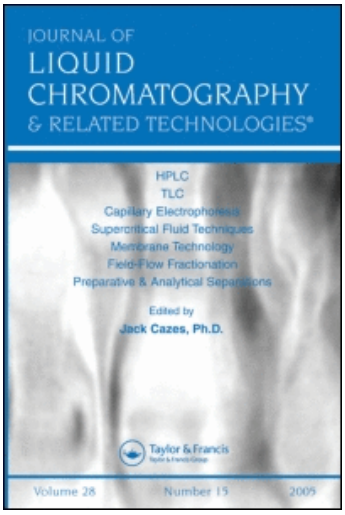

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High-Throughput Approach to 2-Dimensional Fingerprinting of Complex Mixtures such as Beverages

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Abstract: A combination of two or more analytically orthogonal separation modes not only multiplies their differentiation powers but may reveal some new relationships in complex molecular distributions, thus providing analytical information that individual separation dimensions could not deliver. The resulting multidimensional chromatogram may serve as a very sensitive differentiator of complex mixtures. Such a "fingerprint" can be linked to certain application relevant characteristics that are difficult to measure directly, including sensory parameters like taste and smell. Many traditional fields of industry, such as food, chemicals, and materials, could benefit from the power of multidimensional separations. However, lack of ready to go instrumental solutions, long analysis times, and overwhelming data streams, contribute to the relatively slow penetration of the 2-D HPLC and similar separation technology into the industrial and routine analysis labs. In this paper, we report on a high-throughput and low cost per analysis approach to the two-dimensional separations of complex samples. It is based on the ion-exchange fractionation using the 96-well plate followed by the reversed-phase HPLC profiling by an eight channel capillary HPLC instrument. Fingerprinting of common beverages is shown and discussed as an example, with a much broader applicability in mind.

Keywords: High-throughput analysis, Multidimensional separation, 2-D Chromatography, Parallel HPLC, Fingerprinting, Molecular distribution, Food analysis

INTRODUCTION

In recent years, high-throughput experimentation and combinatorial chemistry approaches led to a significant acceleration of development and discovery

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processes in a wide range of industries, making a step from the life sciences into the chemicals and materials.[1–4] Alongside this effort, a variety of new, rapid sample handling and measurement methods and devices became available, including modular high-throughput experimentation platforms[5] and commercial parallel HPLC instruments.[6] While unidimensional liquid chromatography is practically reaching its limits,[7] the combined separation power of multidimensional chromatography still offers numerous yet unexplored opportunities via its unique differentiation and analytical powers.[8–10] However, even the simplest 2-D liquid chromatography needs to deal with a great number of challenges on its path to become a high-throughput technique of a broad industrial significance. Quite often, it takes up to a few hours to complete a single 2-D chromatographic analysis, while desirable turn around time is typically no more than a few minutes. The "high-throughputization" of multidimensional separation processes might be the missing piece in a string of desirables that brings the related techniques closer to the application needs of chemical, food, polymer, petroleum, and other industries.

Earlier, we have reported on the fully automated high-throughput 2-D HPLC screening of a wide variety of random copolymers, consisting of the normal-phase separation in a gradient from hexane through tetrahydrofuran to water, the size-exclusion profiles for 30 fractions, and the re-equilibration back to hexane, all within approximately 20 minutes from injection to injection.[11] In cases where the throughput requirements go to thousands of samples per week and the resolution power of the second dimension cannot be compromised in sake of speed, parallelization of the separation dimensions may become a necessity. Lowering the cost per analysis might be another motivation factor behind the extremely rapid analyses. Monitoring of industrial streams, quality control of multicomponent formulation products, or quick optimization of materials with multiple distributions of molecular features, are examples of the analytical tasks that may benefit from extremely high throughput 2-D separations.

This paper describes a new approach to the 2-D chromatography based on an off-line but well synchronized combination of two parallel separations. After the initial extraction of polar species, the first dimension 96-channel anion-exchange technique separates the original beverage sample into several fractions, each of them to be separated further by an eight-channel capillary reversed-phase HPLC. Examples of the resulting high-throughput "fingerprints" for soft drinks and other beverages are shown and discussed.

EXPERIMENTAL

Samples and Reagents

All chemicals were of highest purity and purchased from Sigma, USA, unless stated otherwise. The beverage samples, namely the coffee, red wine, coke,

cranberry juice, apple juice, ice tea, and lemonade, were obtained from the local stores.

Instruments and Methods

The two-dimensional fingerprinting of the beverage sample discussed in this paper was performed employing an automated sample preparation and analysis workflow, as depicted in the Figure 1. The workflow was composed of the following instrumentation units:

Sample handling robot from Symyx Technologies Inc., Sunnyvale, CA, equipped with the Impressionist® software and customized to accommodate sample vials, balance, and shaker, in order to perform all necessary sample transfer and solvent exchange procedures throughout the workflow. More details on the robotic platform can be found elsewhere.[5]

The Speedisk-96 Positive Pressure Processor from Krackeler Scientific Inc., Albany, NY, with manifold air pressure of 30 psi. It has served as the first dimension separation instrument to extract and fractionate the anions from beverages, using the anion-exchange Oasis MAX 96 well SPE plates from Waters Corporation, Milford, MA, with 30 mg of trialkylamino functionalized polymeric sorbent in each well. Prior to the extraction procedure, the pH

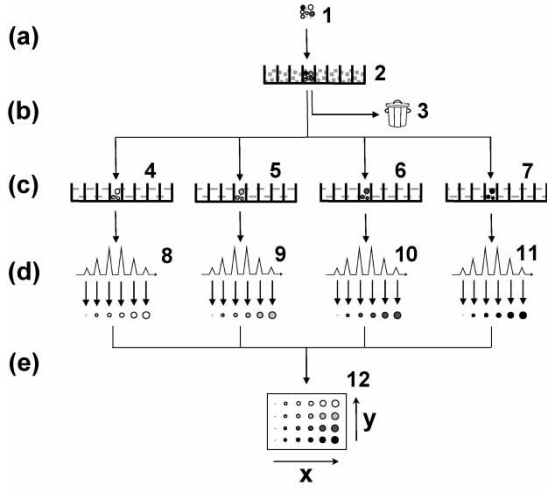

Figure 1. Scheme of the high-throughput 2-D separation workflow, consisting of the following steps—sample loading (a), washing (b), parallel ion-exchange fractionation (c), parallel HPLC of all of the fractions (d) and composing of the 2-D fingerprint (e). The components of the process are the sample (1), the 96-well ion-exchange plate (2), the waste (3), the ion-exchange elution fractions (4–7), the HPLC chromatograms (8–11), and the resulting 2-D fingerprint (12) along the ion-exchange (x) and the HPLC (y) elution axes.

values of the beverages samples were adjusted to 11 by 1 M NaOH, followed by 1:1 dilution with water. For each run, 2 mL of sample was loaded on the sorbent in each well and washed by 10 mL of 50%, vol., methanol in water. Then, 4 fractions were eluted using a series of buffers of gradually increased ionic strength, acid concentration, or organic solvent level. The eluent compositions are shown in Table 1. All the effluents were collected and advanced to the subsequent second dimension HPLC separation.

The 8-channel capillary HPLC ExpressLC-800 from Eksigent Technologies, LLC, Dublin, CA, equipped with UV detectors operating in the range of 200 to 380 nm with the separate traces collected at 230 nm. A series of eight C_{18} capillary columns of the same kind was employed during the analysis. The Zorbax SB-C18 capillary column with the 3.5 μm particle size of the packing material, 0.5 × 35 mm i.d., from Agilent Technologies, Santa Clara, CA, has been used for the method development. For the final 2-D fingerprinting, the X-Terra MS C18 capillary column with the 3.5 μm particle size of the packing material, 0.3 × 100 mm i.d., from Waters

Table 1. Eluent compositions for the first dimension separation

Eluent no.	Composition
Eluent sequence of increasing acid concentration	
1	1 mL of 2.5% formic acid in 50% methanol
2	1 mL of 2.5% nitric acid in 50% methanol
3	1 mL of 2.5% hydrochloric acid in 50% methanol
4	1 mL of 5% formic acid in 50% methanol
5	1 mL of 5% nitric acid in 50% methanol
6	1 mL of 5% hydrochloric acid in 50% methanol
7	1 mL of 10% formic acid in 50% methanol
8	1 mL of 10% nitric acid in 50% methanol
9	1 mL of 10% hydrochloric acid in 50% methanol
Eluent sequence of increasing ionic strength	
1	1 mL of 0.25 M potassium chloride in 50% methanol
2	1 mL of 0.25 M lithium bromide in 50% methanol
3	1 mL of 0.25 M potassium iodide in 50% methanol
4	1 mL of 0.5 M potassium chloride in 50% methanol
5	1 mL of 0.5 M lithium bromide in 50% methanol
6	1 mL of 0.5 M potassium iodide in 50% methanol
7	1 mL of 1 M lithium bromide in 50% methanol
8	1 mL of 1 M potassium iodide in 50% methanol
9	1 mL of 1 M lithium bromide in 50% methanol
Combined eluent sequence	
1	1 mL of 0.1 M potassium chloride in 50% methanol solution
2	1 mL of 0.1 M lithium bromide in 50% methanol solution
3	1 mL of 2% formic acid in 75% methanol
4	1 mL of 2% hydrochloric acid in 75% methanol

Corporation, Milford, MA, was used. Aliquots of 60 nL of each fraction coming from the first dimension anion-exchange separation were injected into the mobile phase composition gradient from 0.2 M phosphate buffer to the acetonitrile in 8 minutes, applied at the flow rate of 5 μL. The details of the data reduction method will be discussed in the next section.

The whole high-throughput 2-D separation workflow was integrated by a proprietary software and methodology from Symyx Technologies Inc., Sunnyvale, CA, including the sample tracking system, results database, and the data reduction software. At the end of the process, each beverage sample produced a 2-D fingerprint with the corresponding response table. Figure 2 illustrates the process of composing a fingerprint.

RESULTS AND DISCUSSION

The high-throughput 2-dimensional separation workflow pictured in Figure 1 has a capacity to process typically two 96-component libraries of samples per day. In the case of the beverage screening reported in this paper, with 4 ion-exchange fractions generated per sample and about 10 minute HPLC runs performed on each fraction, most of the working day would be spent for the sample prep and the ion-exchange steps, with the HPLC running overnight plus a portion of the next day while another library is getting ready. In such a way, the workflow could produce up to about 1000 2-D chromatograms per a regular week, and could be multiplied further if the chromatographic resolution can be lowered in gain of speed or by implementing additional workflow cycles into the initial one.

The resulting 2-D chromatogram represents some kind of fingerprint of the specific complex mixture, which can be used to track and correlate sample properties in many different ways. In our case, the 2-D fingerprint is the abundance distribution map along the two analytically orthogonal separation dimensions—the ion-exchange retention strength representing the y-axis, and the reversed-phase HPLC elution time as a measure of hydrophobicity on the x-axis. The process of combining the HPLC data streams along the ion-exchange elution order into the fingerprints is shown in Figure 2.

The resulting fingerprints can be then digitized by cutting it into several response zones and integrating the abundance responses within each zone (Table 2). The z-axis of the response map can be calibrated against the concentration of known related species, and the x-y position of the response zone can be related to the molecular properties of the calibrants. Unfortunately, the proper calibration standards are often unavailable for unknown and complex mixtures. However, if the main purpose is to get a characteristic fingerprint of a complex sample and, potentially, to correlate the distribution of the chemical species with sensory and other difficult to measure parameters such as taste, it does not necessarily require full understanding of the separation and detection mechanisms, but needs to provide the maximum resolution to effectively differentiate the complex samples.

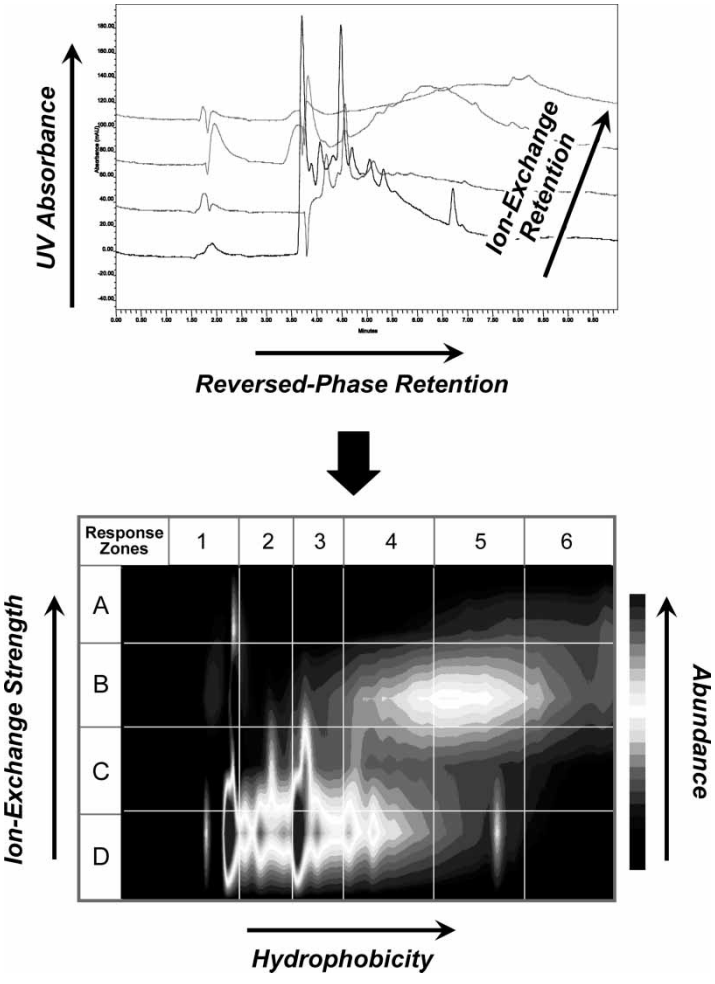

Figure 2. Example of the creation of the 2-D fingerprints by combining of the HPLC traces obtained for four different ion-exchange fractions from the coffee sample. The conditions of the ion-exchange and reversed-phase elutions are described in the Experimental part and the Table 1.

In order to maximize the differentiation power, we have optimized both separation dimensions, first individually, then in their two-dimensional combination. Finding the conditions for rapid HPLC profiling was a relatively easy task, considering the inherent advantages of the multichannel capillary HPLC instrument and quite limited choice of the applicable commercial columns. A sufficiently good compromise between the chromatographic resolution and the time of analysis was achieved using a capillary HPLC column of narrow i.d., medium length, and packed with a material of small particle size. On the other

Table 2. 2-D response distribution and deviations within

Zone	1	2	3	4	5	6
Mean abundance response, % of total response						
A	1.12	0.49	1.46	1.50	4.12	1.02
B	1.02	2.98	8.92	6.15	9.61	1.09
C	0.66	6.92	5.77	2.23	2.76	0.22
D	9.04	17.85	10.24	2.50	2.24	0.09
Standard deviation of the mean abundance response						
A	0.30	0.03	0.07	0.05	0.19	0.04
B	0.76	0.42	0.59	0.31	0.31	0.04
C	0.15	0.89	0.53	0.17	0.20	0.01
D	0.75	1.09	0.99	0.38	0.53	0.03

hand, optimization of the ion-exchange separation is much more complicated, taking into account the huge variety among potential eluents and very small separation efficiency of the minute amount of resin, available in a 96-well plate designed originally for a solid-phase extraction application purposes. Among the numerous possible combinations in the buffer sequences, we have focused on controlling the ion-exchange elution power by increasing concentration of the acid or salt at a constant level of the organic solvent in the eluent. All of the eluents are summarized in Table 1 and listed in the order of their use. As expected, the best differentiation has been achieved by the combination of the increasing acid concentration, ionic strength, and organic solvent level. Results of the corresponding 2-D separations are shown in Figure 3.

The optimized elution conditions have been applied to the high-throughput 2-D fingerprinting of a variety of complex mixtures, including common beverages. Figure 4 shows an example of such a fingerprinting of the coffee, red wine, coca cola, cranberry juice, apple juice, tea, and lemonade samples. The fingerprinting procedure for each sample was repeated five times in order to assess reproducibility of the whole process. By comparing the fingerprint images within the repeats of the same sample, as well as across the whole sample set, it is apparent that the difference among the sample is greater than the difference from run to run, thus indicating that sufficiently high differentiation power has been achieved.

As mentioned earlier, the fingerprints can be digitized into a data stream that could be more easily data based, browsed, reploted, and cross correlated in many different ways. For our purpose of creating a digital fingerprint using a minimum number of values specific for each sample, we have cut the 2-D chromatograms into 24 zones and stored the relative abundance responses, together with links to the sample description, separation process, fingerprint image, and other parameters, into a database. The data allowed the quantitative assessment of the differentiation power and the repeatability of the whole process. For example, the fingerprint reproducibility can be evaluated as a standard deviation of the

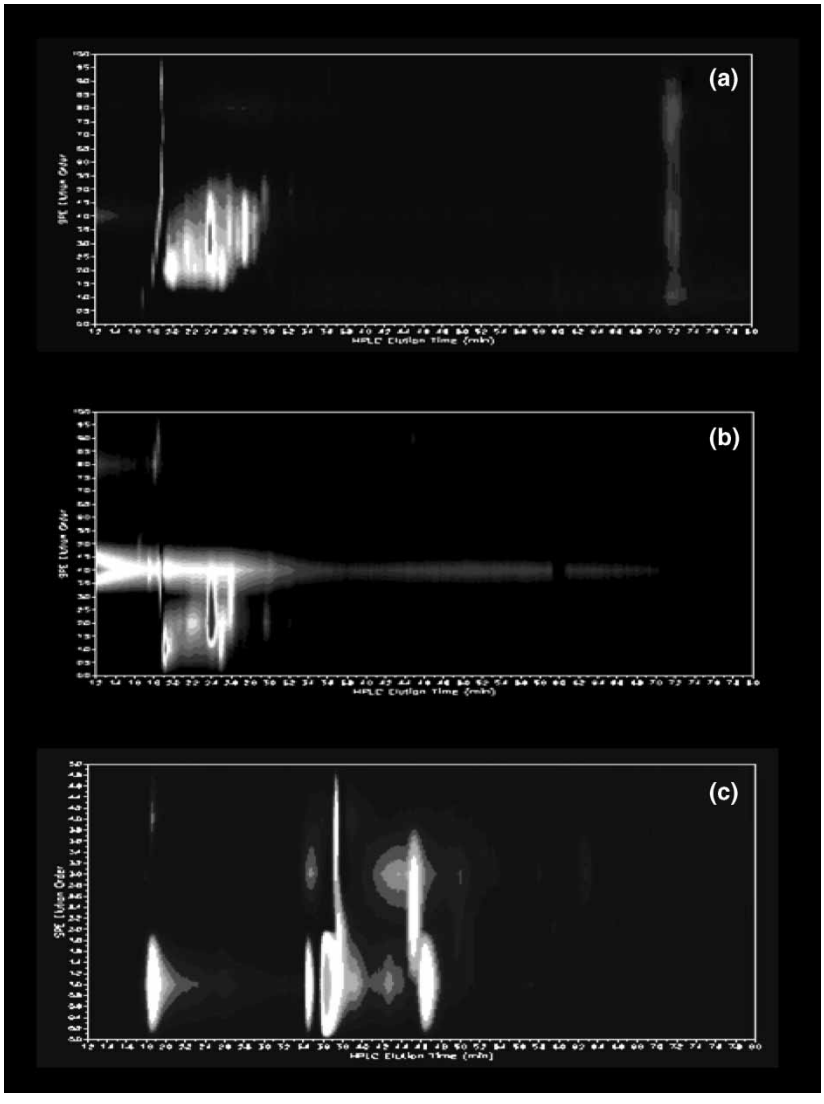

Figure 3. Example of the ion-exchange optimization process. The apple juice sample anions were eluted from the ion-exchange resin by a series of eluents in order of increasing acid concentration (a), ionic strength (b), and by combination of increasing acid concentration, ionic strength and organic solvent level (c). The specific eluent compositions are listed in the Table 1.

relative abundances in each zone within a sufficient number of repeats. Variation of about 1% of total response can be observed for the zones of the maximum response, much lower variation is typical for the other zones. Example of both relative responses and standard deviations is in Table 2.

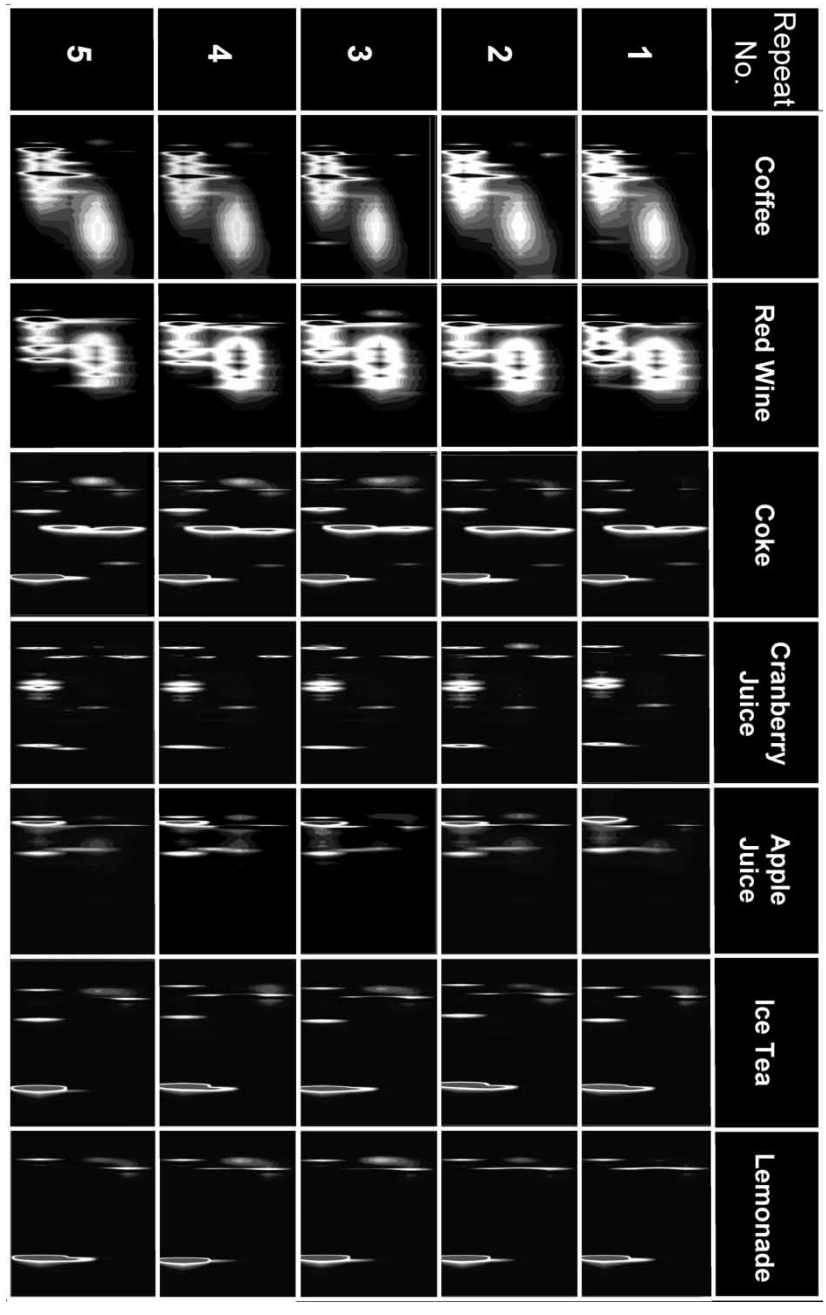

Figure 4. Comparison of the 2-D fingerprints obtained for 7 different beverage samples, with 5 repeats of each. The separation conditions are described in the Experimental part and the Table 1.

CONCLUSION

High-throughput 2-D fingerprinting of complex mixtures provides information on the chemistry distribution in the space of two orthogonal dimensions, in our case the ion-exchange strength and the hydrophobicity. Sufficiently high differentiation power with good repeatability needs to be achieved in order to use the fingerprints in possible further correlations to sensory and other difficult to measure performance properties of complex mixtures. High-throughputization of such a 2-D analysis may help this technique to introduce itself into, penetrate further in, and provide a substantial benefit to a broad range of application fields, including food, chemicals, and materials industries.

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