Development of UHPLC and UHPSFC-MS assays to determine the concentration of Bitrex™ and sodium saccharin in homemade facemask fit testing solutions

Julie M. Herniman* and G John Langley

Faculty of Engineering and Physical Sciences, School of Chemistry, University of Southampton, Highfield Southampton SO17 1BJ, UK

*For correspondence jmh7@soton.ac.uk

ABSTRACT

Rationale

Fast and easily transferable chromatography-mass spectrometry assays were required to detect and quantify the amount of Bitrex™ and sodium saccharin in homemade facemask fit testing solutions.

Methods

Bitrex™ solutions were analysed using reversed phase ultrahigh-performance liquid chromatography coupled with positive ion electrospray ionisation mass spectrometry (UHPLC-MS). Separation was achieved using a mobile phase gradient with an Acquity BEH C18 packed column. Sodium saccharin solutions were analysed using ultrahigh-performance supercritical fluid chromatography coupled with negative ion electrospray ionisation (UHPSFC-MS). Separation

was achieved using isocratic elution with an Acquity UPC^2 Torus Diol packed column and a methanol (25 mM ammonium acetate) co-solvent.

Results

Calibration curves obtained using the ratio of the active compound an internal standard achieved $R^2 > 0.99$. Samples analysed prior to and after an autoclave sterilisation process and bottling give repeatable measurements within 10% of the expected concentration.

Conclusions

The two assays afford a fast robust and quantitative analytical method for the detection of the active components used to test the efficacy of the homemade facemask testing solutions.

1. INTRODUCTION

In extreme environments where airborne pollutants may be present *e.g.* toxic gases, fumes, vapours, and other harmful pollutants it is essential that personal protective equipment is worn and fitted correctly. In hospitals and care homes the use of respirators and face protection ensures that contaminants do not cause potential harm to the wearer. Masks work to protect individuals from spreading their own saliva and bodily fluids into the wider environment. Whilst a respirator face mask works to mitigate and filter out noxious and toxic chemicals/odours, vapours, and other harmful pollutants from the air. [1] All protective equipment must be fitted correctly and adjusted individually to the wearer's face. A Face Fit Test is normally undertaken prior to the respirator or facemask being worn and needs to be checked regularly. There are two tests as defined by Occupation Health and Standard Association (OSHA) in 29 CFR 1910.134. [2] The Qualitative Fit Test (QLFT) is a simple pass/fail test that measures the user's response to a test solution. The Quantitative Fit Test (QNFT) is an assessment of the adequacy of fit of the respirator by measuring the amount of leakage into the respirator. [3] Two different solutions are commonly used to assess disposable and reusable half-masks, denatonium benzoate otherwise known by the brand name Bitrex™ [4] and sodium saccharin. [5] The recent outbreak of COVID-19 in the UK has led to shortages of these commercial spray solutions as demand has significantly increased in line with increased usage of masks and respirators. Chemists at the University of

Southampton have produced homemade solutions [6] following a method adapted from Fakherpour *et al.* [7] and made to the British Standard BS ISO 16975-3:2017 [8] and the US standard set out by OHSA in 29 CFR 1910.134. [2]

An analytical quality control (QC) method was required to speedily assess the quantity of taste test compound in each prepared batch solution. This was required immediately after batch preparation and following an autoclave sterilisation (> 120 ° C) and subsequent bottling process. A minimum of 10 % of the bottles were tested prior to distribution.

The initial aim of the analytical QC methodology was to develop fast, easily transferable, fit for purpose tests that could be undertaken across many analytical laboratories. Reversed phase (RP) high or ultrahigh-performance liquid chromatography (UHPLC) is one of the most common instruments available to a QC laboratory, hence this separation technique coupled to mass spectrometry was initially attempted for the analysis of both solutions adapted from several published methods. [9-14]

Bitrex™ is a quaternary ammonium compound, therefore positive ion electrospray ionisation is required for the analysis and negative ion electrospray ionisation is required for the analysis of the sodium saccharin salt. (Figure 1) Whilst reversed phase UHPLC-MS methods were successfully developed for both the Bitrex™ and sodium saccharin an alternative chromatographic technique, ultrahigh-performance supercritical fluid chromatography - mass

spectrometry (UHPSFC-MS) was also investigated for both assays. This would maximise usage of available instrumentation at the University of Southampton, Chemistry Mass Spectrometry Laboratory. The two QC assays could then run in parallel and both assays could also be undertaken on either instrument. Here the optimum chromatography and mass spectrometry methods are reported for each assay (RP-UHPLC-MS for Bitrex™ and UHPSFC-MS for sodium saccharin).

In its simplest form UHPSFC can be considered as a surrogate normal phase chromatography technique. Supercritical carbon dioxide ($scCO_2$) is the primary mobile phase used and is a supercritical fluid above its critical point of 31.1 °C and 73.8 bar where it has properties intermediate between those of a gas and a liquid. [15-18] For many reasons $scCO_2$ is the most commonly used mobile phase; its critical point is easily obtainable, it is readily available, inexpensive, considered green and relatively safe to use. [19]

UHPSFC can be easily coupled to a mass spectrometer using an atmospheric pressure ionisation source with a flow splitter. A make-up solvent, such as methanol with formic acid, is delivered to the mass spectrometer *via* a splitter configuration to promote ionisation and ensure a stable spray.

2. EXPERIMENTAL

2.1 Chemicals

Acetonitrile, methanol, water (LC-MS grade) and formic acid were purchased from Thermo Fisher Scientific (Loughborough, UK). Denatonium benzoate,

oxybutynin chloride, vanillic acid and ammonium acetate were purchased from Sigma-Aldrich (Gillingham, UK). Sodium saccharin was obtained from Vimto Soft Drinks-Nichols plc (Newton-Le-Willows, UK) and Nutraceutical Group Europe (Redhill, UK) and used without further purification. Food grade carbon dioxide was purchased from BOC Special Gases (Manchester, UK).

2.2 Stock Solutions

The standard compounds (Bitrex™ and sodium saccharin) were prepared volumetrically at a concentration of 10 mg made up to 10 mL (methanol) to give stock solutions of 1mg/mL.

2.3 Internal Standard Preparation

The internal standard compounds (oxybutynin chloride and vanillic acid) were initially prepared at a concentration of 1mg/mL in methanol and then diluted using volumetric dilution to the appropriate concentration.

2.4 Calibration Preparation

Standard calibration solutions were prepared volumetrically for Bitrex™ containing nominally 20, 50, 100, 150 and 200 ng/mL with oxybutynin chloride as the internal standard at 100 ng/L. Standard calibration curves were prepared for sodium saccharin containing nominally 5, 10, 15, 20, 32.5 and 50 µg/mL with vanillic acid as the internal standard at 10 µg/mL. The concentration of the standards was selected to ensure that the calibration curves correspond to the linear ionisation response region of each instrument.

2.5 Sample Preparation

Each batch of Bitrex™ and sodium saccharin is prepared at two concentrations, one for SENSITIVITY (to test the response of an individual) and one TEST solution to test the facemask once it has been fitted to the individual. 1 mL of each batch solution is removed from the bulk and prepared for QC analysis. The Bitrex™ SENSITIVITY solution is prepared at a concentration of 135 mg/mL and this is serially diluted using methanol containing an internal standard oxybutynin chloride. The first step is a 1:1 dilution using the internal standard at 200 ng/mL followed by dilutions using the internal standard at 100 ng/mL to a concentration of 67.5 ng/mL. (x 2000 dilution). The TEST solution is at 1.69 g/L and this is serially diluted using methanol containing the same internal standard to a concentration of 84.7 ng/mL. (x 20,000 dilution) The saccharin SENSITIVITY solution is prepared at a concentration of 8.3 mg/mL and is serially diluted using methanol containing an internal standard vanillic acid. The first step is a 1:1 dilution using the internal standard at 20 µg/mL followed by dilutions using the internal standard at 10 µg/mL to a concentration of 10.4 µg/mL. (x 800 dilution) The TEST solution is at 536 mg/mL and is serially diluted using methanol containing the same internal standard to a concentration of 26.8 ug/mL. (x 20,000 dilution)

2.6 Chromatography

Separations were performed for the Bitrex™ samples using an Acquity ultrahigh-performance liquid chromatograph (Waters, Manchester, UK.) with a Waters BEH C18 column, 1.7 μm particle size, 2 × 50 mm. The column was held at 50 °C in a column oven and 2.0 μL of each sample was injected. Solvent A, water 0.2 % formic acid and solvent B, acetonitrile 0.2 % formic were used for separation at a flow rate of 0.6 mL/min. A gradient elution was performed using the method in Table 1 and a 1-minute isocratic pre-run was used for column equilibration.

Separations were performed for the sodium saccharin samples using an Acquity ultrahigh-performance convergence chromatograph (UPC^2, Waters, Manchester, UK.) with a Waters Torus Diol packed column, 1.7 μm particle size, 3 × 100 mm. The column was held at 40 °C in a column oven and 2.0 μL of each sample was injected. $scCO_2$ with methanol (25 mM ammonium acetate) co-solvent were used for separation at a flow rate of 1.5 mL/min. The $scCO_2$ back pressure of the system was set to 150 bar. An isocratic elution was performed using co-solvent B at 40% for 1.5 minutes.

2.7 Mass Spectrometry

Positive ion ESI mass spectra were recorded using a tandem quadrupole mass spectrometer (TQD) and ESCi multi-mode ionisation source (Waters, Manchester, UK) with the following conditions: capillary voltage 2.5 kV; cone voltage 20 V; extractor 3.0 V; source temperature 150°C; desolvation temperature 600°C; desolvation gas flow 600 L/h (nitrogen) and acquisition and data

processing was achieved using MassLynx™ version 4.1 and TargetLynx. Selected ion monitoring (SIM) was used to detect $[M]^+$ for Bitrex™, nominal *m/z* 325, and $[M + H]^+$ for the internal standard oxybutynin chloride nominal mass *m/z* 358 with a dwell time of 0.088 s.

Negative ion ESI mass spectra were recorded using a single quadrupole mass spectrometer (SQD2) and ESCi multi-mode ionisation source (Waters, Manchester, UK) with the following conditions: capillary voltage 2.5 kV; cone voltage 20 V; extractor 3.0 V; source temperature 150°C; desolvation temperature 500°C; desolvation gas flow 650 L/h (nitrogen) and acquisition and data processing achieved using MassLynx™ version 4.1 and TargetLynx. An isocratic solvent manager (ISM) (Waters, Manchester, UK) was used to introduce the make-up solvent, methanol (50 µM ammonium acetate) at a flow rate of 0.45 mL/min.

Selected ion monitoring (SIM) was used to detect the deprotonated molecules $[M - H]^-$ for saccharin, nominal *m/z* 182, and the internal standard vanillic acid, nominal *m/z* 167 with a dwell time of 0.163 s.

3. RESULTS AND DISCUSSION

3.1 Method development

In the first instance, commonly available reversed phase UHPLC-MS assays requiring minimal modification and customisation were used to facilitate the QC analysis for both compounds. Generic gradients were used, starting at 5%

solvent B increasing to 100% solvent B over 5 minutes with a standard BEH C18 column. The first task was to identify a suitable internal standard for each assay. Oxybutynin chloride was selected for the Bitrex™ assay since this was readily available in the laboratory and is routinely used as a component of the system suitability test used for RP UHPLC-MS (Figure 2A). It has a similar retention time to the Bitrex™ and the positive ion electrospray ionisation efficiency closely matched to that of Bitrex™. To accelerate the analysis, the generic gradient was optimised to 20% solvent B increasing to 100% solvent B in 1.5 minutes with a 1-minute isocratic pre-run (Figure 3). The linear response for the Bitrex™ using this assay was defined using solutions of standard Bitrex™ ranging from 1 ng/mL to 1000 ng/mL. Single ion monitoring (SIM) for the molecule was used to improve peak profile and ensure that the methods could be transferred to other analysers. Once the linear response region has been defined, calibrations solutions were prepared (20-200 ng/mL) containing the internal standard at a concentration within the middle of the curve (100 ng/mL). A 5-point calibration curve was constructed automatically with TargetLynx using the ratio of the Bitrex™ to the internal standard, to give R^2 values of > 0.99. (Figure 4)

The same assay was attempted for the analysis of the sodium saccharin using negative ion electrospray ionisation, however the peak shape for sodium saccharin was initially poor. This was somewhat improved by changing to water

as the internal standard diluent to give a fit for purpose assay. Saccharin analysis was developed using an in-house UHPSFC-MS assay, 10% co-solvent (methanol 25 mM ammonium acetate) increasing to 40% solvent co-solvent over 5 minutes using a Torus Diol column. Vanillic acid was selected as the internal standard for this assay since this was readily available in the laboratory, the negative ion electrospray ionisation closely matched to that of sodium saccharin and gave similar good chromatographic peak shape (Figure 2B). To accelerate the analysis, the assay was optimised using an isocratic 40% co-solvent method for 1.5 minutes (Figure 5) hence removing the need for a pre-run column equilibration method. The linear response for the sodium saccharin using this assay was defined using solutions of standard sodium saccharin ranging from 1 µg/mL to 1000 µg/mL using SIM for the deprotonated molecule, to improve peak profile. Once the linear response region has been defined, calibrations solutions were prepared (5-50 µg/mL) containing the internal standard at a concentration in the middle of the curve (20 µg/mL). A 5-point calibration curve was constructed automatically with TargetLynx using the ratio of the sodium saccharin to the internal standard, to give R^2 values of > 0.99.

3.2 Application of methodology

Following preparation of the bulk solutions by the University of Southampton chemists, 1 mL of solution is removed ready for QC analysis. A calibration curve is created with the standards over the linear response range of the

instrument prior to analysis and this must give an R^2 value > 0.99 before proceeding. If the R^2 value is < 0.99 then the calibration curve must be repeated and adjusted if necessary. Each solution is diluted with the pre-prepared internal standard solution in methanol to the appropriate concentration (Bitrex™, 67.5 and 84.75 ng/mL SENSE and TEST respectively, sodium saccharin 10.4 and 26.8 µg/mL SENSE and TEST respectively). These pre-autoclave solutions are then measured in triplicate ensuring a solvent blank is analysed between each sample to monitor and prevent carry over. The data are plotted against the calibration curve using TargetLynx and the results recorded. If the required concentration is calculated to be 10-12% of the required concentration, then the batch solution can proceed to the bottling and autoclave stage. Following the autoclave stage, a minimum of 10% of bottles are randomly selected and 1 mL of solution is removed for QC analysis. These solutions are diluted to the appropriate concentration using the internal standard solution in methanol. These samples are analysed in triplicate ensuring a solvent blank in between each analysis to monitor and prevent carry over. The data are plotted against the calibration curve using TargetLynx and the results recorded. If the required concentration is calculated to be within 10% of the required concentration, then the bottled solutions can be released for distribution. (Tables 2 and 3)

CONCLUSIONS

The reversed phase UHPLC-MS and UHPSFC-MS assays developed here deliver fast, robust and quantitative for the analysis of homemade Bitrex™ and sodium saccharin solutions. Calibration curves acquired using SIM methods over the linear response range for each instrument give R^2 values > 0.99. If UHPSFC-MS is not available, then an alternative reversed phase UHPLC-MS method could be utilized providing calibration and samples solution are made up using water rather than methanol as a solvent.

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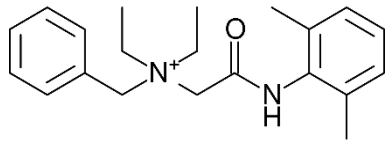
doi:1021/acs.energyfuels.5b00103

FIGURES

A 

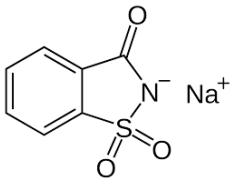
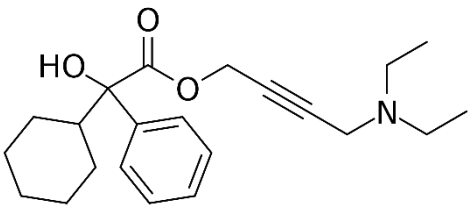
B 

Figure 1. Chemical structures of A) Bitrex™ (Denatonium benzoate) and B) Sodium saccharin

A 

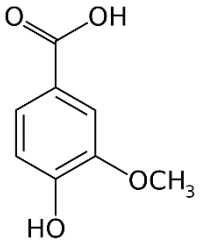
B 

Figure 2. Chemical structures of internal standards A) Oxybutynin chloride and B) Vanillic acid

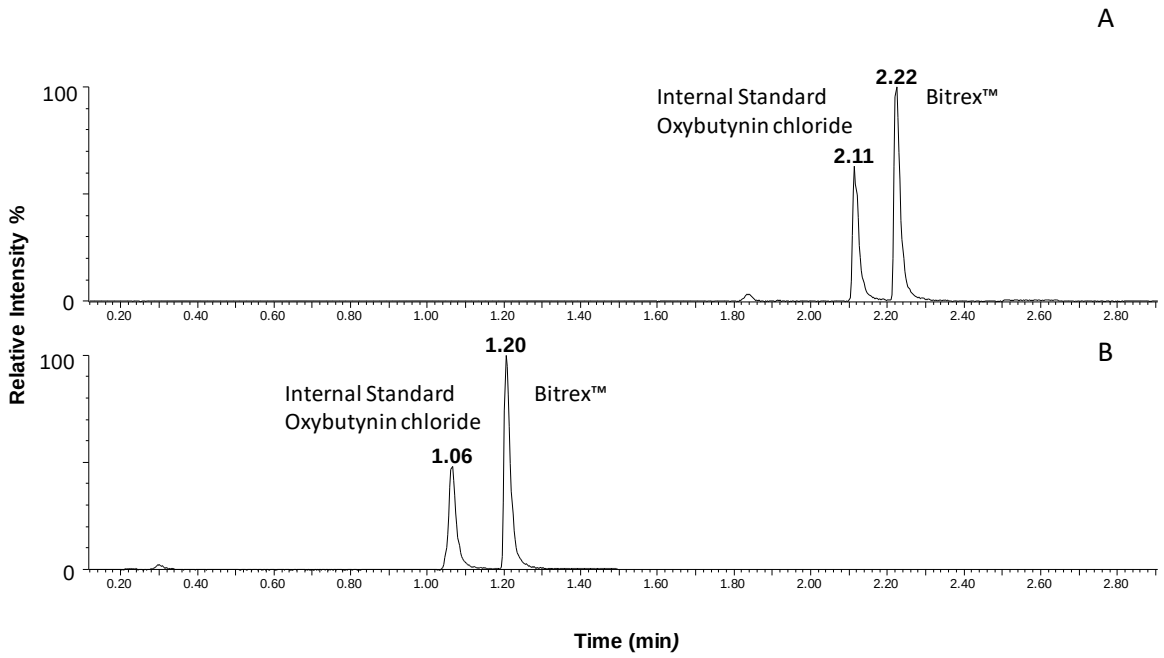


Figure 3. Reversed Phase UHPLC-MS SIR chromatograms for Oxybutynin chloride and Bitrex™ using A) 5-minute gradient and B) 1.5-minute gradient

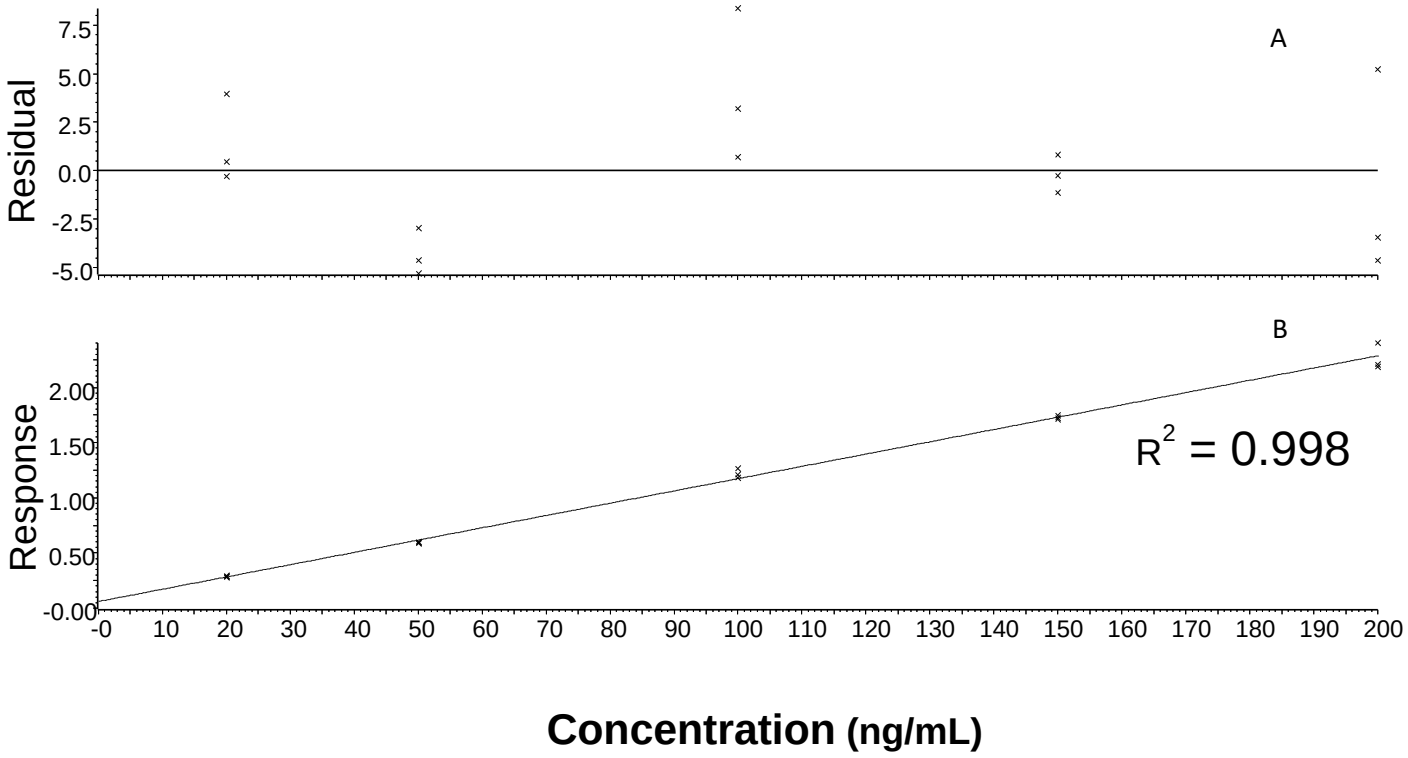


Figure 4. Calibration measurements for Bitrex™ using oxybutynin chloride as the internal standard A) residuals and B) calibration curve produced using TargetLynx.

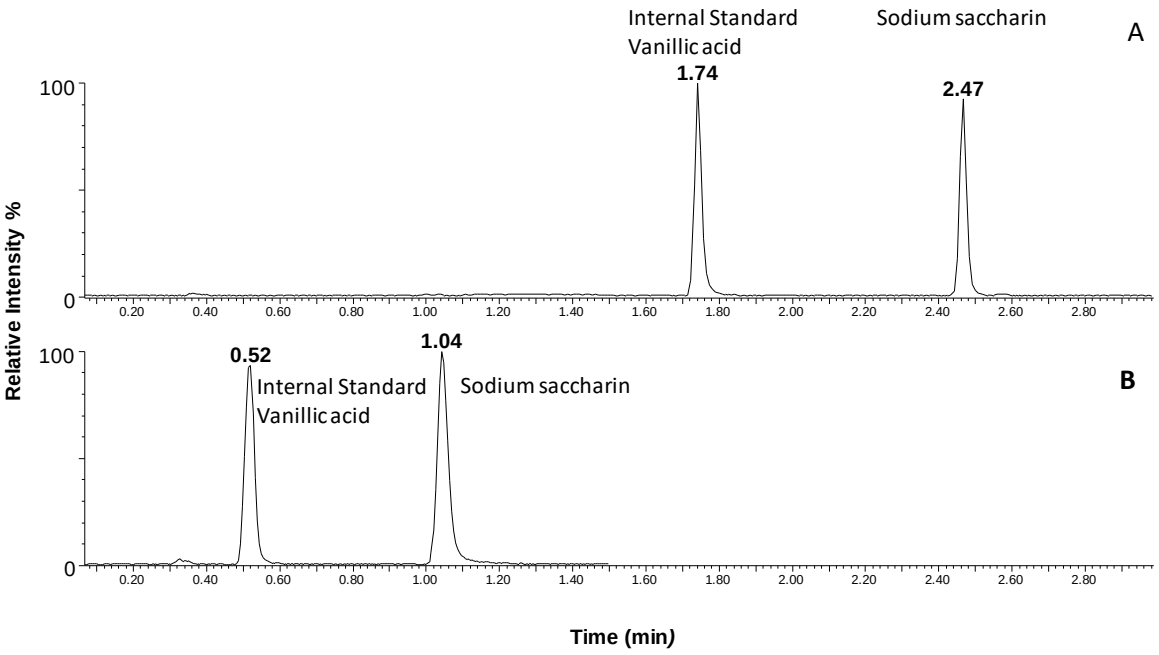


Figure 5. UHPSFC-MS SIR chromatograms for Vanillic acid and Sodium saccharin using A) 3-minute gradient and B) isocratic elution

TABLES

Time min.	**Solvent A %**	**Solvent B %**
0.00	80	20
1.30	0	100
1.35	0	100
1.50	80	20

Table 1. UHPLC gradient for the analysis of Bitrex™

Bitrex™ Batch	**Bulk Prepared Concentration g/L**	**Diluted Concentration ng/mL**	**Pre-Autoclave Measured ng/mL**	**Pre-Autoclave Calculated g/L**	**Post-Autoclave Measured ng/mL**	**Post-Autoclave Measured g/L**
TEST	1.69	84.7	75.5±0.4	1.51	75.7±5.5	1.51
SENSE	0.135	67.5	67.2±0.9	0.134	62.9±4.2	0.126

Table 2. Quality control measurements and calculations for one batch of Bitrex™ TEST and SENSITIVITY (5 L)

Sodium saccharin Batch	**Bulk Prepared Concentration g/L**	**Diluted Concentration µg/mL**	**Pre-Autoclave Measured µg/mL**	**Pre-Autoclave Calculated g/L**	**Post-Autoclave Measured µg/mL**	**Post-Autoclave Measured g/L**
TEST	536*	26.8	26.6±1.1	532	25.0±2.0	501
SENSE	0.008	10.4	9.3±0.7	0.007	10.7±0.4	0.008

*calculated applying theoretical adjustment of solute volume change

Table 3. Quality control measurements and calculations for one batch of sodium saccharin TEST and SENSITIVITY (5 L)