ANALYSIS OF PHYTOCHEMICALS IN PROCESSED COCOA USING ION MOBILITY MASS SPECTROMETRY

[1]M McCullagh, [2]Sara Stead, [1]Gitte Barknowitz and [2]Clare Mills.
[1] Waters Corporation, Stamford Avenue, Altrincham Road, Wilmslow, SK9 4AX. UK. [2] Manchester Institute of Biotechnology, The University of Manchester, Princess St, Manchester M1 7DN.

OVERVIEW

- Analysis of a reference cocoa powder library using liquid chromatography and ion mobility mass spectrometry.
- Characterisation of the impact of manufacturing process upon phytochemical make-up using principal components analysis (retention time, drift time and accurate mass).
- Identification and differentiation of phytochemical isomers using accurate mass, retention time and collision cross section values.
- Discovery of procyanidin isomers producing charge-bearing conformers and generation of procyanidin isomer $^{TW}CCSN_2$ fingerprints.

INTRODUCTION

Ion mobility mass spectrometry (IM-MS) based measurement and separation of compounds, is based on molecule size, shape and charge differentiation. IM provides an added dimension of separation to that of liquid chromatography (LC) hydrophobicity and mass spectrometry (*m/z*), in addition to collision cross section (CCS) values which provide a complimentary identification descriptor.

Cocoa beans are the seeds of the tropical tree Theobroma cacao L. and contain a high concentration of bioactive compounds. However, several studies have established that food manufacturing processes (including alkalization, roasting, fermentation and drying), of cocoa bean produce significant changes in the phytochemical make-up of food commodity products such as cocoa powder and chocolate.[1] Nutritional interest in phytochemicals has increased due to the relation between their consumption and health benefits, such as the reduction of the risk factors of cardiovascular disease and certain types of cancer.[2,3]

The combined peak capacity of liquid chromatography ion mobility (LC-IM-MS) and CCS can be used to produce routine unequivocal identification of phytochemicals. Importantly, IM separation can often resolve LC coeluting and *m/z* coincident isomeric species, which makes it a powerful tool in complex mixtures analysis.[4-6] The enhanced specificity of the approach has been utilised to profile and differentiate the phytochemical make-up, of processed “off the shelf” cocoa powder food commodities (including vegan, organic, alkalized, low fat, supermarket home-brand, high-end brands, natural cocoa powder and unsweetened).

METHODS

A reference library of cocoa powders, representing a variety of processing and manufacturing conditions were sourced from various suppliers (*n=67*). Cocoa powder (0.5 g) was extracted with 10 mL of ethanol:water (70:30 *v/v*). The extract was shaken for 5 minutes and centrifuged at 6,000 rpm for 5 mins. A 1:10 dilution of the supernatant was performed using methanol + 0.1% formic acid. The cocoa library extracts were analysed in triplicate with positive and negative ion electrospray precursor/product ion data acquisition using a Synapt™ G2-*Si* mass spectrometer (Resolution (R)~40). Reverse phase LC separation was performed using a C_{18} (1.7 µm, 2.1x100 mm) analytical column.

The chromatographic conditions were comprised of a 17 minute (0.1% *v/v* formic acid in H_2O) and (0.1% *v/v* formic acid in acetonitrile) reverse phase gradient at 0.75 mL/min. Column temperature 45ºC. Sample injection volumes of 10 µL were used.

RESULTS AND DISCUSSION

Sixty-seven cocoa powder food commodities have been analysed using a non-targeted screening strategy to determine the influence of manufacturing processing and production methods upon phytochemical make-up. LC linear travelling wave ion mobility (TWIM) data were acquired for the cocoa extracts. Data was screened against natural product libraries incorporating positive and negative ion mass spectrometry detection results for precursor ion, ion mobility product ions, retention time and $^{TW}CCSN_2$ values. Ion mobility separated single component precursor ion/product ion spectra were generated for the $[M+H]^+$ and $[M-H]^-$ phytochemicals identified. Examples of phytochemicals identified are presented in Figure 1. The ESI+/ESI$^-$ base peak ion chromatograms shown in Figure 2, illustrate the differentiation in sample complexity and phytochemical profiles generated when using ESI+/ESI$^-$. Using positive ion mode, abundant phytochemicals identified across most of cocoa powder food commodities analysed include (detection values illustrated for sample 1): methylxanthine alkaloids which have been identified based on accurate mass (precursor ion/product ions), retention time, and $^{TW}CCSN_2$: caffeine (ref $^{TW}CCSN_2$ 137.4 Å^2, Δ $^{TW}CCSN_2$ 0.5 %); theobromine (ref 130.8 Å^2, Δ $^{TW}CCSN_2$ 0.3 %) and theophylline (ref CCS 132.2 Å^2, Δ $^{TW}CCSN_2$ 0.3 %).

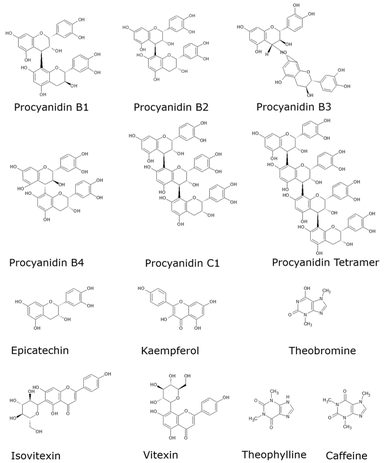


Figure 1. *Phytochemicals identified in cocoa powder and characterised using ion mobility.*

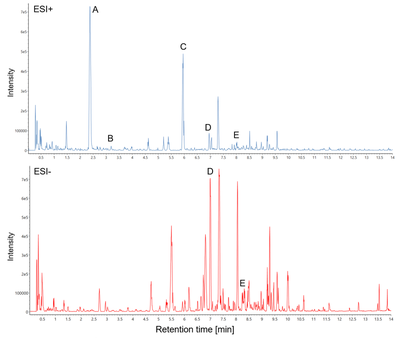


Figure 2. *ESI $^+$ and ESI$^-$ base peak ion chromatogram for organic cocoa powder sample 1 ((A) theobromine, (B) theophylline, (C) caffeine, (D) procyanidin B2 and (E) procyanidin C1).*

Additionally, CCS profiling of polymeric flavan-3-one procyanidins was performed. In the case of procyanidins, initial reference CCS values were determined using CCS prediction, to facilitate identification. Dimer (isomers), trimer and tetramers have been distinguished using ion mobility spectrometry. Interestingly, distinctive $[M+H]^+$ and charge bearing (potassimer $[M+K]^+$ and sodimer $[M+Na]^+$) conformer ratios have been observed, for which corresponding $^{TW}CCSN_2$ values have been determined (see Figure 3 and Table 1). A combination of charged isomer species produce a highly specific $^{TW}CCSN_2$ fingerprint, to differentiate polymeric procyanidins.

In negative ion mode, abundant phytochemicals identified across many of cocoa powder food commodities analysed include: Flavanols: (−)-Epicatechin (ref 158.9 Å^2, Δ $^{TW}CCSN_2$ 0.7 %) and (+)-catechin (ref 158.9 Å2, Δ $^{TW}CCSN_2$ -0.64 %). Also, quercetin, kaempferol, quercetin-3-glucoside and isoquercitrin. Other minor polyphenolic compounds identified include flavonoids (vitexin, isovitexin, isoorientin, apigenin and apigenin-7- O glucoside), flavones (luteolin) and flavanones (naringenin and eriodyctiol). The combination of retention time, accurate mass and CCS affords enhanced specificity to identify low intensity phytochemical components, as well as differentiate isomeric phytochemicals.

Additionally using ESI$^-$, procyanidin B1, B2, and B3 were observed to have $^{TW}CCSN_2$ ~ 222 Å^2, procyanidin C1 ($^{TW}CCSN_2$ 275.2 Å^2) and procyanidin tetramer ($^{TW}CCSN_2$ 322 Å^2). $^{TW}CCSN_2$ values have been determined, from detection and identification within the analysed samples. However, using negative ion mode, broad ATD were observed for the detected procyanidin isomeric compounds, which may suggest unresolved $[M-H]^-$ conformer species have been observed.

The summed phytochemical intensity for cocoa commodity group type is shown in Figure 4. However, from the PCA score plots presented in Figure 5, organic and non-alkalised samples cluster differentially in comparison to the other sample group types indicating differences in the underlying phytochemical make-up, determined by manufacturing process. In Figure 6 the intensity spread for theophylline and procyanidin C1 in alkalized cocoa and organic raw cocoa commodities are compared, indicating procyanidins and methylxanthines are impacted by manufacturing process.
Compared to theobromine, theophylline is more effective in cardiac stimulation, coronary dilation and smooth muscle relaxation.[7] Procyanidins possess a variety of chemo-preventive biological effects including anticancer and anti-inflammation.[8,9] Both example phytochemicals discussed have isomeric constituents in the food commodities profiled. Ion mobility mass spectrometry has been used to enhance the specificity of profiling the impact of manufacturing process upon phytochemical make-up.

Compound	$[M+H]^+$ $^{TW}CCSN_2$ Å^2	$[M+H]^+$ Conformer A $^{TW}CCSN_2$ Å^2	$[M+H]^+$ Conformer A $^{TW}CCSN_2$ Å^2	$[M+Na]^+$ Conformer A $^{TW}CCSN_2$ Å^2	$[M+Na]^+$ Conformer B $^{TW}CCSN_2$ Å^2	$[M+K]^+$ Conformer A $^{TW}CCSN_2$ Å^2	$[M+K]^+$ Conformer B $^{TW}CCSN_2$ Å^2	Retention time (mins)
Caffeine	137.4							5.95
Theobromine	130.8							2.40
Theophylline	132.5							3.52
Procyanidin B1		229.0 >	252.9			231.7		5.24
Procyanidin B2		227.4 >	248.1	230.2		226.6 >	239.0	6.98
Procyanidin B3*		226.6 <	244.8	244.7		227.6 <	248.4	9.28
Procyanidin B4*		228.8 >	248.6	228.0		234.0		4.99
Procyanidin C1	283.7			274.3	291.1	278.4		8.03
Procyanidin Tetramer	337.9			331.8		331.7		8.30
Vitexin	196.6							9.00
Isovitexin	198.6							9.08
Quercetin	163.8							11.58
Apigenin	156.5							12.66

Table 1. Subset of $^{TW}CCSN_2$ values determined for phytochemicals identified in processed cocoa powder using LC-IM-MS (ESI$^+$).

Tentative assignment/<> indicates conformer intensity ratio.

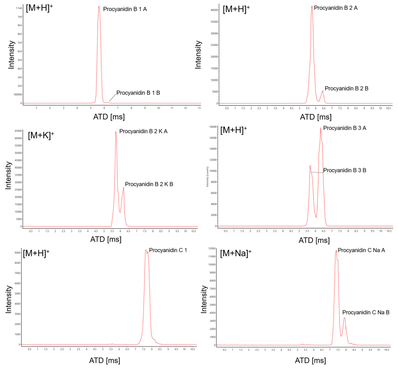


Figure 3. *Arrival time distribution (ATD) of procyanidin B isomer and procyanidin C1 conformer species.*

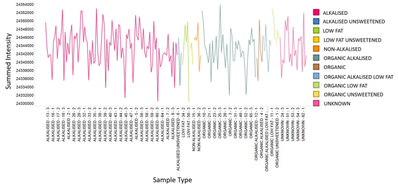
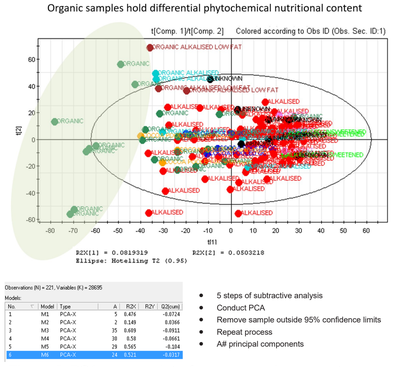


Figure 4. *Total ESI$^+$ summed intensity of phytochemical make-up for cocoa powder group type.*



- 5 steps of subtractive analysis
- Conduct PCA
- Remove sample outside 95% confidence limits
- Repeat process
- A# principal components

Figure 5. *PCA scores plot of cocoa powder group type illustrating differential phytochemical make-up.*

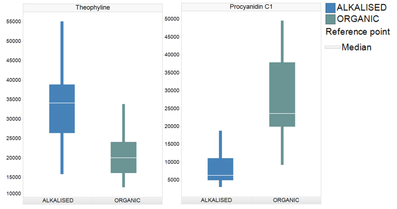


Figure 6. *Box Plot comparing intensity spread for theophylline and procyanidin C1 in alkalized cocoa and organic raw cocoa powder.*

CONCLUSION

- Using LC-IM-MS, a combination of accurate mass measurement, retention time and CCS values has facilitated identification of abundant and trace level phytochemicals present in processed cocoa powders, enabling impact of manufacturing process upon food commodity nutritional value to be assessed.
- Phytochemical isomers have been distinguished using CCS and identified at low intensities without reliance upon product ion information.
- Procyanidin isomers have been observed to produce distinctive conformer CCS fingerprints and characteristic conformer isomerising species ratios.
- Further investigations using high resolution cyclic ion mobility are required to determine if unresolved procyanidin conformer species have been observed, to enhance identification specificity using dual polarity CCS fingerprints.

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