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Liquid Chromatography Detectors for the Analysis of Compounds With a Weak or No Chromophore for Food QC and Composition Testing

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INTRODUCTION

High Performance Liquid Chromatography (HPLC) is a widely adopted analytical tool for the routine analysis of food and beverage products for quality control, label claim confirmation, in-process monitoring, and raw material consistency and authenticity. There are several benefits in using HPLC for routine food and beverage testing, including the ability to increase throughput and automate parts of the workflow, through the use of autosamplers and chromatography data systems. HPLC can also allow for the quantitation of multiple analytes in a single method, increasing laboratory efficiency.

A common system configuration for routine food and beverage testing methods is HPLC coupled to an ultraviolet-visible light (UV-Vis) detector. The wide adoption of UV-Vis detectors for routine liquid chromatography (LC) analysis is mainly due to its ease of use and reliability, however a key requirement for UV-Vis detection is that the compound(s) of interest contain a strong chromophore (group of atoms in the molecule which absorb light). For compounds which have a weak chromophore or do not contain one, derivatization either pre- or post- column is required. The use of derivatization might not be suitable for all laboratory workflows as the off-line (pre-column) approach can involve several time-consuming steps and the on-line (post-column) approach requires a more complicated LC system set-up. Use of alternative detectors can be a more efficient approach for compounds with a weak or no chromophore and these detection techniques can include refractive index, evaporative light scattering and mass detection.

The objective of this white paper is to compare and contrast these three detection techniques, introducing each technique and using sucralose in beverages as a case study of a compound requiring alternative detection to UV-Vis.

DETECTOR TECHNOLOGY BACKGROUND

Refractive index detector

A refractive index detector (RID) measures the changes in the bulk properties of the solvent and the solutes. This detector takes advantage of the solvent and targeted solutes having a different refractive index. As the target analytes, which have been chromatographically separated, pass through the detector flow cell there are several things that occur, including:

- A change in the composition of the sample solution in the flow cell
- The RI of the solution changes
- The light beam passing through the sample solution is refracted

The position of the light beam is detected by the RI detector, creating a signal which is different from the baseline signal. Figure 1 illustrates how refraction by the sample in the detector flow cell changes the proportion of light on each of the elements of the photodiode.

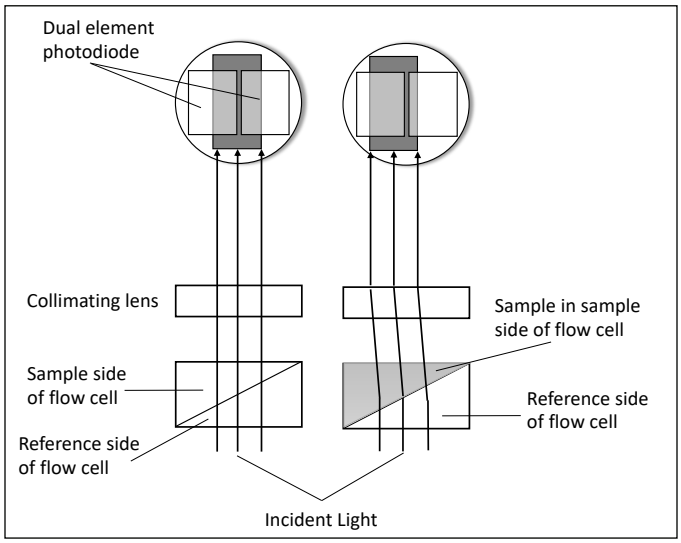


Figure 1. The presence of sample changes the photodiode signal

The amount of light reaching the elements of the photodiode is determined by the external angle of deflection (Φ), as shown in Figure 2. The Φ determines the magnitude of the shift (Δx) of the image cast on the photodiode by the light beam.

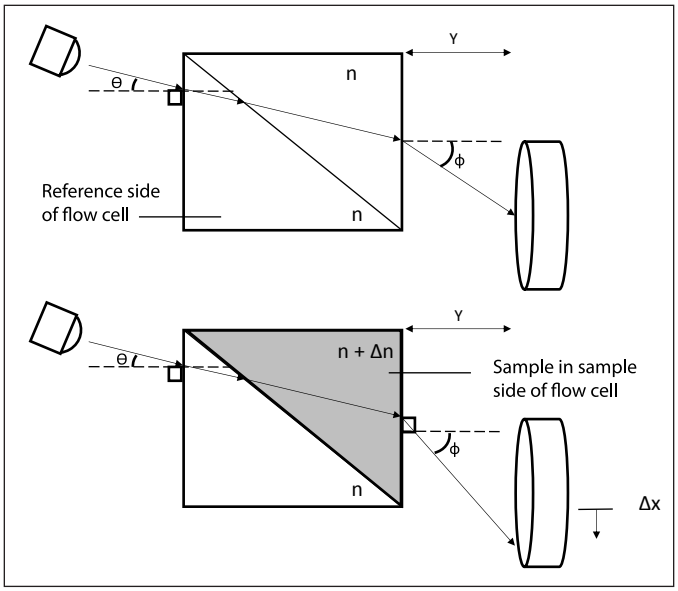


Figure 2. How refraction changes the external angle of deflection

Moving along the light path towards the photodiode, the beam of light encounters and is refracted by the air in the optics bench assembly, the fused quartz walls of the flow cell, the solvent in the reference side of the flow cell, and the solution in the sample side of the flow cell. Of these refractors, only the solution in the sample side of the flow cell should change over the course of an analytical run. The result is that the reference external angle of deflection does not alter until a change in the RI of the sample, such as a target analyte entering the flow cell, causes the light beam to be refracted from its zero position.

The 2414 RI detector uses a dual-pass optics bench assembly (Figure 3), so the light beam passes through the flow cell twice before reaching the photodiode, doubling the image shift.

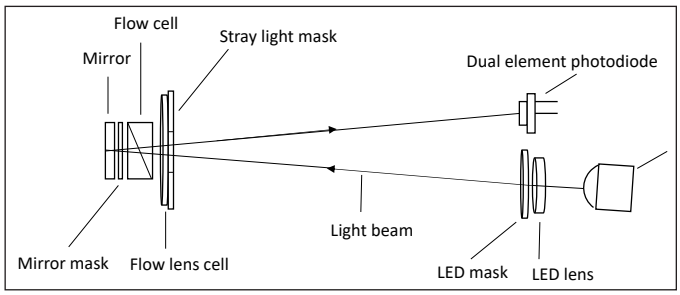


Figure 3. 2414 RI optics bench assembly light path

The change in the external angle of deflection (Φ) determines the shift (Δx) of the light beam on the photodiode. By detecting how far the image shifts (Δx), the refractometer measures the difference in RI (Δn). It is the shift in the amount of the light beam striking each element of the dual-element photodiode which results in a change in output voltage from the RI detector, this is registered in the Chromatography Data System (CDS) software and results in detected analyte peaks.

Keeping the mobile phase, temperature, and pressure constant means the changes in RI measured are caused only by the changing sample concentration. A high concentration of solute in a solution will refract a beam of light more than a dilute solution, so higher concentrations will give larger signals (peaks). RI detectors such as the 2414 RI detector can measure extremely small changes in refractive index to detect the presence of analytes. Because of this RI is limited to isocratic methods, as the mobile phase must stay a constant composition. A difference in RI between a reference solution and a sample solution is referred to as Δn, and this is expressed in refractive index units (RIU).

Evaporative light scattering detection

In evaporative light scattering detection (ELSD), the eluent from the LC column undergoes nebulization and then is carried along as droplets in a gas stream. Mobile phase is then evaporated from the droplets in a drift tube. Analytes which are less volatile than the mobile phase remain in the gas stream as dry solute particles and then enter the detection region where a light beam is scattered by those solute particles. Analytes which have a similar volatility to the mobile phase cannot be detected as it is impossible to evaporate the mobile phase from the droplets without also evaporating the analyte. The amount of scattered light is measured and displays a relationship to the concentration of the eluting analyte.

As described above, ELS detectors such as the Waters 2424 ELSD consist of three separate regions, which are:

- Nebulization
- Desolvation
- Detection

Nebulization

In the nebulization region, chromatographic eluent is mixed with a carrier gas (usually nitrogen) developing small droplets that form an aerosol which can enter the narrow orifice of the drift tube. The concentric flow nebulizer allows for the control of carrier gas flow rate which has an impact on the droplets size. By setting the flow rate to create smaller droplets, less heat is required to evaporate the solvent. Figure 4 shows a representative illustration of the nebulization region and drift tube for an ELSD.

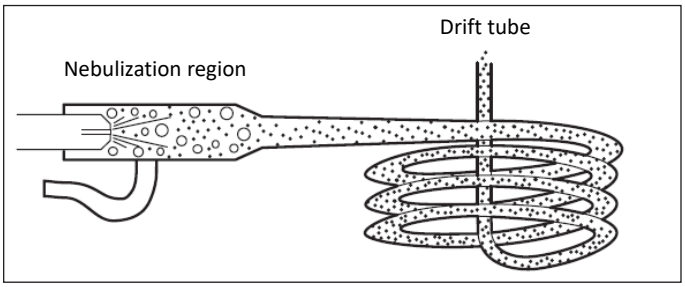


Figure 4. Nebulization region and drift tube for an ELSD

Desolvation

As the fine aerosol enters the drift tube it is now in the desolvation region of the detector. In this region the mobile phase is evaporated, resulting in dried solute particles which are carried by the gas to the detection region. Several factors have an effect on evaporation, including time, temperature and the carrier gas pressure. It is important to use mobile phase solvent conditions that easily and quickly evaporate and use of solvents with fairly low boiling points and low viscosities are best. These include commonly used LC solvents such as methanol, acetonitrile and ethanol which are employed in an isocratic or gradient elution method with water. If insufficient evaporation of the mobile phase occurs this will result in background noise, decreasing the analyte signal response, thereby reducing sensitivity and limits of detection. Any particle can interfere with the analyte signal, including particulates in low grade chromatographic solvents as the detector responds equally to all particulates, so the quality of the solvent is also important for ELSD and should be correctly selected. If additives and buffer salts are used in the mobile phase to aid separation, these also need to be volatile.

Detection

After desolvation, the dry solute particles which are suspended in the gas stream enter the detector region and pass into the center of the light scattering chamber. Light from a lamp is focused through a slit using two condensing lenses. The light from the slit is then relayed to the centre of the scattering chamber by a third lens. The amount of stray light reaching the scattering chamber is minimized through the use of a baffle between the slit and the relay lens. Only light which is scattered at a 60° angle relative to the incident light is channelled through the snout and collector lens. The combination of the snout design and two light traps minimize the stray light that can be detected. The collector lens focuses the light onto a mirror to change its direction, reflecting it onto the photomultiplier tube (PMT) which converts the light into a signal, creating the chromatogram in the CDS software. The remaining gas is vented to waste. Figure 5 is a representative illustration of the Waters 2424 ELS detection process.

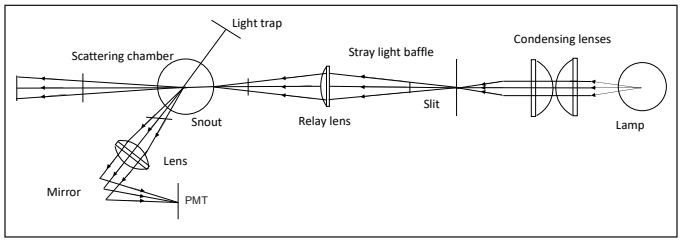


Figure 5. Representative illustration of the Waters 2424 ELS detection process

The size (diameter) of the analyte particles determines how the light is scattered and particles of different sizes exhibit different angular distributions of the scattered light. The scattered light is a rough measurement of the mass of the dry solute material, which is then represented by the chromatographic peak. Generally, larger particles will scatter more light, leading to more intense analyte peak responses. This mass response can be compound independent to some degree, but several factors can also affect the mass response including the size and density of the analyte. However, the output of the ELS detector has no direct relation to the molecular weight of an analyte.

Types of light scattering

There are three possible regimes of light scattering which are:

- Rayleigh
- Mie
- Refraction-reflection

Figure 6 shows representative illustrations of light scattering direction.

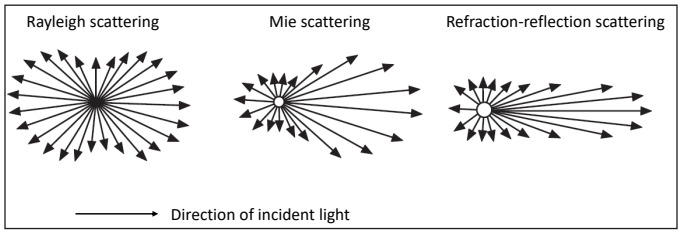


Figure 6. Representation illustration of light scattering direction

The light scattering type depends on the size of the solute particles going through the light beam. The ratio of the diameter (D) of the particle, to the incident of light wavelength (λ) or D/λ defines the resulting light scattering type.

As a chromatographic peak elutes from an analytical column, the response of the analyte it represents changes. The response goes from near zero at the baseline to a maximum that corresponds to column efficiency, injection volume, retention time and the analyte concentration of the sample injected. If the concentration of analyte is high enough, the diameter of the dry particle can vary through light scattering regimes. It is this variance which results in ELS exhibiting nonlinear calibration curves.

Mass detection

Mass Spectrometry (MS) detectors such as the ACQUITY™ QDa™ provide a selective tool for analyte determination for a variety of applications. The increased selectivity achieved using mass detection provides more confidence in compound identification due to a greater level of analyte discrimination. A mass spectrometer can analyze a sample only after target molecules are converted to gas-phase ions. To accomplish this an electric charge is imparted to the molecule which results in charged ions. Modern MS technologies tend to use atmospheric pressure ionisation (API) methods, with the most commonly used being electrospray ionization (ESI). The ACQUITY QDa Mass Detector is a bench top single quadrupole mass spectrometer designed with a small footprint and ease-of-use features which enables more efficient implementation when compared to previous generations of single quadrupole mass spectrometers. Key regions of a mass detector are shown in Figure 7.

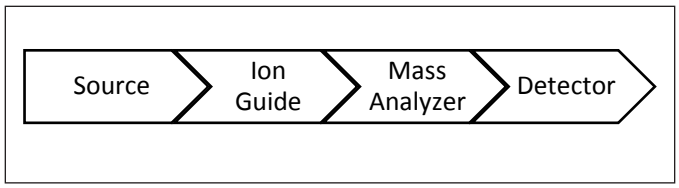


Figure 7. Key regions of the ACQUITY QDa Mass Detector

Electrospray ionization

Electrospray ionization is considered a soft ionization technique in contrast to other techniques such as electron ionization (EI), which is typically used when gas chromatography is coupled to mass spectrometry. ESI is a commonly used technique because it ionizes a wide range of analytes, working best for compounds that are slightly polar to highly polar. In ESI the liquid chromatography flow is introduced via a probe which is fitted to the source and contains a narrow metal capillary. A common way that ions are formed is in solution via a series of electrochemical reactions. The ion containing solution is then sprayed from the capillary tip in the form of tiny droplets. To enhance the creation of very small droplets, the end of the probe is heated, and a nebulizer gas is used. The combination of high temperature and gas flow leads to the evaporation of solvent from the droplets (desolvation) and the transfer of ions from the liquid into the gas-phase. Typically, the ions produced under aqueous conditions are $[M+H]^+$ or $[M-H]^-$ but it is also possible for ESI to form ions with multiple charges, for example $[M+2H]^{2+}$ and adducts such as $[M+Cl]^-$. Positive and negative ions are both formed in solution, but it is the potential difference applied to the capillary and the source cone that determines whether positive or negative ions enter the mass spectrometer. Figure 8 shows a simplified schematic of the ESI probe and MS ion inlet.

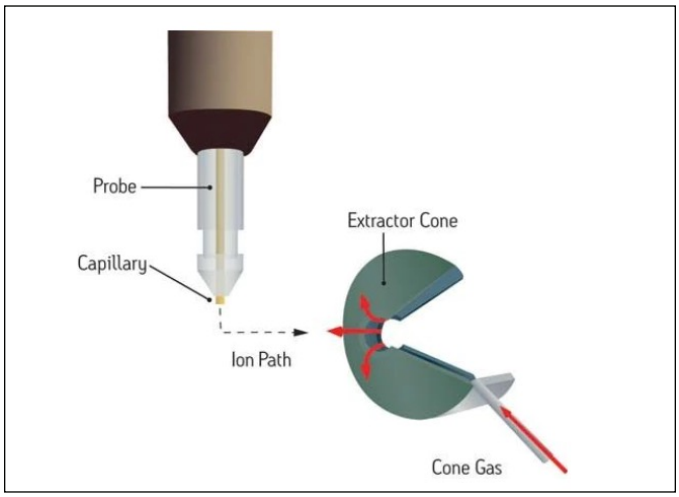


Figure 8. ESI probe and MS ion inlet

One consideration when using ESI is the potential for matrix effects which can result in ion suppression or enhancement. If the analytes of interest are not either isolated from other matrix co-extractives through sample clean-up or chromatographically resolved, then competition for ionization between molecules occurs. Use of matrix-matched calibration curves if practical, or stable isotope labelled internal standards can be used to compensate for matrix effects, improving accuracy and precision for quantitation methods. The quality of the solvent used is also important as contaminants in the solvent can not only result in increased instrument cleaning but also compete in the formation of charged ions.

Ion guide

Ions are transferred from the ESI source into the low-pressure mass analyzer via a series of ion guides. The ACQUITY QDa Mass Detector uses a two-stage ion guide which operate under different pressures. The first stage is a stacked ring assembly with a larger ring section which captures the incoming diffuse ion cloud. This guide uses an alternating radio frequency (RF) voltage to contain the ions and a direct current (DC) voltage to move the ions through the guide. A differential voltage is applied to transfer and focus the ions into a smaller ring section, allowing ions to be moved through an orifice into the second stage of the ion guide. During this step, most neutral species are removed by a backing pump, which helps to improve instrument robustness. The second stage is a segmented quadrupole where the ion beam is further focused for optimal transmission through a very narrow orifice into the analyzer region.

Mass analyzer

Ions are separated in the mass analyzer according to their mass to charge ratio *(m/z)* in an electric field between four rods, known as quadrupoles (Figure 9). Then the ions are transferred to the detector.

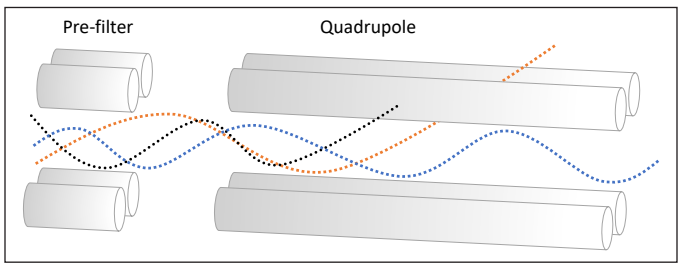


Figure 9. Quadrople mass analyzer

A specific combination of voltages is required for each *m/z* value, and these are changed rapidly and sequentially over time to produce a mass spectrum. The ACQUITY QDa Mass Detector can operate in either full scan mode, which is a scanning experiment or selective ion recording (SIR), which is a static experiment. When MS scan mode is used, a programmed range of masses are scanned allowing the collection of data across a range of *m/z* values, this is most typically used for qualitative analysis. SIR mode fixes the *m/z* value for a defined period of time (dwell time). Use of SIR mode allows for increased specificity and sensitivity and is most often used for targeted, quantitative experiments. Multiple masses can be acquired in SIR mode in a single acquisition method.

Detector

The ACQUITY QDa has a low noise, off-axis, long life photo-multiplier detector. After entering the detector region from the mass analyzer, ions strike a dynode causing an electron emission, which in turn strike phosphor leading to the generation of photons. The photons are converted in a photomultiplier to an electronic signal which is recorded in the CDS software.

CASE STUDY SECTION – SUCRALOSE

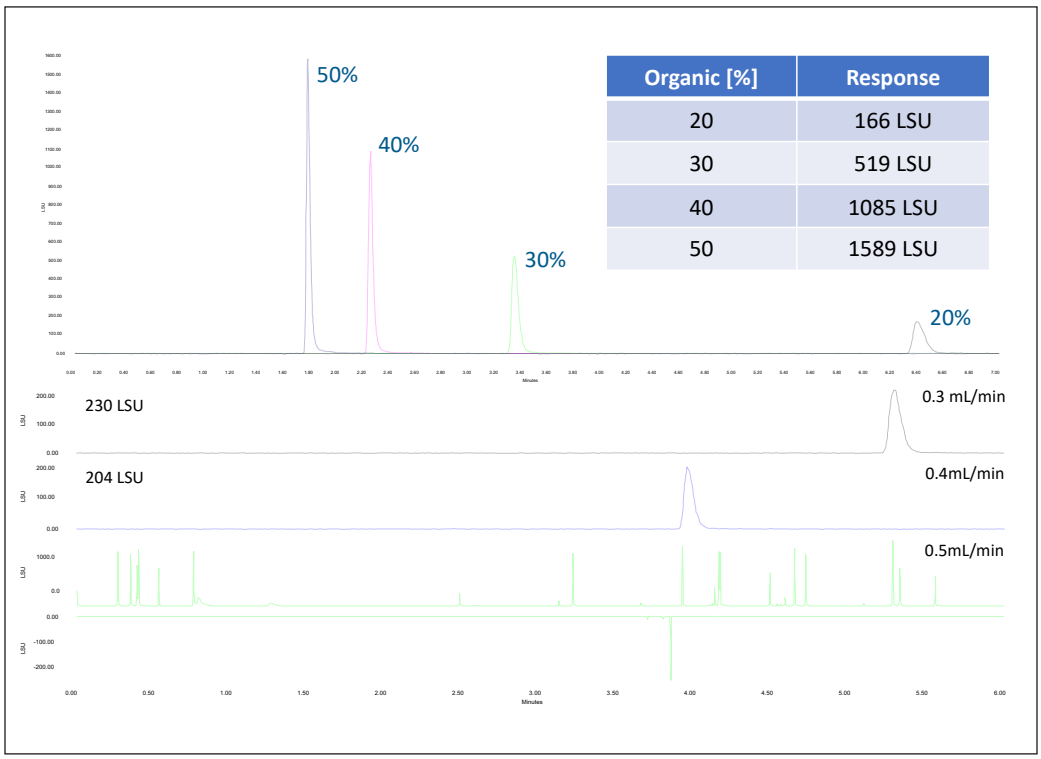
With the continued focus from consumers and national governments on reducing sugar consumption, food and beverage manufacturers are continuing to reformulate existing products or develop new reduced or no sugar products to appeal to the consumer and reduce the impact from sugar taxes. To achieve the decrease in sugar content or replace sugar, but still maintain the level of sweetness associated with brands and consumer taste preference, sugar substitutes such as sucralose are used.

Sucralose is a high intensity artificial sweetener which can be found in several food and beverage products. Sucralose is derived from sucrose through the selective replacement of three hydroxyl groups which are substituted with three chlorine atoms. Similarly, to sucrose, sucralose does not have a strong chromophore so requires an alternative detection technique to UV-Vis.

This section will explore the performance of RID, ELSD, and the ACQUITY QDa Mass Detector for the analysis of sucralose in beverages by LC.

Liquid chromatography and detector experimental conditions

The liquid chromatography system used in this study was an ACQUITY UPLC™ H-Class PLUS, coupled with a CORTECS™ T3 2.7 µm 3.0 mm x 100 mm Column. This set-up achieved acceptable retention and separation of sucralose from other ingredients in the tested beverage samples. The mobile phase consisted of HPLC grade methanol and water (20:80). For RI detection, the flow rate was 1 mL/min, run under isocratic conditions, which is a limitation of RI discussed previously. The same isocratic conditions were used for the ACQUITY QDa. To aid with efficient nebulization, a lower flow rate of 0.3 mL/min and a gradient LC method was required for the ELS detector with 50:50 methanol and water used as the mobile phase composition for elution of sucralose to achieve optimal performance. See Figure 10.



Organic [%]	Response
20	166 LSU
30	519 LSU
40	1085 LSU
50	1589 LSU

Figure 10. Flow rate and % methanol optimization for the ELS detector

For both RID and ELSD a 10 µL injection volume was used, but this was reduced to 5 µL injection on the ACQUITY QDa Mass Detector due to the increased sensitivity of this system in order to avoid detector saturation. Table 1 provides an overview of the LC conditions used for each detector.

To reduce baseline fluctuations, the RI detector temperature was also set to 50° C. This temperature also gave a better response for sucralose when compared to 30° C. The ELSD drift tube temperature was set to 55° C to evaporate the solvent. The ACQUITY QDa Detector settings can be found in Table 2.

DETECTION:	RID	ELSD	ACQUITY QDa
Column:	CORTECS T3	CORTECS T3	CORTECS T3
Column Temp:	50° C	50° C	50° C
Sample Temp:	25° C	25° C	25° C
Injection Volume:	10 µL	10 µL	5 µL
Flow Rate:	1.0 mL/min	0.3 mL/min	1.0 mL/min
Mobile Phase:	80:20 Water: Methanol (isocratic)	Line A Water B: Methanol (gradient)	80:20 Water: Methanol (isocratic)

Table 1. LC conditions used for each detector

DETECTION:	ACQUITY QDa
Mode	Performance
Ionization Mode	ESI -
Capillary Voltage	-0.8 V
Probe Temp.	600° C
Cone Voltage	15 V
Sampling Rate	2 pts/sec

Table 2. ACQUITY QDa detector settings

Detector comparison for the analysis of sucralose

Linearity was assessed for each detector using solvent standards, using the method conditions described above. The RI detector gave excellent linearity (R^2 >0.9999) over the widest concentration range which was 7.8 ppm to 500 ppm. As expected, the ELS detector required a quadratic curve fit (R^2 >0.994) over a concentration range of 6.1 ppm to 200 ppm. The ACQUITY QDa, due to its sensitivity, required a calibration curve over a lower concentration range of 0.244 ppm to 1.95 ppm. The curve was a linear fit (R^2 >0.995) over this range. Example calibration curves are shown in Figure 11.

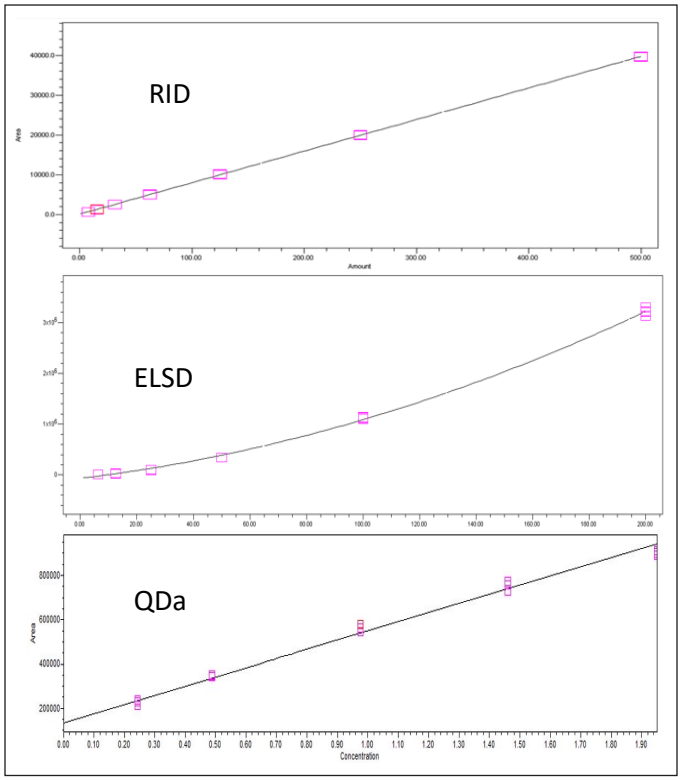


Figure 11. Example solvent calibration curves for each detector (RID top, ELSD middle, ACQUITY QDa bottom)

Three samples of shop purchased energy drinks were diluted with mobile phase and filtered using a 0.2 µm PTFE CE Acrodisc Minispike syringe filter. Each sample was run with six replicate injections and the results can be found in Table 3. Example chromatograms for each of the samples, for the three detectors are shown in Figure 12.

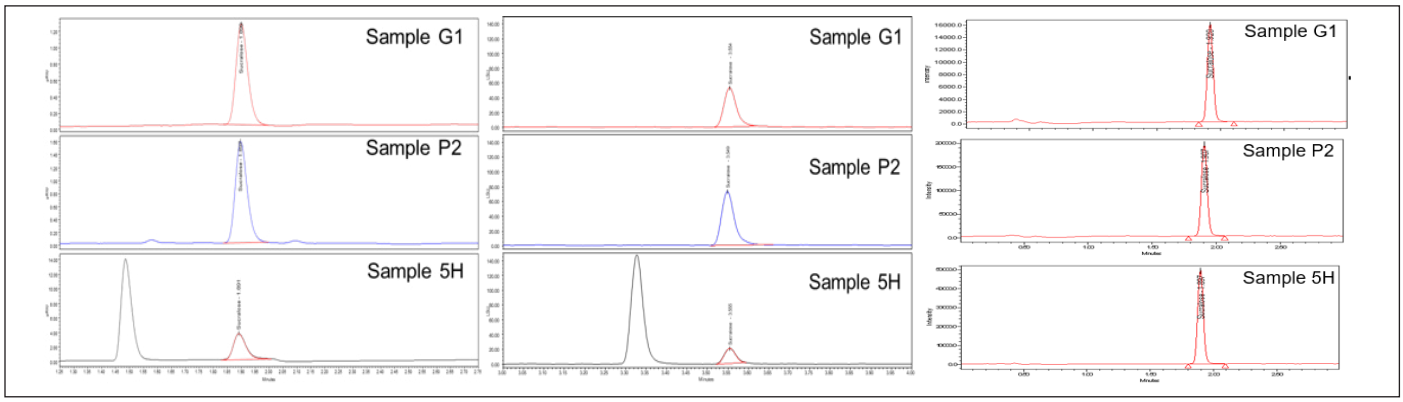


Figure 11. Example solvent calibration curves for each detector (RID left, ELSD middle, ACQUITY QDa right)

The ACQUITY QDa data was not corrected through use of a stable isotope labelled standard such as sucralose d6. The use of stable isotope labelled standards can correct for ESI response variation resulting from matrix effects (signal suppression or enhancement), which can be observed when using solvent calibration curves to measure analytes in samples. Due to the greater sensitivity, achieved through better specificity, a higher level of sample dilution was also required to bring the sample concentrations into the calibration range used for the ACQUITY QDa Mass Detector. The measured concentrations for each sample on each detector can be found in Table 3.

SAMPLES	RETENTION TIME	AMOUNT	RETENTION TIME	AMOUNT	RETENTION TIME	AMOUNT
N=6	RID	RID	ELSD	ELSD	ACQUITY QDa	ACQUITY QDa
	min (%RSD)	min (%RSD)	min (%RSD)	ppm (%RSD)	ppm (%RSD)	ppm (%RSD)
Sample G1	1.90 (0.1)	57.3 (1.1)	3.55 (0.0)	57.3 (1.1)	1.92 (0.0)	72.4 (4.0)
Sample P2	1.90 (0.1)	73.0 (0.6)	3.55 (0.0)	74.0 (0.9)	1.91 (0.0)	90.9 (2.7)
Sample 5H	1.89 (0.1)	1423 (1.6)	3.55 (0.0)	1570 (3.2)	1.90 (0.0)	1689 (2.1)

Table 3. Sample results from each detector showing concentration and retention time (n=6 for each sample)

As discussed in an earlier section, the ACQUITY QDa can be used in both SIR and full scan mode. The use of full scan can allow for the collection of MS spectra which is useful for troubleshooting and peak identification. Figure 13 shows the mass spectra from the sucralose standard and from the suspected sucralose peak found in sample 5H. The observed *m/z* for sucralose in ESI negative mode are 395 *m/z* and 397 *m/z* which are a result of chlorine isotopes. Sucralose has three chlorine atoms which gives the isotopic pattern shown in Figure 13.

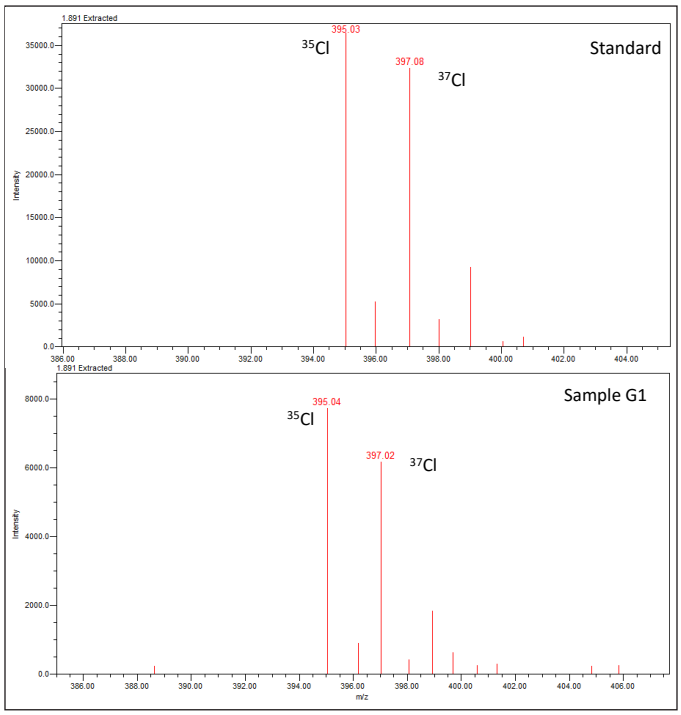


Figure 13. Mass spectra from the sucralose standard (top) and from the suspected sucralose peak found in sample G1 (bottom)

Choosing a detector

All three of the detectors discussed in this white paper would be suitable for the detection of sucralose in energy drinks. Various factors including sample complexity, sample throughput and method performance criteria such as limits of quantification, accuracy and precision will factor in determining which detector best meets a laboratory's requirement. Table 4 provides some strengths and considerations for each of the detectors discussed in this white paper.

Detector	Strengths	Considerations
2414 RI Detector	• Lowest purchase and running costs • Simple isocratic methods with no need for re-equilibration between injections • Excellent linearity and precision	• Not compatible with gradient methods • Limited selectivity and sensitivity
2424 ELS Detector	• Compatible with gradient methods • Near-universal detection of non-volatile and semi-volatile sample components, independent of pH • Mass response independent of analyte's optical properties	• Requires clean nitrogen supply and removal • Can exhibit a non-linear response • Not compatible with non-volatile buffers
ACQUITY QDa Mass Detector	• Highest selectivity and sensitivity • Compatible with gradient methods • Mass confirmation data • Less complex chromatograms with reduced impact from chromatographic co-elutions	• Requires clean nitrogen supply and removal • Not compatible with non-volatile buffers • May require use of stable isotope labelled standards for quantification methods

Table 4. Strengths and considerations for each of the detectors discussed in this white paper

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