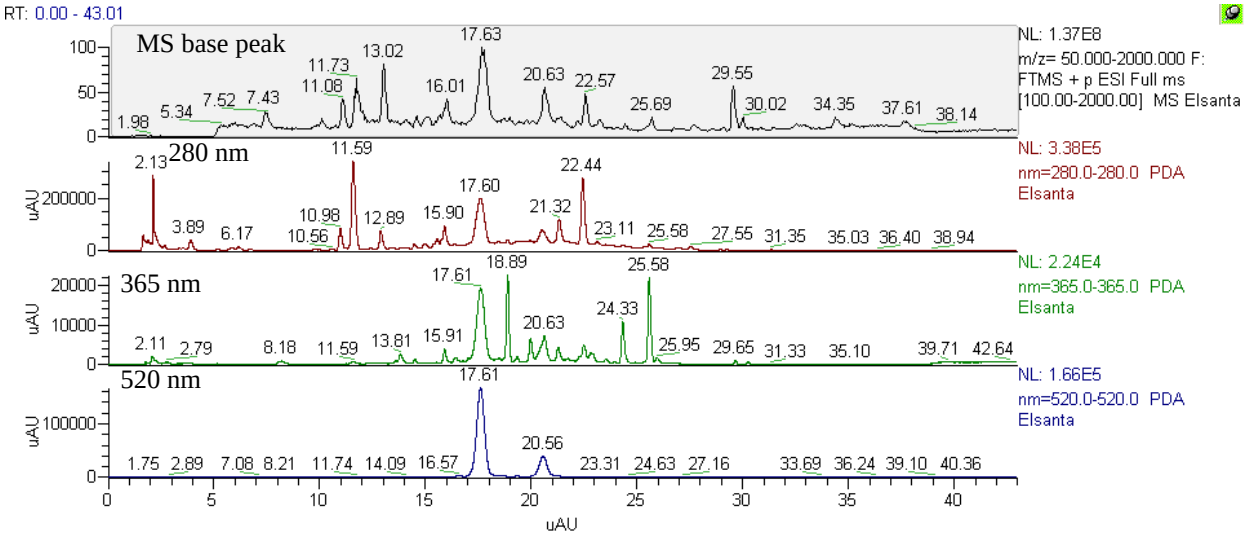
2.3.1.5 Strawberry polyphenols

Strawberry contains large amounts of proanthocyanidins and less anthocyanins (analysed in positive ion mode) and ellagitannins (negative ion mode). Flavonols (negative and sometimes positive ion modes) are minor components.
The following table gives a list of polyphenols found in strawberry and expected masses.

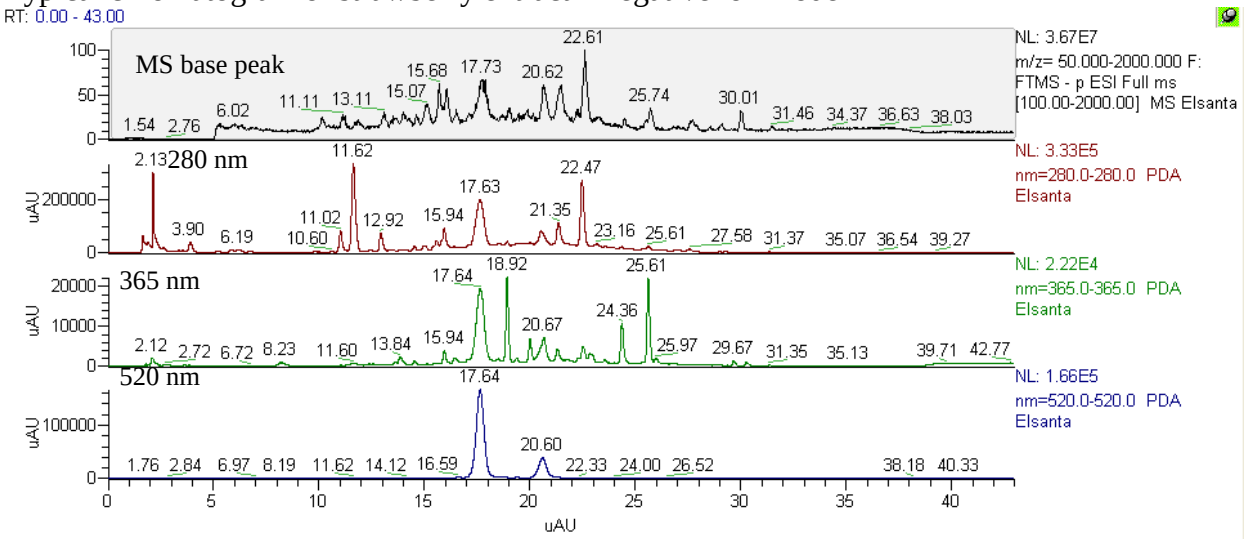
Compound	**Formula**	**MS**	**MS2**
Anthocyanins		**$[M+H]^+$**	
Cyanidin 3-sophoroside	$C_{27}H_{30}O_{16}$	611.161	287
Cyanidin 3-glucoside	$C_{21}H_{20}O_{11}$	449.108	287
Cyanidin 3-rutinoside	$C_{27}H_{30}O_{15}$	595.166	449, 287
Pelargonidin 3-glucoside	$C_{21}H_{20}O_{10}$	433.113	271
Petunidin 3-glucoside	$C_{22}H_{22}O_{12}$	479.119	317
Pelargonidin 3-rutinoside	$C_{27}H_{30}O_{14}$	579.171	433, 271
Pelargonidin 3-(malonyl)glucoside	$C_{23}H_{22}O_{11}$	519.114	433, 271
Pelargonidin 3-(6'-acetyl)glucoside	$C_{24}H_{22}O_{13}$	475.124	271
Flavonol		**$[M-H]^-$**	
Quercetin 3-glucuronide	$C_{21}H_{18}O_{13}$	477.067	301
Kaempferol 3-coumaroylglucoside	$C_{30}H_{26}O_{13}$	593.130	447, 285
Kaempferol 3-glucoside	$C_{21}H_{20}O_{11}$	447.093	285
Kaempferol 3-acetylglucoside	$C_{23}H_{22}O_{12}$	489.103	285
Kaempferol 3-glucuronide	$C_{21}H_{18}O_{12}$	461.072	285
Ellagitannins/ellagic acid derivatives		**$[M-H]^-$**	
Bis-HDDP-glucoside	$C_{34}H_{24}O_{22}$	783.068	301
Galloyl bis-HHDP-glucoside 1	$C_{41}H_{28}O_{26}$	935.079	633, 301
Galloyl bis-HHDP-glucoside 2	$C_{41}H_{28}O_{26}$	935.079	633, 301
Sanguiin-H6	$C_{82}H_{54}O_{52}$	$[1869.150]^{-1}$ $[934.071]^{-2}$	1697, 1567, 1407, 897, 783, 633, 301
Lambertianin C	$C_{123}H_{80}O_{78}$	$[1401.107]^{-2}$	2019, 1869, 1567, 1402, 935, 897, 633, 301
Ellagic rhamnoside 1	$C_{20}H_{16}O_{12}$	447.056	301
Ellagic rhamnoside 2	$C_{20}H_{16}O_{12}$	447.056	301
Flavan-3-ols/proanthocyanidins		**$[M-H]^-$**	
Catechin	$C_{15}H_{14}O_6$	289.071	
Dimer (Cat-Cat)	$C_{30}H_{26}O_{12}$	577.135	
Dimer (Cat-Afz)	$C_{30}H_{26}O_{11}$	561.140	
Trimer (Cat-Cat-Cat)	$C_{45}H_{38}O_{18}$	865.198	
Trimer (Cat-Cat-Afz)	$C_{45}H_{38}O_{17}$	849.203	
Trimer Cat-Afz-Afz)	$C_{45}H_{38}O_{16}$	833.208	
Tetramer (Cat-Cat-Cat-Cat)	$C_{60}H_{50}O_{24}$	1153.261	
Tetramer (Cat-Cat-Cat-Afz)	$C_{60}H_{50}O_{23}$	1137.266	

The following chromatograms show typical strawberry (Elsanta) profiles and identification of some major components.

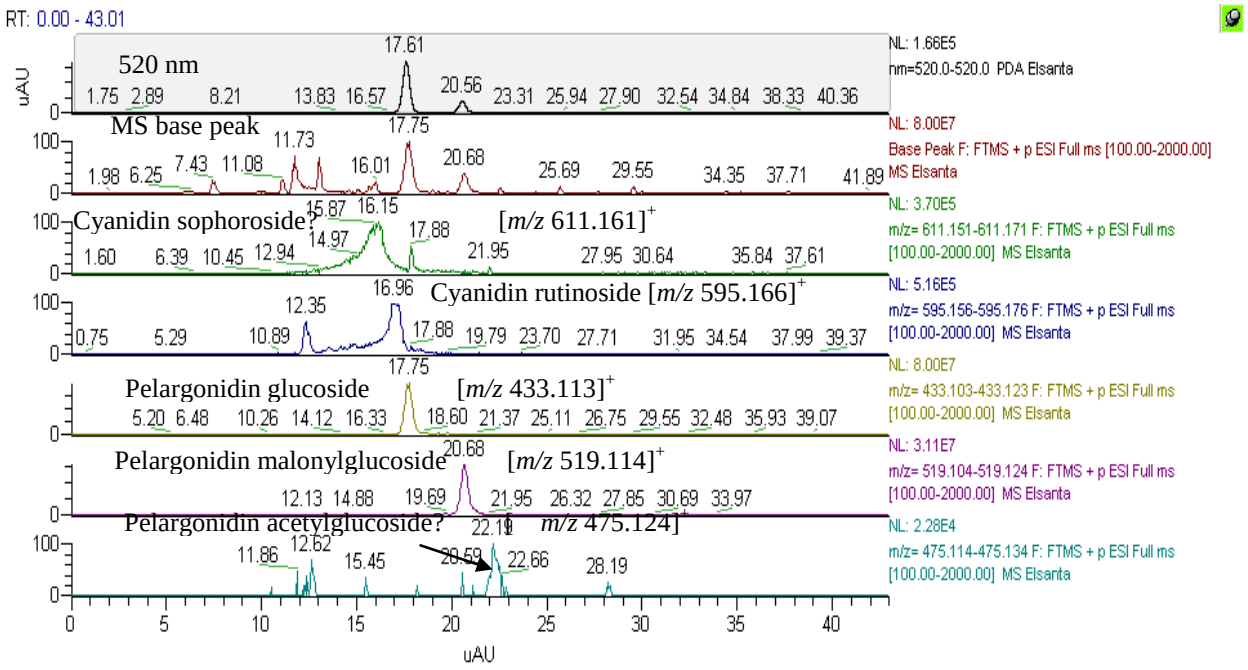
Typical chromatogram of strawberry extract – positive ion mode



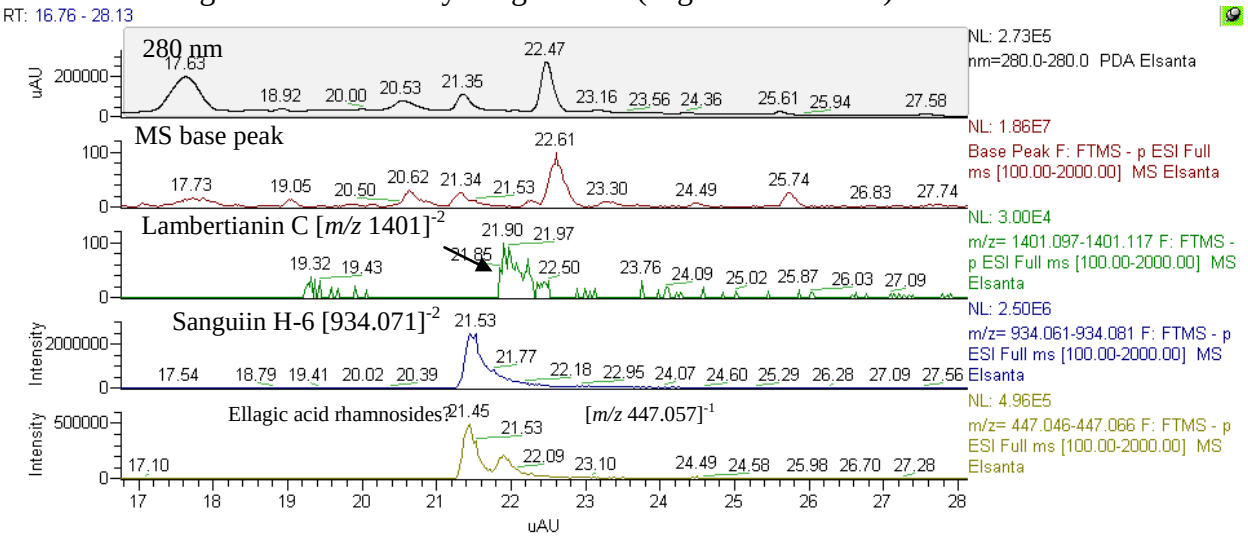
Typical chromatogram of strawberry extract – negative ion mode



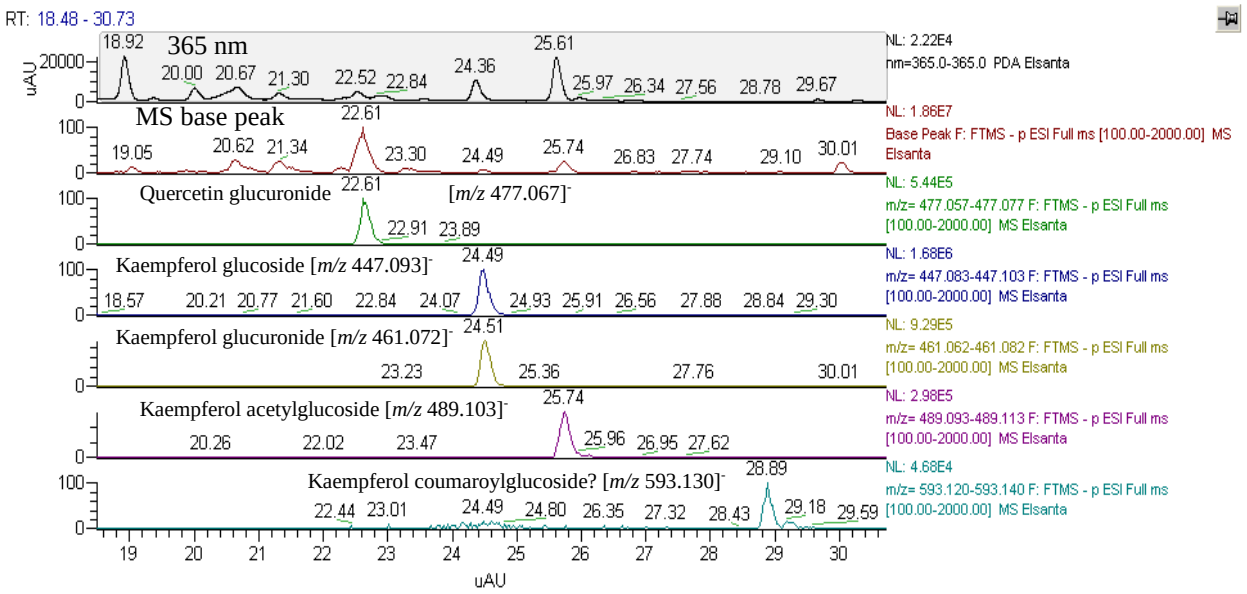
Mass chromatograms of strawberry anthocyanins (positive ion)



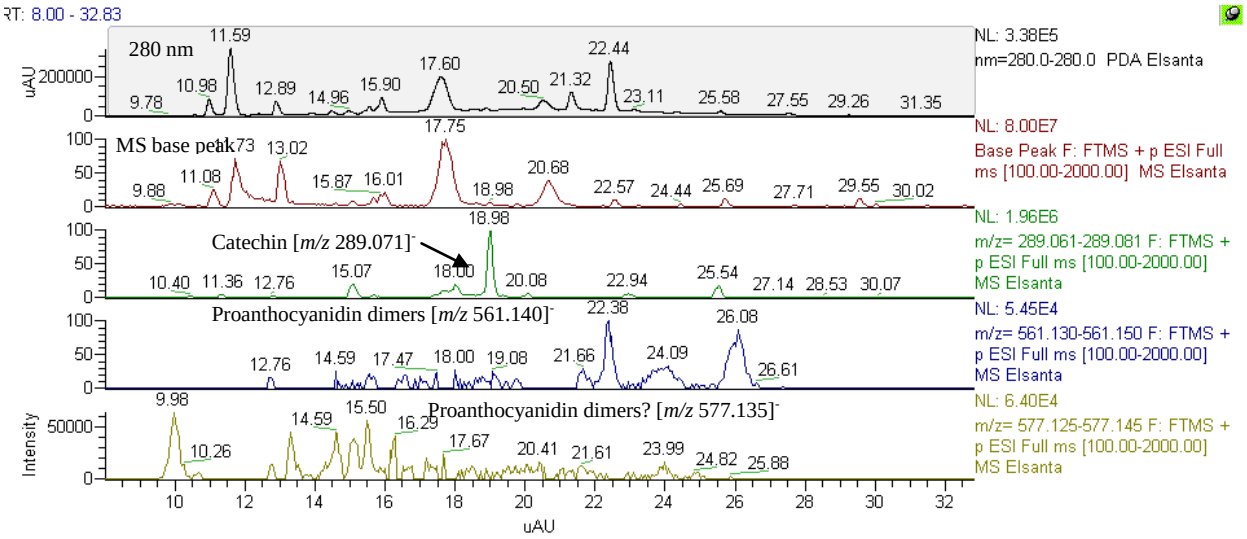
Mass chromatograms of strawberry ellagitannins (negative ion mode)



Mass chromatograms of strawberry flavonols (negative ion mode)



Mass chromatograms of strawberry flavan-3-ols (negative ion mode)



2.3.2 Quantification

For quantification of compounds, morin is added to the juice sample at a concentration of 0.1 mg mL^{-1} (add 100 μl of 1 mg mL^{-1} morin to 900 μl juice). Suitable external standards similar to the compounds of interest are prepared to cover the concentrations in the juice. Morin is added to each standard at the same concentration (0.1 mg mL^{-1}) as in the juice and the standards are analysed together with samples. Samples and standards are processed so that the ratio of the area of a specific ion of the compound of interest to that for morin internal standard (*m*/*z* 301 and 303 for negative and positive mode, respectively) is calculated. In Excel, calibration curves of response ratios against concentration are plotted for the standards and best-fit equations are used to calculate the concentrations in the juices.