Theoretical description of a new analytical technique: Comprehensive online multidimensional fast Fourier transform separations

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ABSTRACT

Comprehensive multidimensional separations are today dominated by systems that are fundamentally limited to highly asymmetrical online separations sacrificing separation space, or to lengthy, time consuming offline separations. With the exception of pulse-modulated methods, separations have thus been limited to two dimensions. It is proposed that some of the limitations and shortcomings of these methods may be ameliorated or overcome by employing multi-dimensional detection whereby each analyte is effectively labelled in the frequency domain by a series of pulsed-injections, and a symmetrical, comprehensive online analysis performed with the resulting signal processed by sequential Fourier analysis. A semi-empirical computer model of this system was developed and its feasibility positively demonstrated in simulations of high-efficiency separations in two dimensions. Separations of higher dimensionality were shown to be possible but involved signal-processing challenges beyond the present work. By eliminating wrap-around effects and enabling the separation of physically unseparated peaks, the technique facilitates significant improvements in peak capacity per unit of analysis time as well as greatly improved signal to noise ratios. Because these comprehensive online multidimensional Fourier transform separations depend heavily upon the practical lifetime of imposed injection pulses, it is envisaged that this method will leverage emerging high-efficiency micro- and nanoscale separations technologies.

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1. Introduction

Online comprehensive separations have been demonstrated in multi-dimensional gas chromatography via the use of inter-dimensional pulse modulation [1,2] and statistical analyses of the resulting signal. Other mathematical methods have also been applied to chromatography and analytical signal processing for many years, and of the transform methods, the Fourier transform is the most important and finds considerable application [3], albeit in various guises and differing purposes. The advantages of pulse-modulation and frequency domain analysis to achieve comprehensive online multidimensional separations have not hitherto been combined.

Allegri et al. [4] used Fourier analysis as a means of deconvolution to resolve partially overlapped peaks in time-dependant (detector) signals. Fourier transforms have also found use in improvements to signal-to-noise ratios, peak resolution and the speed of in-process chromatography by either analysing the results of multiple overlapped sample injections (correlation chromatography [5,6]) or measuring analyte column migration speeds by multiple-point detection such as in Shah Convolution Fourier Transform (SCOFT) detection [7] and combinations of such methods [8]. Over time, these methods have been refined to include the application to correlation chromatography of Hadamard transforms (a discrete Fourier transform in two variables) by employing complicated sample-injection protocols of pseudo-random binary sequences [9,10]. Further enhancements include the use of multiple parallel sample injection as in Fourier Transform Capillary Electrophoresis (FTCE) [11] to achieve the same result as that generated by SCOFT, but without the need for multiple-point detection: translation of the migration speed of an analyte into a frequency domain signal. Each of these applications has achieved notable improvements in signal to noise ratios and, in some cases, improved chromatographic resolution [11,12]. Each one replaces time-domain detection with frequency-domain detection. Most of these methods are used in process control analyses, in industrial environments and are unknown by most laboratory chromatographers.

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The present work suggests a further enhancement to the repertoire of frequency domain methods by simultaneous application of both time- and frequency-domain detection to pulse-modulated separations in multiple dimensions, using only one single-point detector.

1.1. Purpose of the proposed method

Multidimensional chromatography has arisen as an increasingly important technique in fields requiring the analysis of highly complex mixtures, usually of natural origin. The fields of proteomics, metabolomics, and natural drug discovery, for example, deal with samples containing hundreds or even thousands of compounds that cannot be adequately or practically separated in one dimension (D_1). In such cases, further separation may be achieved by serial application of separations in further dimensions ($D_2 \cdots D_n$), which may be carried out in either time (S_t) or space (S_s) or combinations of both (such as $S_t \times S_s$).

1.1.1. Current methods of implementation of multidimensional chromatography

In practice, it has been found that multiple time-domain dimensions ($S_t \times S_t$) provide the simplest and most efficacious separations [13]. The analytical requirements, constraints and limitations placed (by choice or necessity) on multidimensional chromatography may be summarised as follows:

1) The separation must be "comprehensive" in that all components pass through all dimensions.
2) No separation achieved in the series of previous dimensions ($D_1 \cdots D_n$) may be lost in any of the subsequent separation dimensions ($D_2 \cdots D_{n+1}$)
3) In the most common case of multiple time-dimension separations ($S_t \times S_t$), practical application may be achieved in a variety of modes:

a. Online:
 N-fractions per peak in D_n are collected, transferred to the next separation level, and directly subjected to analysis in D_{n+1} in real-time. In this case, the analysis time (T_{n+1}) in D_{n+1}, must be less than the time between subsequent fractions transferred to the second dimension, ideally less than one standard deviation of the peak distribution eluting from the first dimension [14]. Otherwise chaotic band displacement may result. In HPLC, this may require D_{n+1} run times of only a few percent of the D_n run time.
b. Offline:
 N-fractions in D_n are stored temporarily until subjected to analysis in D_{n+1} such that the run rime of D_{n+1} is no longer subject to the same constraint as in (a), but analyses are time consuming.
c. Stop–Start:
 After an eluate plug has been delivered directly to D_{n+1} from D_n, the flow in D_n is stopped and the separation is then run in D_{n+1}. Upon completion of the D_{n+1} separation, the flow is resumed in D_n to deliver another eluate plug, and so on. The need to collect, store and manage large numbers of fractions, free of contamination, is thereby obviated. There is, however, no reduction in the overall analysis time compared with (b).

In offline analysis, relaxation of the constraint on D_n run times comes at the cost of a very large increase of the total analysis time, which is given by the series:

$$T_A = T_1 + N_1 T_2 + N_2 T_3 + \ldots \tag{1}$$

where T_A is the total analysis time, $T_1, T_2, \ldots, T_n$ represent the analysis time in each respective dimension and N is the number of fractions transferred to each dimension. This drawback has been

addressed by subjecting only specific fractions of interest from D_n to analysis in D_{n+1}: 'Heart-cutting' [15] – but this method can no longer be termed a 'comprehensive' separation. For comprehensive separations, the online method 3a is preferred due to its simplicity and speed of analysis. Unfortunately, the constraint placed on the analysis time in the second dimension is severe, and necessitates highly optimised, asymmetrical analyses that sacrifice much of the separation space that could otherwise be available in a multidimensional system [13]. Parallelisation of online second-dimensions has been employed as a means of achieving rapid symmetrical comprehensive separations [16], but remains limited in its D_1 retention time resolution and is a very expensive approach that necessitates significant optimisations.

Now, the crux of these difficulties – and the reason for the development of the above techniques – lies in the problem of being able to identify in D_n, the results of separations that have taken place in $D_{<n}$, such that requirement (2) may be met. In time-domain separations (such as in liquid chromatography and electrophoresis), components are identified by their retention time. If there were another way to persistently 'label' the components previously separated and thus to identify and maintain the achievements of the $D_{<n}$ time-domain separations, the analysis-time constraint for online D_{n+1} could be removed and there would be no need for time-consuming offline separations.

1.1.2. Introduction of the frequency domain in multidimensional chromatography

To date, the frequency domain has been applied in chromatography essentially as a simple translation of retention time to migration speed through one dimension, providing improvements in signal to noise ratios. In multidimensional separations it would be possible for two components that would otherwise be separated in the time domain, to present the same frequency at the detector. Similarly, components migrating at different speeds may elute at the same time in later dimensions. Rather than using the frequency domain or time domain alone, if we were to separate components in both the time and frequency domains, the separation of coincident components in subsequent dimensions becomes a problem of the separation of the frequencies that are coincident in time. This may be treated as a purely mathematical manipulation of the resultant signal by Fourier analysis (Eq. (2))[17].

$$G(f) = \sum_{k=1}^{N} g(t) \cdot \cos\left(\frac{2\pi k}{N}\right) - j \cdot \sum_{k=1}^{N} g(t) \cdot \sin\left(\frac{2\pi k}{N}\right) \tag{2}$$

where $G(f)$ is the discrete Fourier transform of a real, time dependant function ($g(t)$): N is the number of data points and $k = 1, 2, \ldots, N$ [17]. Solution of the discrete Fourier transform is highly computationally intensive and, in practice, is achieved by using Fast Fourier Transform (FFT) algorithms that exploit Euler's theorem [18] (Eq. (3)) and the transform's symmetries. These are known as radix-2 algorithms and the Cooley–Tukey FFT algorithm (Eq. (4)) is probably the best known and most widely used example [19,20].

$$e^{i\theta} = \cos\theta + i\sin\theta \tag{3}$$

$$X_k = \sum_{m=0}^{N/2-1} x_{2m} e^{-(2\pi i/(N/2))mk} + e^{-(2\pi i/N)k} \sum_{m=0}^{N/2-1} x_{2m+1} e^{-(2\pi i/(N/2))mk} \tag{4}$$

where N is the number of data points and must be an integer power of 2, i.e.: $N = 2^x$ where x is a positive integer. The Fourier transform is symmetrical about the Nyquist limit, N_f (Eq. (5)):

$$N_f = \frac{N}{2} + 1 \tag{5}$$

where N_f is the number of frequencies that may be represented by a Fourier transform using a given number ($N = 2^x$) of data points and the '1' represents 0 Hz [17]. The FFT (Eq. (4)) thus calculates only the first half of the transform. Furthermore, Euler's relationship
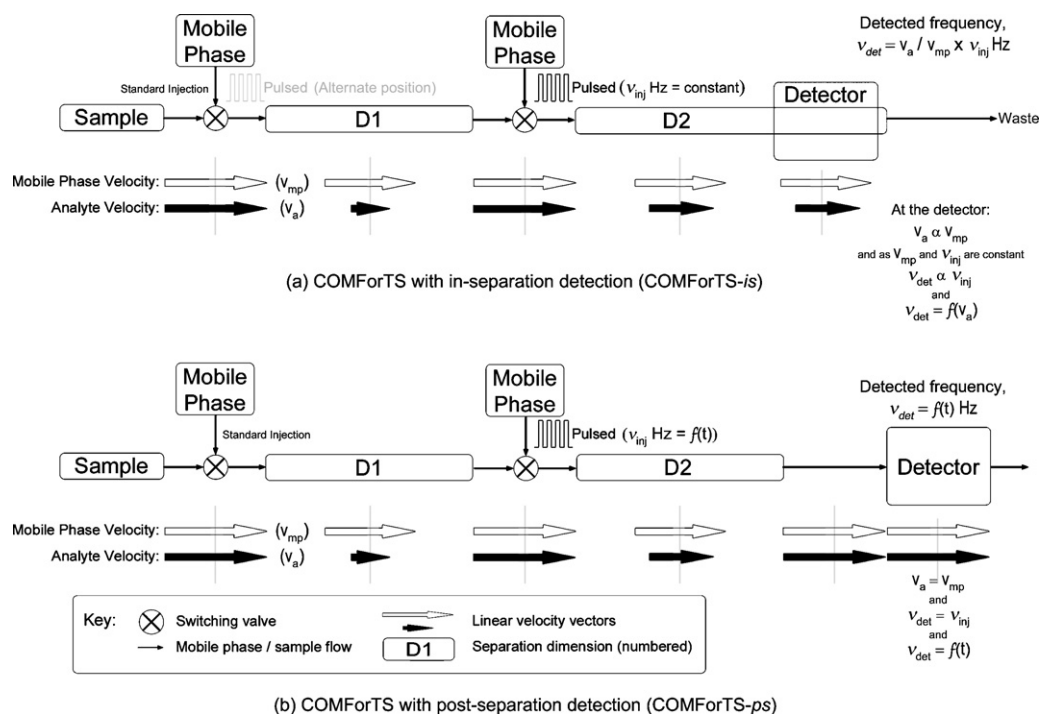
Fig. 1. COMForTS detector configurations and their influence on measured pulse frequencies. The detected frequency of pulses and the regime used to impose those pulses on the eluate are necessarily different when the detector measures a signal whilst the analytes are in the separation phase (a) or post separation (b).

(Eq. (3)) allows a further division of the transform into its odd ($m+1$) and even (m) terms. Eq. (4) may be applied recursively on its own output until $N=1$. The result of this 'divide and conquer' approach is a massive decrease in the total number of calculations, and hence the computational time required to perform the transform, allowing today's personal computers to perform FFT's on most analytical signals in real time.

1.2. Principle of the proposed method

Having established a means of separating overlapping frequencies, we must find some means of introducing these frequencies into the chromatograms without affecting the relative amplitudes of the component signals. The imposition of a frequency domain signal would be most readily achieved by the application of pulses at a defined frequency to the eluate flow between separation dimensions.

1.2.1. Pulse modulation in multidimensional chromatography

The use of pulse-modulated flow between separation dimensions to achieve online comprehensive multidimensional separations has already been established, with the identity and quantity of individual components in each dimension determined by the solution of multiple linear differential equations [21] and/or parallel factor analysis (PARAFAC) [1,22,23]. Such methods however, utilise very low frequency pulses (0.15–3 Hz [1]) whereby detection and deconvolution of overlapped peaks are achieved by statistical methods confined solely to the time domain. These chemometric algorithms are suitable only for target analytes and when multiple runs are combined for a single analysis all require alignment and pre-processing to enhance co-linearity of separations data. In combining multiple runs (though not in single runs), PARAFAC also requires an identical shape for a particular analyte peak in each run if it is not to output artificially generated peaks [24]. They also cannot resolve components that are not physically resolved in at least one dimension – unless there is a marked variation in peak shape between dimensions [1].

If pulse modulation combined with detection in the frequency domain is to be employed to overcome these difficulties, the effects of the relative velocities of the frequency source and the velocity of pulses in the medium relative to the detector must be considered: the Doppler effect.

1.2.2. Influence of detector configuration on detected frequencies

Chromatographic detectors may be used in two distinctly different physical configurations: either 'on-column' where analyte signals are recorded whilst the analyte is in the separation process or, more commonly, 'post-column' where the separation is complete and the analyte signal is recorded as it passes through the detector with the same velocity as the mobile phase. The technique proposed here of online inter-dimensional pulsed injections followed by time-based sequential Fourier analyses has been called Comprehensive Online Multidimensional Fast Fourier Transform Separation (COMForTS). Because these two detector configurations and their effects on the detected frequencies have fundamental differences, COMForTS must be operated in two correspondingly different modes: COMForTS-*is* for in-separation detection and COMForTS-*ps* for post-separation detection (Fig. 1).

In the case of in-separation detection (Fig. 1a), the differing relative velocities of the analyte and the mobile phase with respect to the detector entail that the frequency presented to the detector by any particular analyte (ν_a) is proportional to its linear migration velocity (V_a) and related to the injection pulse frequency (ν_{inj}) and the mobile phase velocity (V_{mp}) in the Doppler relationship of classical physics [25] (Eq. (6)):

$$\nu_a = \nu_{inj} \times \frac{V_a}{V_{mp}} \tag{6}$$

This expression may be re-written in terms of the retention factor of the analyte (k_A):

$$\nu_a = \nu_{inj} \times \frac{1}{k_A + 1} \tag{7}$$

From Eq. (7), it is clear that the pulse frequency chosen for the injection (ν_{inj}) must be sufficiently high that late-eluting peaks (with high values of k_A) present to the detector frequencies that remain above and are distinguishable from low and zero-frequency components (from 0 Hz to about 5 Hz) that are often strong in the Fourier transform of such data [3].

When post-separation detection is employed (Fig. 1b), analytes experience the same linear velocity as the mobile phase – which is also the velocity at which they were travelling when the pulses were applied. There is therefore no Doppler shift in the detected frequencies and the detected frequency is the applied pulse frequency. If analytes are to carry different frequency 'labels' that are related to their retention time in the previous dimension, pulses must be applied at different frequencies as some function of time ($f(t)$):

$$\nu_t = f(t) = \nu_0 \pm f(n) \cdot t \tag{8}$$

where ν_t is the pulse frequency at any given time ν_0 is the initial pulse frequency, t is time and $f(n)$ is either constant or a function to optimise the distribution of applied frequencies over time.

1.2.3. Frequency domain processing of pulsed signals

Pulsed (on/off) injection would be expected to produce a waveform that approximates a square wave and it is known [26] that such waves cannot be represented by a single sinusoidal function but must rather be represented by the sum of a number of such functions as shown in Eq. (9). As each term in the FFT takes the form of $ze^{-i\theta}$ (see Eq. (4)) with no zero offset, the FFT of a square wave of frequency f will thus contain a signal at the fundamental frequency f as well as signals with harmonic frequencies of $3f$, $5f$, $7f$, . . ., $n_o f$, where n_o is a positive, odd integer [26].

$$y = \frac{4}{\pi} \cdot \left(\sin(x) + \frac{\sin(3x)}{3} + \frac{\sin(5x)}{5} + \frac{\sin(7x)}{7} \ldots \right) \tag{9}$$

Square wave harmonics contain significant real data at predictable frequencies ($n_o f$) and with predictable magnitudes (M_n): the calculation of the magnitude (M_n) of the nth harmonic in the FFT of a square wave with fundamental magnitude M_f, pulse length ($t_2 - t_1$) and a repetition period of T [26] is given by Eq. (10).

$$M_n = \left| \frac{2M_f}{\pi n} \cdot \sin\left(\frac{\pi n(t_2 - t_1)}{T} \right) \right| \tag{10}$$

Any pulse-modulated separation process that is to be analysed in the frequency domain should therefore also make appropriate compensation for the presence of square wave harmonics.

1.2.4. Separations utilising more than two dimensions

When higher dimensionalities are considered, Eqs. (7) and (8) may be applied to assign to each dimension a unique range of frequency labels. Alternatively it is also possible to introduce multiple or multiplexed detectors or time-specific binary coded sequences [27,28] embedded in dimension-specific 'carrier' pulse frequencies; however, the complexities of the data analysis are beyond the present discussion.

1.3. Testing the principles of the proposed method

For the present, by the expedience of the introduction of a single frequency component, and the use of a single detector, very rapid comprehensive online two dimensional separations may now be achieved in real time without the lengthy analysis time concomitant with symmetric offline comprehensive separations. Moreover, components that are not resolved in the time domain in any dimension may be resolved in the frequency domain provided that the overlapping components are different in at least one dimension.

The only constraint on the type of time-based separations to which COMForTS may be applied is the facility to apply pulses to the eluate flow. In other words, COMForTS is equally applicable to, amongst others, liquid and gas chromatography and capillary electrophoresis.

The COMForTS technique combines the essentially digital physical process of 'on–off' pulsing and the entirely mathematical process of the Fourier transforms of the resulting signal. Because these processes are complicated and the lifetime of pulses within a separation will be limited by band-broadening, it was expedient to test the above conceptualisation of COMForTS by computational modelling and simulation before attempting to construct a practical system.

2. Experimental simulation

The feasibility of COMForTS was assessed by construction of a software package (the 'COMForTS package') that involved the semi-empirical modelling of band-broadening within a separation system and the simulation of time-domain separations based on defined retention data and the band broadening model. Separations in the second and higher dimensions were treated by repeating the above process using the output of the previous dimensions' separation as the input for the next dimension. The package allowed programmed pulse modulation of the signal before 'injection' into any given dimension.

The liquid chromatographic separation of an entirely arbitrary mixture of six components was simulated in both COMForTS-*is* and COMForTS-*ps* modes and the resulting simulated signal was then processed by a series of Fourier transforms of small time-slices of the simulated signal. The intent was to provide only a relatively uncomplicated demonstration of the main features, principles and possibilities of the proposed method and as such is not intended to represent a "best-case" separation at the limits of possibility which are the subject of further investigations.

3. Materials and methods

All data processing and calculations were performed by the COMForTS package, written by us in Microsoft Visual Basic .NET 2010 (Microsoft Corp., Redmond WA) as a proprietary executable program. Plotting functions were performed by the COMForTS package, Microsoft Excel 2007 and Mathematica 7 (Wolfram Research Inc., Champaign, IL).

The COMForTS package runs natively on Microsoft Windows 32- or 64-bit operating systems from Windows XP on, and requires the Microsoft .NET Client Framework 4.0 or above. The work presented here was conducted with a 64-bit version of the COMForTS package running under Microsoft Windows 7 64-bit on a personal computer equipped with an AMD Phenom II X6 (six-core) CPU @ 3.4 GHz (Advanced Micro Devices, Sunnyvale, CA) and 8 GB RAM.

3.1. Generation of simulated detector signals

Band-broadening was modelled using a constant area, point-by-point Gaussian distribution biased for peak tailing and fronting. At each point in time, the standard deviation of the distribution was empirically defined as a function of the empirical rate at which an injected plug broadened in time within a given separation system. The retention times and tailing factors of each of the components were defined arbitrarily (or empirically) and elution was assumed to be isocratic. The performance of the band-broadening model and separation simulation was assessed by using empirical band-broadening rates, retention times and tailing factors to reproduce the empirical chromatogram from which these data were derived
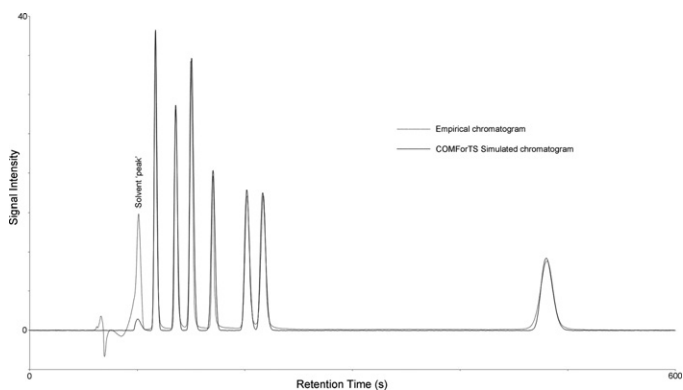
Fig. 2. Comparison of COMForTS-simulated data to the original empirical chromatogram. The COMForTS simulation (solid line) used the empirical retention times, peak tailing (symmetry) factors and the band-broadening rate measured from the empirical data (dotted line).

(Fig. 2). The simulated chromatogram produced peaks at the correct retention times with peak shapes that differed only slightly on the tails of peaks below the level of about 5% of the peak height. The behaviour of the model with respect to changes in flow rate and column dimensions was as expected by chromatographic theory. Samples of chromatographic noise were taken from a conventional HPLC-DAD system and were added to the final simulated signal, along with a linear baseline drift. The behaviour of the 'solvent peak' was also simulated (as an additional, unretained 'component' of the original injection) in order to facilitate visualisation of column void times and the relationships of retention time and detected frequencies to the pulse program. The resulting synthetic chromatograms thus contained real noise and close to real chromatographic distortions.

The retention characteristics of a simulated six component 'sample' were construed, for the purposes of demonstration, such that components 'A', 'B' and 'C' exhibited low retention and low selectivity in the first dimension (Fig. 3a) and *vice versa* in the second (Fig. 3b). The inverse was held to be the case for components 'D', 'E' and 'F'. The result of such behaviour was that, of the six components, only component 'A' was fully resolved by a conventional symmetrical online separation (Fig. 3b).

This elution pattern also served to illustrate that even if an additional detector were used immediately after the first dimension, the expected result would be the resolution of no more than two of the six components. Peak tailing factors (see [29]) were varied between 1.2 and 1.7 such that convoluted peaks consisted of components with both similar and dissimilar peak shapes.

Thus, the simulation conditions represent a set of circumstances under which no existing chromatographic method could achieve resolution of each of the six components within a comparable analysis time.

The COMForTS simulations were performed within the following liquid chromatography conditions and parameters:

- Column size: 25 mm × 3 mm (in 1st and 2nd dimensions).
- Overall rate of band broadening within the separation system: 0.005 s/s.
- Injection volume: 10 μL.
- Eluent flow rate: 2.0 mL/min.
- Run times: 11 s in the first dimension and 11 s in the second.
- Total analysis time: 22 s.
- Detector sampling rate: 256 Hz.
- Signal generation resolution: 1024 Hz (1st dimension), 512 Hz (2nd dimension).
- COMForTS-*ps* pulse function: 14.0 Hz + 0.3 Hz/0.05 s, t_0 = 5.3 s.
- COMForTS-*is* pulse frequency: 30 Hz.
- Total instrument noise was set to 1% RMS (using a sample of noise recorded from an HPLC-DAD instrument).
- A 10% sloping baseline was also incorporated into the COMForTS-*ps* detector signal.

Note that whilst the rate of band broadening is actually unitless, we have specified units of "s/s" to emphasise that it describes the increase in band width (in seconds) per second of retention.

3.2. Frequency and time domain processing of the detector signal

Within the range of frequencies used in the simulated pulsed injection regimes, the lowest square wave harmonic frequencies would fall partially within the range of the analyses. If the magnitudes of the harmonic components (which are legitimate signals) were to be summed with the magnitude of the fundamental frequency component, it would be possible to realise a significant improvement in the ratio of the detector signal to the background noise. For the sake of simplicity, however, the COMForTS package was programmed with the facility to only identify (using Eq. (9)) and remove these harmonics at the lesser of either their measured levels or the mathematically predicted level (Eq. (10)).

The COMForTS package was also equipped with some common apodization routines [17,18] to minimise the effects of performing Fourier transforms (which assume a continuous signal) on discrete data sets. Detector signals produced by separations in time also contain large tracts of data, such as the baseline, that contain virtually no frequency component. A sampling function [18] was used to mitigate the otherwise overwhelming near-zero frequencies contained in their transforms.

4. Results and discussion

4.1. Separation of physically unresolved components

Comprehensive online two-dimensional Fast Fourier Transform separations were simulated with both in-separation and post-separation detection. The total simulated analysis time in both cases was 22 s and the actual signal processing was completed in less than two seconds – sufficiently quickly for real-time processing. When detection employed both the time and frequency
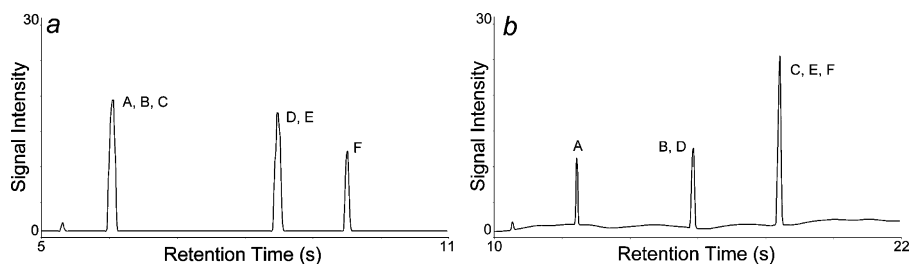

Fig. 3. Simulated conventional online two-dimensional separation of six arbitrarily defined components. The first dimension separation result (a) and final two-dimensional result (b) show that components B, C, D and E are not resolved in either dimension.
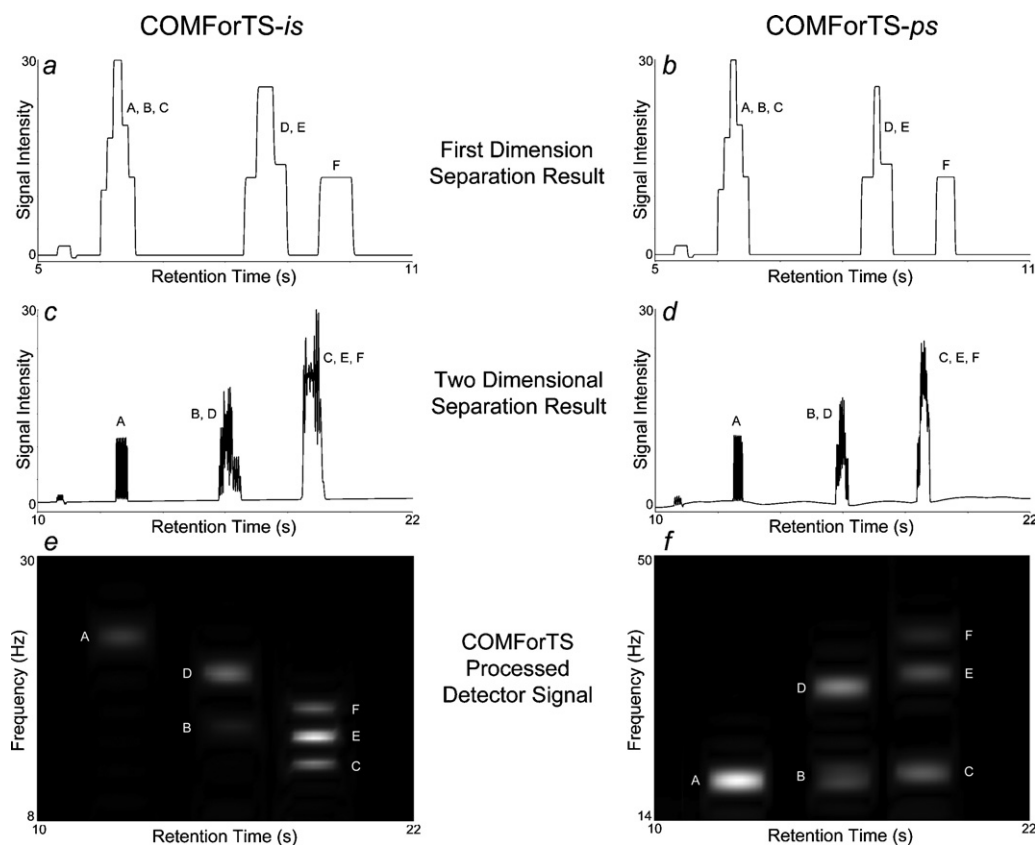
Fig. 4. Simulation separation results (a–d) and 2D density plots of the COMForTS processed detector signal (e, f) for the arbitrary six-component example. The eluate flow from the first dimension (a, b) was pulsed, according to each COMForTS mode, into the second dimension resulting in the final detector signal (c, d).

domains, all six components were completely resolved in both COMForTS modes (Fig. 4) even though components B, C, D and E were not physically resolved in either dimension.

The roughly rectangular peaks observed in the first dimension (Fig. 4a and b) are the natural result of employing a relatively large (10 μL) injection plug and the effects of band-broadening in a high-efficiency system do not become immediately apparent until these peaks are divided into smaller pulses as they pass into the second dimension (Fig. 4c and d).

4.2. Frequency labels and the reconstruction of separations in individual dimensions

Whilst there is absolutely no difference between the physical separations achieved in either COMForTS mode, as simulated analytes passed through an in-separation detector at a velocity that was lower than that of the mobile phase, it was expected that in this mode (COMForTS-*is*), that apparent widths of peaks (Fig. 4a and c) would be greater than those observed with post-column detection (COMForTS-*ps*, Fig. 4b and d). Differing patterns of detected frequency with respect to overall retention time are clearly evident in Fig. 4e and f, which is also a direct result of the differing mechanisms of frequency labelling, described by Eqs. (7) and (8), applicable to each of the COMForTS modes.

Considering first, the frequency-retention pattern of the COMForTS-*is* separation (Fig. 4e) in which a constant pulse-modulation frequency of 30 Hz was applied between the first and second dimensions: because the detector is situated in the second dimension of the separation, the observed frequencies are proportional to the analyte velocity (Eq. (6)), and hence retention time (Eq. (7)) in the second dimension, where 30.0 Hz corresponds to the velocity of the mobile phase (i.e.: a retention time equal to the void

time (5.3 s)). Component D, for example, with a measured pulse frequency of 20.9 Hz has a retention time of 7.6 s in the second dimension (30.0/20.9 × 5.3 s). With an overall retention of 15.8 s (Fig. 4c and e), the first dimension retention time of Component D must have been 8.2 s (15.8–7.6 s) (Fig. 4a). These relationships illustrate how the separation in any dimension may be determined and reconstructed by a series of such calculations based only on the signal from a single detector.

In the case of separations run with post-separation detection (COMForTS-*ps*), the relationship between the detected frequency and retention time is simpler because there is no difference between the linear velocities of the analytes and the mobile phase, and there is therefore no Doppler shift involved in the detected frequencies. The pulse frequencies are arbitrarily defined and related to the retention time in the dimension immediately prior to the application of pulses, by the pulsing regime defined by Eq. (8). If we examine the behaviour of component C, we observe an overall retention time of 18.4 s (Fig. 4d and f) with an observed pulse frequency of 20.4 Hz (Figs. 4f and 5). Because the pulses were applied with a defined regime as a function of time (14.0 Hz + 0.3 Hz/0.05 s, t_0 = 5.3 s), a detected frequency of 20.4 Hz corresponds directly to a retention time in the first dimension ((20.4 − 14.0)/0.3 × 0.05) + 5.3 s = 6.4 s (Fig. 4b). As the overall retention time was 18.4 s, the retention time in the second dimension alone must have been 12.0 s and we again demonstrate that the time-space separations in individual dimensions may be reconstructed from the COMForTS detector signal.

4.3. Detection in single and multiple domains

Fig. 4c and d describes the expected overlap of component peaks if detection were to be made in the time domain alone, producing
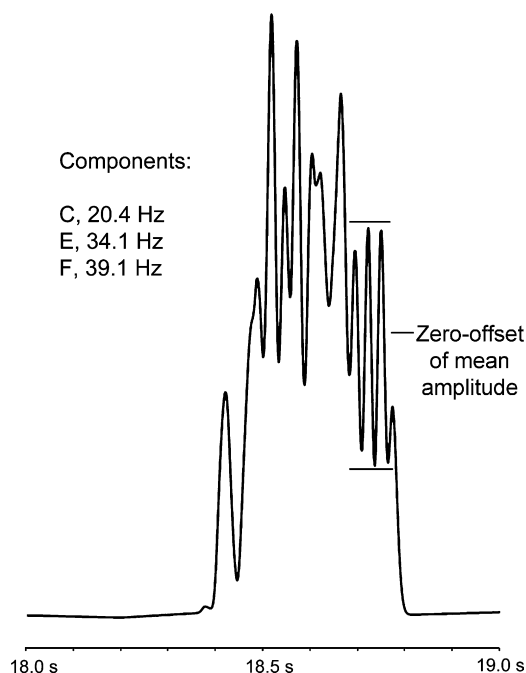
Fig. 5. Detail of the simulated COMForTS-*ps* detector signal of the latest eluting peak consisting of the co-eluting components C, E and F. The zero-offset of the mean pulse amplitude is also shown.

only one pure peak and two convoluted peaks with overlapping pulses of varying frequency (Fig. 5). Transforming the time domain signal to the frequency domain alone (Fig. 6, main image) produced equally inadequate results with, for example, only three main frequency bands detected in the COMForTS-*ps* signal (Figs. 4f and 6, inset). In terms of resolution, however, maximum temporal resolution is found in the pure time-domain signal and maximum frequency resolution (Fig. 6, inset) is attained in the Fourier transform of that entire signal. Using a single detection domain provides minimal, but high resolution, information about the signal.
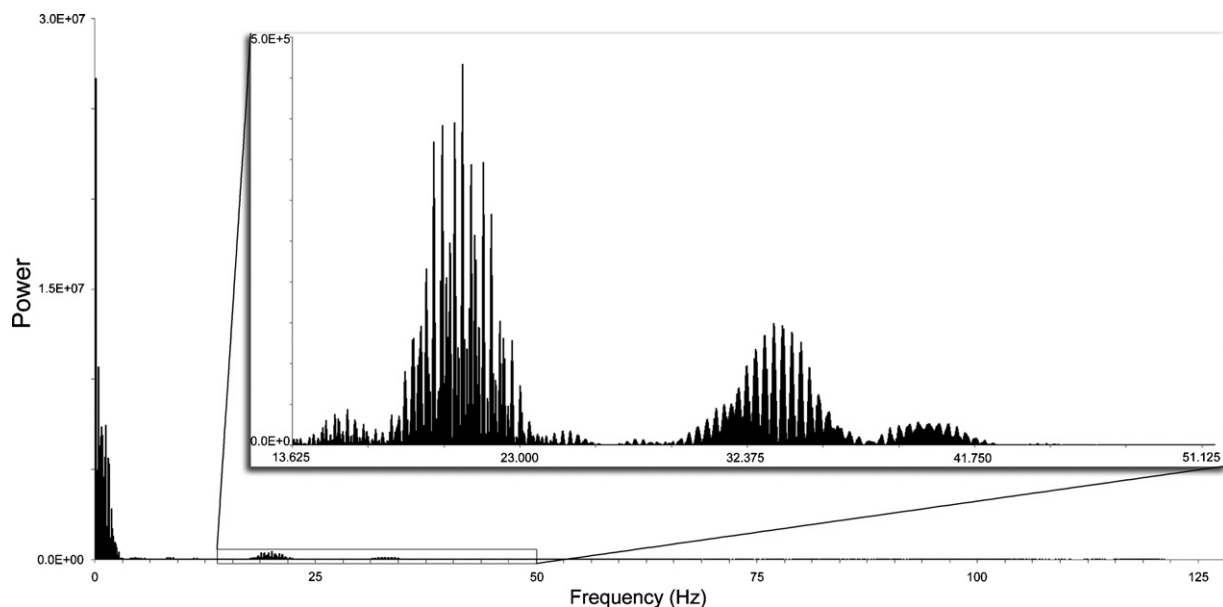
4.4. COMForTS and digital signal processing

Because the distinguishing factor in COMForTS processing of detector signals is the combination of information from both domains by measurement of the frequencies present in small time-segments, it is necessary that some resolution is lost in both domains. The frequency resolution (*df*) of the Fourier transform is given by Eq. (11) [26]:

$$df = \frac{1}{N} \times R \tag{11}$$

where N is the number of data points used in the transform and R is the data sampling rate, from which it is evident that increasing N simultaneously reduces the resolution in time whilst increasing resolution in frequency (i.e.: *df* is small). Whilst Fourier transforms were performed at 200 ms intervals, 256 data points (corresponding to a 1 s sample of the detector signal) were needed to provide adequate resolution in the frequency domain. Because the length of data used for transforms exceeded the length of data contained in a single transform interval, there is some overlap of transforms and an associated increase in apparent peak width. This effect is most clearly evident in the time width of COMForTS-processed peaks when compared to the widths of peaks in the raw detector signal as shown in Fig. 4.

Evident also, in Fig. 6, is some broadening of the component peaks along the frequency axis that, in this high-frequency-resolution plot, are resolved into discrete frequency components. This is undoubtedly the result of a lack of optimisation of the conditions chosen for the Discrete Fourier transform (DFT) and is a phenomenon known as 'leakage': a discrete Fourier transform uses a discrete number of data points (extracted from what is assumed to be a continuous data set) to return a discrete set of discrete frequencies. If any particular frequency actually present in the signal does not exactly match any of the discrete frequencies represented by the transform, the signal will be reported as the sum of two or more of the frequencies that are represented by the transform. This results in a 'spread' in the range of reported frequencies. These aspects of the DFT are well known and their effects are readily mitigated by appropriate processing techniques [30].

Whilst the function of our square-wave filter had been confirmed with pure rectangular pulses, little difference was observed

Fig. 6. High resolution power spectrum of the COMForTS-*ps* detector signal showing frequencies from zero Hertz to the Nyquist limit and (inset) the frequency range of applied pulses; 14 Hz to about 44 Hz. The detector sampling rate was 256 Hz and the sampling time was 22 s.
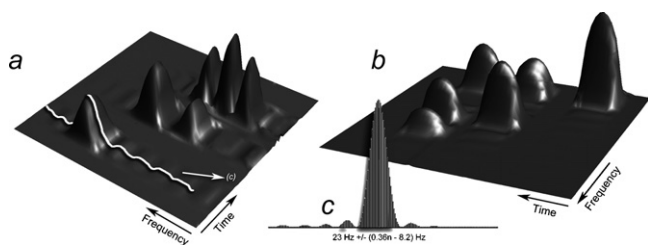
Fig. 7. Surface plots of the COMForTS processed signals resulting from COMForTS-*is* (a) and COMForTS-*ps* (b) simulations of the same six-component separation. A single frequency spectrum (c) obtained from the Fourier transform of the detector signal centred at the indicated retention time, displays a uniform series of overtones.

in the filtered and unfiltered COMForTS results. In practice, the essentially square form of the injection pulses was distorted to a significant extent by diffusion, resulting in a signal that more closely resembled a sinusoidal waveform (see Fig. 5) thereby greatly reducing the contributions of square-wave harmonics. This observation would be supported by Crabtree et al. [7] who, whilst not then able offer an explanation, did note that observed harmonic contributions, when using SCOFT, were of both a lower order and a lower magnitude than those predicted in their own simulations. Nonetheless, the rudimentary processing methods applied in this experiment revealed low-level spurious frequencies (Fig. 7) that were not anticipated by the authors.

These processing artefacts were found to be of a uniform nature (Fig. 7c) and of greater amplitude in the COMForTS-*is* chromatogram (Fig. 7a) compared to COMForTS-*ps* chromatogram (Fig. 7b). The reasons for this and whether the relationship would hold in all cases are yet to be established. However, in both COMForTS modes, the arithmetic mean of the applied pulses was not zero, and as the pulses decay, this mean value increases, as is evident in Fig. 5. At the same time, the 'baseline' of a given signal segment does not necessarily have a slope of zero. These factors represent a constant or near constant contribution to the pure frequency component of the signal, creating an offset from the expected mean amplitude of zero. As for a square wave, this constant offset is represented by the Fourier transform as the sum of a series of frequency components as previously described in Section 1.2.3. Furthermore, standard apodization windows assume that whilst the data is finite, the signal is continuous, which is certainly not the case in chromatographic signals. That most of the interfering frequencies appear to take the form of overtones (Fig. 7c) similar to those observed in un-windowed samples [18,26], and that the standard apodization windows employed appeared to have little effect, it is assumed that these interferences are due largely to inadequate apodization of the signal segments with smaller contributions from the zero-offset.

Even though the application of frequency analysis to discontinuous pulsed separations signals is entirely new, Felinger [3] describes a method that, by mirroring a signal sample, the time-broadening of COMForTS peaks would be halved and the effect of a sloping baseline is negated. Further reduction of this time broadening may also be achieved by cross-correlation with the pure time-domain signal and/or the pure frequency domain signal, whilst the effect of a constant zero offset is easily dealt with by adjusting the mean of the detector signal sample to zero (removing the zero offset), which has no affect on the absolute amplitude of the frequency components. It would be reasonable to expect that these improvements, together with the application of state-of-the-art digital signal processing techniques could result in a doubling of the resolution in both the time and frequency domains as well as significant gains in signal to noise ratios afforded by filtering and/or compensation for the observed harmonics.

4.5. Sensitivity and noise

By imposing full pulse modulation, the pulse amplitude of component peaks is not appreciably altered and as the Fourier transform reports this same amplitude, there is no real sacrifice in terms of detector sensitivity. And though the digital signal processing methods applied in this simulation were of a very basic nature, the ratio of the signal to RMS noise in the COMForTS-*ps* chromatogram was about 2500:1 whereas the same ratio in the simulated detector signal was set to about 100:1; more than an order of magnitude improvement. This is not altogether surprising, given that other chromatographic applications of the Fast Fourier Transform (for example: [31–34]) have been designed for this sole purpose. Three dimensional fully modulated GC separations processed using PARAFAC also improve signal to noise (by a factor of about ten) [1]. In contrast, methods employing partial pulse modulation have shown both a decrease in sensitivity and an increase in noise [21].

The performance of current COMForTS processing on signals with and without added noise was not found to be significantly different; indicating that the 'noise' in the COMForTS chromatograms consisted almost entirely of the uncompensated harmonics (Fig. 7) already discussed. Removal of these harmonics with appropriate signal processing methods would yield a further significant improvement in the already very high signal to noise of COMForTS separations.

4.6. Quantitative aspects of COMForTS

Within the context of the current examination of the principles of a proposed separations method based on the Fourier transform, it is not possible or justified to provide a detailed description or evaluation of the quantitative performance of COMForTS in practice. Nonetheless, it is possible to infer that because the Fourier transform is a linear operation and the amplitude of a sine wave is directly related to its Fourier magnitude [18], the amplitude (height) of pulsed signals will also be directly related to the Fourier magnitude. This provides some direct relationship, whether linear or otherwise, to raw signal intensity and a quantitative measure of analyte concentration. Other aspects of the signal processing, including apodization and sampling functions may also affect the efficacy of quantitative analysis. In each of these cases, the mathematical processes are not only reversible and linear but also are applied uniformly and with perfect reproducibility to the raw detector signal. There is little doubt that a stable and uniform relationship between analyte concentration and the height or area of COMForTS peaks will be established in practice.

4.7. COMForTS in three and more dimensions

The introduction into the analysis of frequency domain information by pulsed injection is by no means limited to two dimensions. It is possible, for example, to pulse injections between higher dimensions, utilising specific frequency ranges for each dimension or even to apply 'carrier' signals for binary coded dimension and time specific data that would then be carried through the separation by individual components. Such a scheme may be likened to the coding of digital FM radio signals and would afford the distinct possibility of rapid separations with much higher dimensionality. The Hadamard transform detection developed by Kaneta et al. [27] and used in capillary electrophoresis, to greatly increase sensitivity, is a cogent example of the practical application of a similar rationale that has seen a considerable range of applications extending into liquid and gas chromatography and beyond (for more recent developments and applications, see also [35–37]).

With a single detector, however, COMForTS is limited in the total separation time available due to the degradation of pulses over time. Nonetheless, as the retention time in a given dimension is related to the detected pulse frequency, the use of multiple inter (or intra-) dimensional detectors is no longer a moot point as it is with conventional online separations and the dimensionality of a system is only limited by the number of available detectors and the total analysis time only by the sum of the lifetime of pulses within each individual dimension. Even though the COMForTS simulation package was programmed with the capacity to simulate separations in higher dimensions, models of three dimensional separations produced detector signals with measureable frequencies but of a complexity that was beyond the processing methods employed for this test of basic concepts. Such complexities may well provide an opportunity for the application of PARAFAC to frequency-domain data and exemplifies the growing need for collaboration between chemists and signal-processing professionals [38,39].

4.8. Analysis time, resolution and peak capacity

COMForTS processing was performed by calculating the Fourier transform of segments of the detector signal at prescribed intervals, which correspond to fractions collected for offline comprehensive analysis. With an effective sampling rate of five samples per second, a conventional offline comprehensive two-dimensional separation would require, by comparison, an analysis time nearly thirty times longer (see Eq. (1)) – if indeed it were possible to collect injectable fractions at 200 ms intervals. Using this offline analysis as a basis for comparison, we can begin to examine the more important aspect of specific peak capacities (peak capacity per unit of analysis time, also known as peak capacity production) of some multidimensional separations methods. Specific peak capacities were calculated relative to an equivalent comprehensive separation assuming that each method utilises separation dimensions with the same efficiency and peak capacity as the current example (Fig. 3 and Section 3.1). The comparative results are listed in Table 1.

Both the method presented here and other pulse modulation methods [1,21,40] sacrifice some peak capacity due to the need to make unusually long injections such that a minimum number of pulse cycles are applied to each peak. These losses, which are greater for COMForTS, are more than compensated by the enhanced capacity afforded by higher dimensionality and the time-advantage gained by online separations. The COMForTS examples presented here have a specific peak capacity that is only slightly less, in two dimensions, than would be expected of a typical asymmetric pulse modulated separation with PARAFAC. If, however, only modest (50% of the expected) improvements are achieved by optimisation of COMForTS signal processing, the specific peak capacity of COMForTS has the potential to reach extraordinarily high levels. Furthermore, because COMForTS is able to resolve completely overlapped peaks as well as peaks that are not physically resolved in any dimension (provided only that no two specific peaks co-

elute in all dimensions), a significantly greater proportion of this theoretical peak capacity is available to COMForTS systems. Given that statistical overlap theory predicts that a reasonably high percentage of components in multidimensional separations will elute as doublets or triplets [40,41], the facility of COMForTS to resolve these components is a marked advantage.

The primary disadvantage of the proposed method is that total peak capacity is, except for the first dimension, limited by the need for short, high efficiency separations. The alternative is to implement either multi-point detection or high total peak capacity (asymmetric) first dimension separations combined with shorter, symmetrical separations in succeeding dimensions.

4.9. Signal acquisition and processing requirements

COMForTS necessitates detectors that exhibit extremely fast (ms) response and allow sampling rates (possibly) in the kHz range (e.g.: MS-TOF and perhaps FTMS [42]). Some detectors may require miniaturised flows cells (e.g.: UV and IR) in order to produce sufficiently rapid response times. Upgraded analogue to digital conversion capabilities will be needed in order to meet the required signal-sampling rates. It is also evident that, whilst not often used with such short columns, the effects of gradient elution must be considered. By varying the migration speed of the analytes, solvent gradients will, when using COMForTS-*is* (but not COMForTS-*ps*), have a significant impact on the range of frequencies recorded over the width of individual analyte peaks. Separations systems such as GC in which eluent flow rates vary along the length of the column, will exhibit similar uniform changes in absolute linear velocities (in both COMForTS modes). Such behaviour is compatible with the demonstrated theory of COMForTS. In the case of COMForTS-*is* separations the result is a distortion of peak shapes along a time–frequency vector dictated by the solvent or flow-rate gradient and does not represent a material problem. COMForTS-*ps* separations are still achieved in variable flow-rate systems but the relationship between detected frequencies and first dimension retention times (Eq. (8) and Section 4.2) is complicated but not unresolvable.

Given the range and extent of processing requirements, COMForTS may appear to be computationally onerous; but not so much so that it might strain the capabilities of current personal computers. For example, the Fourier analyses performed in this experiment were executed in less than one tenth real time. There will be an increased workload concomitant with higher retention and frequency resolution, harmonics-compensation and detection and decoding of binary sequences. Nonetheless, the workload is not anticipated to increase by more than a factor of four and most current multi-core CPU's would be capable of real-time signal processing and generation of two-dimensional COMForTS chromatograms.

Table 1
Estimated relative specific peak capacities of multidemsional separations.

Separation method	Relative specific peak capacity[a]	
	Two dimensional	Three dimensional
Comprehensive offline[b]	1	0.52
Comprehensive online pulse modulated with PARAFAC[c]	3.8	14
COMForTS with the current rudimentary processing[d]	3.7	26
COMForTS with optimised processing[e]	8.2	87

[a] Separation efficiencies are assumed to be the same as the simulated example.
[b] Based on 5.1 peaks per second in each dimension and 5 'cuts' per second.
[c] Based on 4 peaks per second per asymmetrical [1,21] dimension.
[d] Based on 1.8 peaks per second per symmetrical dimension.
[e] Based on 2.8 peaks per second (50% of the expected improvement).

5. Conclusions

This simulation has identified a number of the advantages and constraints that its practical application will enjoy or should deal with. At present, the main obstacles to the assembly and construction of an instrument are the need to produce programmatically defined pulses of relatively high frequency and to be able to detect and measure those pulses in high efficiency separations. That is not to say that this requisite technology does not already exist as does the technology for high-speed, high-data rate analogue to digital signal conversion but that it has simply not yet been applied in this field. Our work shows that these practical limitations may be overcome and that it is possible to achieve, with the aid of advanced signal processing methods, extremely rapid, sensitive and powerful separations.

The method of Comprehensive Online Multidimensional Fast Fourier Transform Separations has been demonstrated by semi-empirical modelling and simulation to be theoretically sound and probably practically feasible within the very near future. Calculations show that it meets all the self-imposed requirements of comprehensive multidimensional separations. Due to its ability to numerically resolve components that are not physically resolved in any dimension, the method exhibits the potential for very high peak capacities per unit of analysis time together with high sensitivity, low noise and the facility to correlate peaks detected with multiple detectors. Even though the total peak capacity of these first simulations may currently appear to be fairly limited, these unique advantages of the COMForTS method nonetheless define its potential as an attractive possibility for very high speed multidimensional separations, where physical separation of the analytes is not required. Further explorations of this potential as well as its inherent possibilities for increased total peak capacity are undoubtedly warranted.

It is expected that the earliest practical implementations of COMForTS will be on the 'lab-on-a-chip' scale wherein the physical limitations of pulse generation, pulse persistence and detection will be minimised. Further investigations of the construction of a simple COMForTS instrument, the optimisation of the parameters of its operation, and the possibilities to compensate for the effects of the harmonics are currently under investigation and the results will be reported later.

Acknowledgements

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