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Solid-phase microextraction-comprehensive two-dimensional gas chromatography-time of flight-mass spectrometry (SPME-GC×GC-TOF-MS) of non-steroidal anti-inflammatory drugs from water

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Abstract

Solid-phase microextraction with comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (SPME-GCxGC-ToFMS) was used for the qualitative analysis of nine non-steroidal anti-inflammatory drugs (NSAIDs) from water. This work focused on the selectivity of the extraction and on the added selectivity provided by the addition of the second column in the chromatography for the analysis of underivatized NSAIDs. Selectivity due to fiber selection, solution pH and extraction temperature is discussed. The final extraction conditions obtained were polydimethylsiloxane/carboxen/divinylbenzene fiber, pH 3.2, temperature 70°C and extraction time 30 minutes. The extracted NSAIDs were then analyzed by GCxGC-ToFMS and the chromatographic selectivity using a combination of a 5% phenyl polydimethyl siloxane column in the first dimension and a trifluoropropyl polysiloxane column in the second dimension is discussed. This method can be applied to determine the NSAIDs in complex matrices such as urine, blood for clinical toxicology for trace level analysis.

Keywords: Non-steroidal anti-inflammatory drugs (NSAIDs); solid phase microextraction (SPME); comprehensive two-dimensional gas chromatography (GCxGC); drugs; qualitative analysis.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are drugs which have analgesic, anti-inflammatory and anti-pyretic effects[1]. NSAIDs are easily available as over-the-counter (OTC) drugs which include ibuprofen, naproxen, ketoprofen, etc. NSAIDs can be misused for suicidal overdose due to easy availability. The residues of drugs are also emerging pollutants in water which enter the environment while they are manufactured, during improper disposal of drugs and also through human and animal excretion[1,2]. These are difficult to detect due to their acidic, highly polar and hydrophilic nature and cause adverse effects to the aquatic life and are a potential risk to human health at low concentration (ng/L) but these studies have not determined long-term toxicological effects[2,3]. Figure 1 shows the structures of the nine NSAIDs used in this study, including the most common of all: aspirin, along with other common NSAIDs. A variety of functionalities, including the characteristic aromatic and acid groups, ketones, nitrogen and chlorine.

Trace analysis of NSAIDs is mostly done using high performance liquid chromatography (HPLC) due to their low volatility and acidic nature; it is challenging to perform the trace analysis of these drugs using gas chromatography (GC)[3-5] Mostly the analysis of NSAIDs using GC is done by incorporating derivatization techniques such as methylation and other methods to make them volatile and heat resistant with the limit of detection at ng/L[2-7]. For the extraction of NSAIDs from water, techniques such as solid phase extraction (SPE) and solid phase micro extraction based on coatings with sol-gel and carbon nano-tubes have been employed[1-9]. However these methods can be time consuming, may utilize large amounts of organic solvents, are not automated or are not readily or commercially available.

Solid-phase micro-extraction (SPME) is a solvent free extraction technique that has been in common use for drug analysis for about two decades. SPME combines analyte extraction and pre-concentration into a single step due to the equilibrium established between

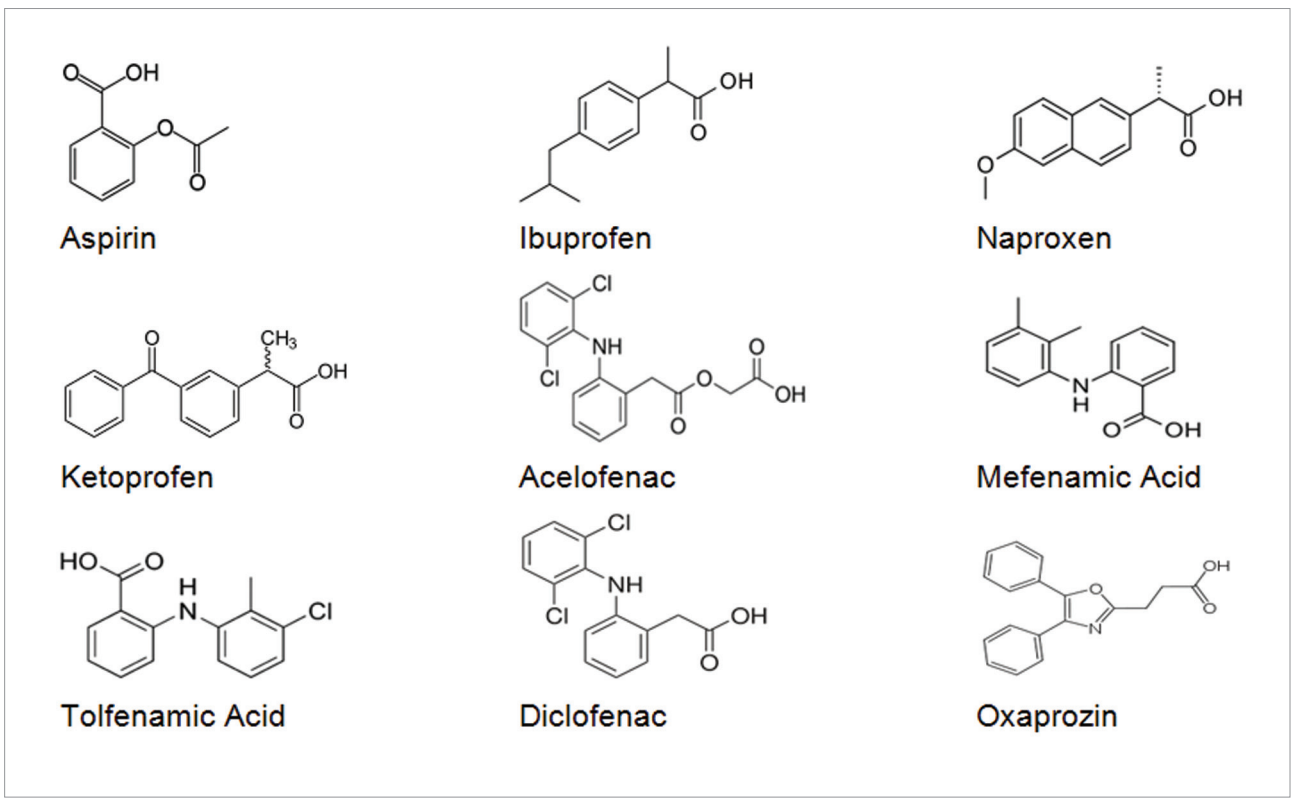

Figure 1. Structures of the nine NSAIDs used in this study.

the analyte in the sample and on the fiber by the aid of agitation. The desorption of analyte from the fiber takes place at high temperature in the GC inlet. The amount of analyte extracted depends on the partition coefficient between the sample and the sorbent layer (fiber) and their respective volumes. SPME is a technique which can be applied to a wide range of volatile analytes in complex matrices such as food, biological fluids and environmental samples. SPME combined with GC and GC-MS can be used for the analysis of a large variety of volatile and semi-volatile substances[10-14].

Comprehensive two-dimensional gas chromatography (GCxGC) is multidimensional separation technique in which two columns are in a series which are coated with different stationary phases. Commonly, the first dimension column is a non-polar and second dimension is a polar column. This combination of nonpolar-polar columns is considered as an orthogonal configuration which increases the resolving power and enhances sensitivity for the trace level analysis of co-eluting components from complex matrices. For detection in GCxGC, the detector must be fast and sensitive due to the rapid eluting from the second dimension; hence GCxGC is most often coupled to a flame ionization detector or a time of flight mass spectrometer (TOF-MS). The high sensitivity and selectivity of ToFMS makes it an ideal detector for GCxGC. GCxGC has been used for the analysis of drugs, especially drugs of abuse in clinical settings almost since its inception[15-22].

In this study, non-steroidal anti-inflammatory drugs (NSAIDs) are extracted from water using SPME and separated using GCxGC-ToFMS, without derivatization. GCxGC-TOF-MS provides a different approach to multidimensional separations. Upon analysis of these parameters, this method can also be applied to determine the NSAIDs in complex matrices such as urine, blood for clinical toxicology and the determination of NSAIDs concentration in drug formulations with easy sample preparation technique.

2. Experimental

2.1. Materials and chemicals

The NSAIDs used in this study were obtained from Sigma-Aldrich (St.Louis, MO): Ibuprofen, Naproxen, Ketoprofen and VWR (Randor, PA): Diclofenac, Mefenamic acid, Oxaprozin, Tolfenamic acid, Acelofenac. A Milli-Q Plus purification system, (Millipore, Milford, MA) was used to obtain Ultra-pure water in the laboratory.

2.2. Instrumentation

A Pegasus 4D comprehensive GCxGC-TOF-MS (LECO, St. Joseph, MI) equipped with an auto-sampler with SPME capability (Gerstel, Columbia, MD) was used in this work. SPME fibers: PA – polyacrylate, PDMS – polydimethylsiloxane, PDMS/DVB - polydimethylsiloxane/ divinylbenzene, PDMS/CAR/DVB – polydimethylsiloxane/carboxen/divinylbenzene were obtained from Sigma Aldrich (Supelco, Bellfonte, PA).

2.3. Sample preparation

NSAIDs standards at 1000 ppm were prepared individually in methanol. Then a mixture of NSAIDs was prepared by spiking 20 µL of standard to 20 mL ultra pure water which was pH adjusted using hydrochloric acid. Specific pH conditions are described in the discussion with the final pH selected to be 3.2.

2.4. SPME conditions

SPME experiments in this study were performed in direct immersion mode. A 20 mL screw cap vial was pre-incubated in the agitator for 10 min, followed by extraction for 30 min and desorption into a splitless inlet for 3 min with a post extraction bake in the inlet under split conditions for 10 min. Extraction temperature was optimized as discussed below with the final temperature selected at 70°C.

2.5. GCXGC-TOF-MS conditions

GCxGC with a primary RTX-5MS, 5% phenyl polydimethyl siloxane, (15 m x 0.1 mm x 0.08 µm) and a secondary RTX-200, trifluoropropyl/methyl polysiloxane (1.5 m x 0.1 mm x 0.1 µm) column, (Restek, Bellefonte, PA) was performed with a constant flow of 1 mL/min. GC inlet was maintained at 230^{0}C, oven was programmed at temperature 100^{0}C for 1 min, 10^{0}C/min to 180^{0}C for 2 min, 4^{0}C/min to 200^{0}C for 10 min with the secondary oven maintained 5^{0}C higher than the primary. The second dimension time was 5 sec. The transfer line was maintained at 280^{0}C and ion source at 250^{0}C. Modulator parameters were maintained at: hot pulse for 0.60 sec and cold pulse for 1.90 sec with a temperature offset at 20^{0}C. The acquisition delay was 120 sec. Masses were scanned from 40-400 amu with an acquisition rate of 100 spectra per second.

3. Results and Discussion

3.1. Solid phase microextraction (SPME)

The SPME conditions were optimized using three representative NSAIDs for simplicity: ibuprofen, naproxen and ketoprofen. When the full mixture was later used, extraction performance was satisfactory for all nine NSAIDs. Fiber phase, extraction pH and temperature were all optimized for a 30 min direct immersion extraction. The extraction time was later confirmed using the optimized conditions. While selectivity in GCxGC-ToFMS methods is most often discussed in terms of column selection and detection, it is clear that the choice of extraction phases (or solvents in a classical extraction) can also greatly impact selectivity.

3.1.1. Fiber Selection

In an SPME method, the greatest impact on selectivity is generated by selection of the appropriate extraction fiber. To date, most SPME methods employ the classical non-polar PDMS fiber or polar PA fiber. Figure 2 shows the extraction results for ibuprofen, naproxen and ketoprofen on three fiber phases: polydimethylsiloxane/carboxen/divinylbenzene (PDMS/CAR/DVB), polydimethylsiloxane/divinylbenzene (PDMS/DVB) and polyacrylate (PA). For ibuprofen, which is the smallest of the three analytes, with the least hydrocarbon backbone, the more polar PA fiber provided the highest response. For naproxen, which includes two fused aromatic rings, indicating a large potential for pi-pi interactions, the mixed phase PDMS-DVB provided the highest response, indicating stronger interactions between the rings on the analyte and on the stationary phase. Finally, for ketoprofen, which has the two aromatic rings separated by a ketone, the mixed PDMS/CAR/DVB phase provided the highest response. This is likely due to the presence of the ketone group interfering with pi-pi interactions between the aromatic rings on ketoprofen and the DVB rings in the fiber. Overall, the best performance was obtained using the mixed-phase fiber: PDMS/CAR/DVB. This is a moderately polar phase that can be effectively used for direct immersion SPME of drugs and other moderately polar, semi-volatile compounds[5].

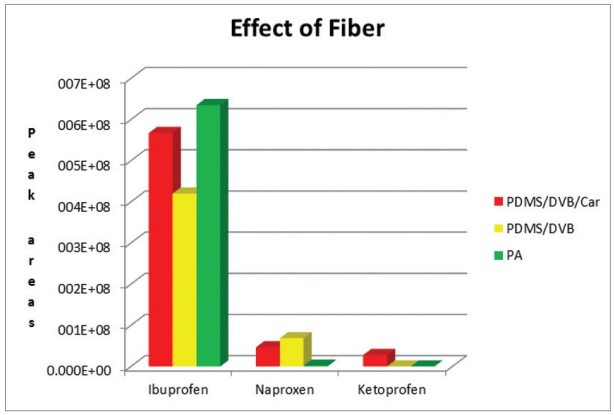

Figure 2. Effect of fiber selection on the extraction of naproxen, ibuprofen and ketoprofen.

3.1.2. Effect of pH

NSAIDs all have a characteristic carboxylic acid group at one end of the molecule, therefore all nine analytes in this study are weak acids. The extraction pH was studied across a wide pH range to both determine

the optimum pH for the extraction and to determine the pH range for which the extraction can be done and an instrument response still be seen. This can provide insight to the utility of SPME to extract NSAIDs from samples in which no sample preparation is possible, such as *in vivo* blood sampling. The pKa's of the NSAIDS are between 4-5, indicating that a lower pH of about 3 or lower should maximize the proportion of the ionizable NSAID that is in the neutral form and therefore available for extraction[13].

Figure 3 shows the effect of pH on the extraction of the three representative NSAIDs from water at 70ºC using the PDMS/DVB/CAR fiber. As expected, the highest response for all three NSAIDs is seen at pH 3.2. At pH 2, there may be some fiber degradation or fouling due to the higher ionic strength of the solution. As the pH increases, as expected, the response drops. However, finite responses were seen at pH as high as 7.7, indicating that, with sensitive instrumentation, NSAIDs could be extracted directly from solutions at physiological pH without further sample preparation. For ionizable compounds, the combination of fiber selection and pH are the major drivers of extraction selectivity. If the extraction goal is to ultimately maximize the instrument response, both should be optimized to provide the maximum response. If simplified or no additional preparation is required, then selectivity can be used to predict whether the extraction is possible at the given non-optimum conditions.

3.1.3. Effect of extraction temperature

Extraction from an aqueous solution into an organic extraction phase that does not contain any analyte is a spontaneous process. If the partition coefficient for the process is greater than one, the standard Gibbs' free energy change involved is negative and the extraction process is most likely exothermic. In the case of SPME, with $K > 1$, extraction must be exothermic. Analytes are concentrated into the fiber therefore the entropy change ΔS must be negative. ΔH must therefore also be negative and larger than $T\Delta S$ for ΔG to remain negative, indicating a spontaneous process. Therefore, all other variables being equal, an increase in extraction temperature will decrease the amount of analyte extracted, at equilibrium. However, for weak acids, such as NSAIDs, the acid dissociation equilibrium will also be affected by temperature. Generally, weak acid dissociation is endothermic, meaning that increased temperature will drive that process in the direction of ionization, reducing the amount of the neutral molecule in solution available to extract. Temperature also affects kinetics; increased temperature reduces the time required to reach equilibrium.

Figure 4 shows the effect of extraction temperature on the responses for the three representative analytes using the PDMS/DVB/CAR fiber at pH 3.2 and a 30 minute extraction. It is seen that 70ºC provided

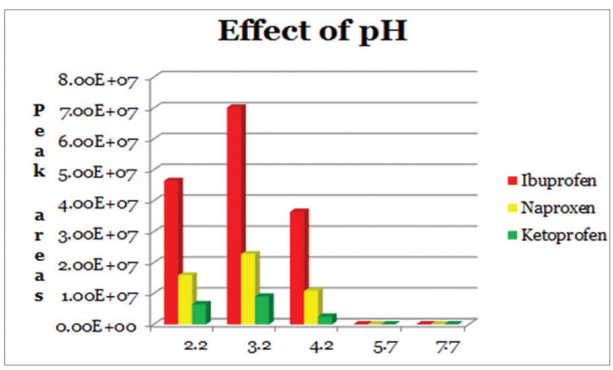

Figure 3. Effect of pH on extraction of naproxen, ibuprofen and ketoprofen.

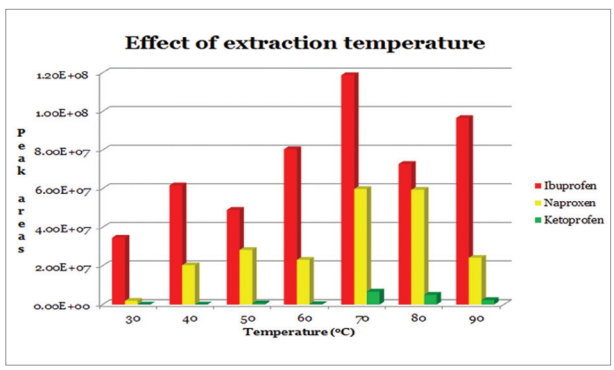

Figure 4. Effect of extraction temperature on extraction of ibuprofen, naproxen and ketoprofen.

the highest response for all three, so this was chosen for all further work. Responses are seen to increase until 70°C, likely a kinetic effect, indicating that equilibrium has not been fully reached, with a decrease above 70°C, likely a thermodynamic effect as exothermic extraction pushes that process back toward the aqueous phase and endothermic acid dissociation pushes that process toward the ionic form.

The final optimized extraction conditions were PDMS/CAR/DVB fiber, pH 3.2, 30 min direct immersion extraction at 70°C.

3.2. GCxGC-TOF-MS

Figure 5 shows a total ion chromatogram of a standard containing the three representative NSAIDs (Ibuprofen, Naproxen and Ketoprofen) extracted using SPME and analyzed using GCxGC-ToF-MS under the optimized conditions. In the first dimension, a 5% phenyl polydimethyl siloxane column was used. This stationary phase separates mainly based on a combination of dispersive interactions on the PDMS backbone with limited pi-pi interactions generated by the phenyl groups. Broadly, it is considered a non-polar stationary phase. These three analytes are well-separated, as expected, due to the addition of the second fused aromatic ring on naproxen and the second ring with ketone on ketoprofen. The second dimension column was 100% trifluoropropyl polysiloxane, a moderately polar phase. This stationary phase separates based on a combination of dispersive interactions and interactions between the lone pair electrons on fluorine with lone pair electrons on the analytes. It is especially selective for compounds containing nitrogen, oxygen and halogens[23]. As the selectivities of the two columns are quite different, pi-pi versus lone pair interactions, this column set may be considered orthogonal. In the second dimension, for the three representative NSAIDs, it is seen that ketoprofen is more strongly retained than ibuprofen and naproxen, mainly due to the presence of the ketone.

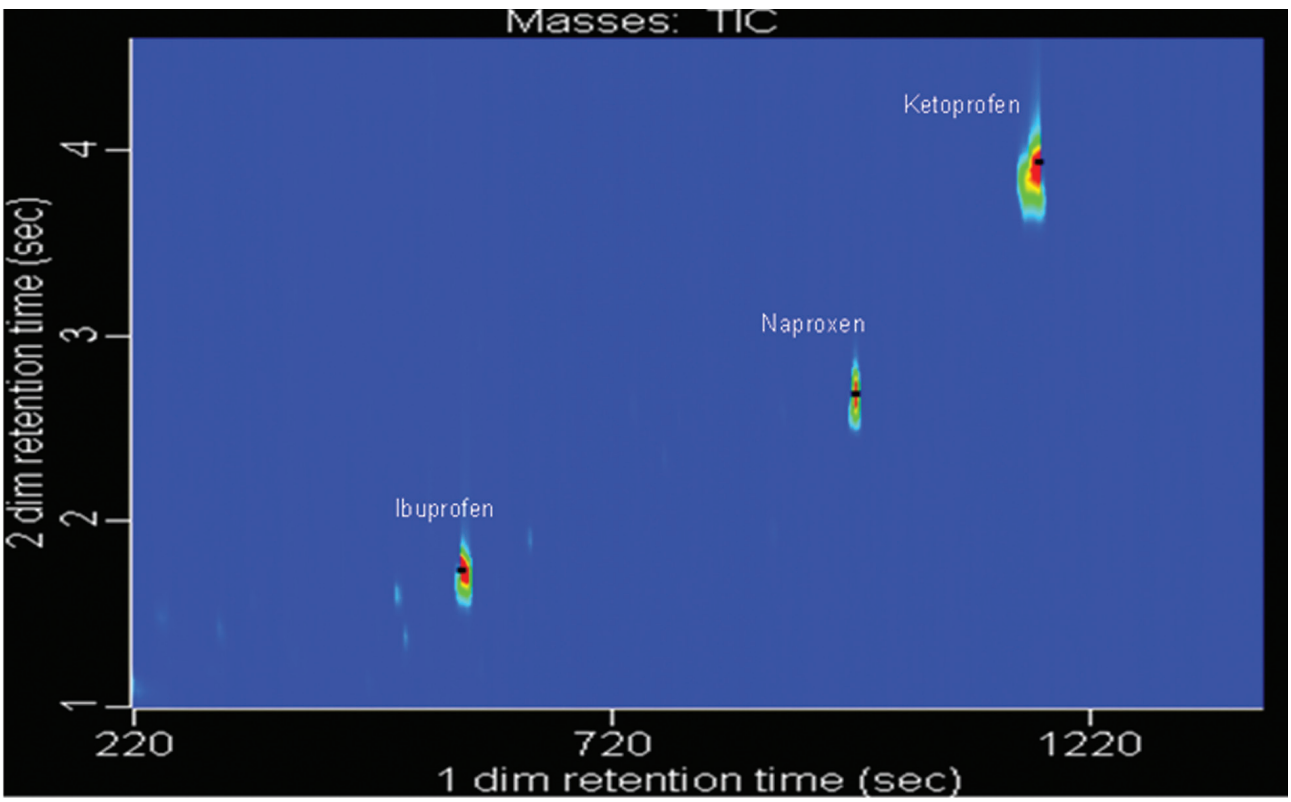

Figure 5. Total ion chromatogram of the three representative NSAIDs.

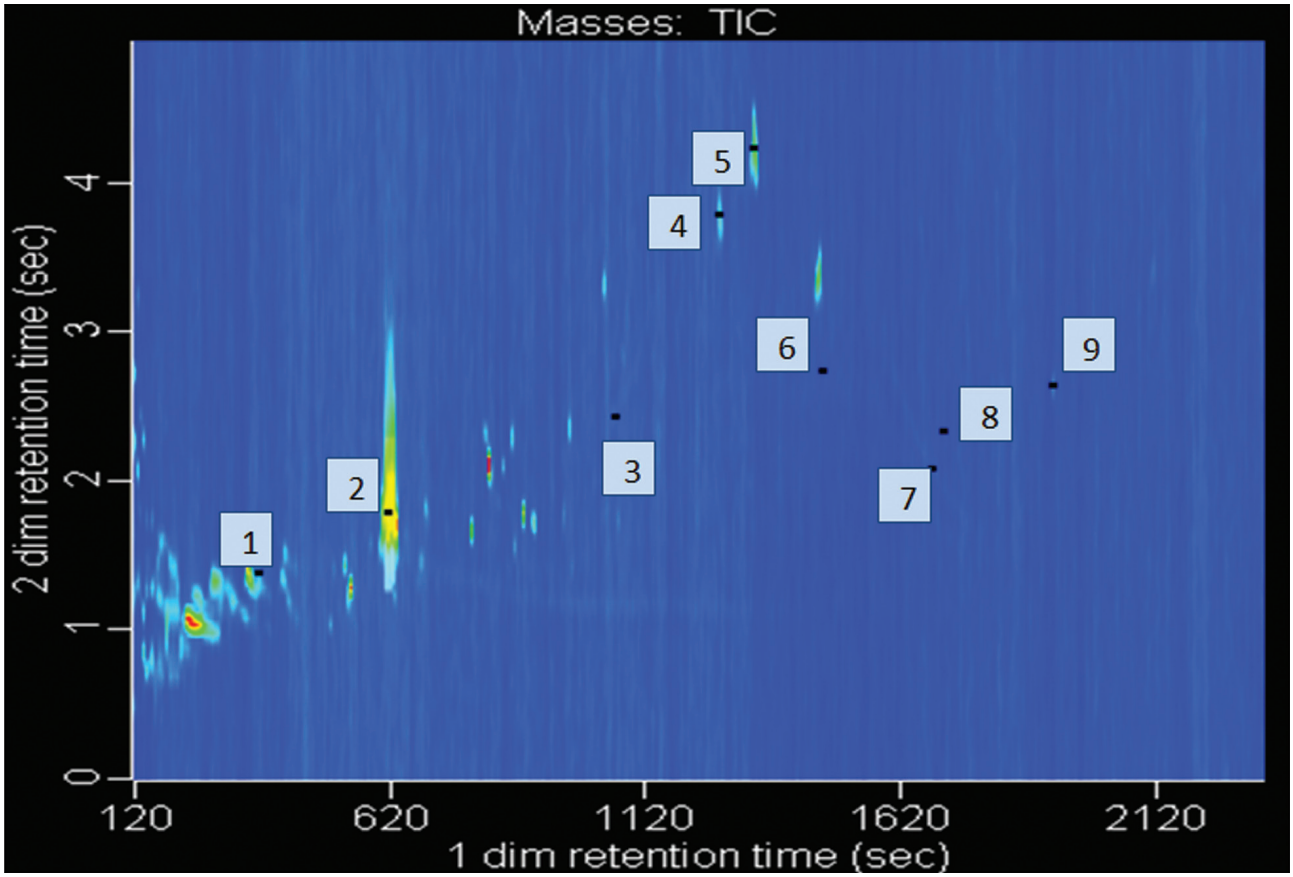

Figure 6. Total ion chromatogram of 9 NSAIDs extracted from water. List of NSAIDs: 1: Aspirin; 2: Ibuprofen; 3: Naproxen; 4: Ketoprofen; 5: Acelofenac; 6: Mefenamic acid; 7: Tolfenamic acid; 8: Diclofenac; 9: Oxaprozin.

Figure 6 shows a total ion chromatogram of all nine NSAIDs used in this study, extracted from water under the optimized SPME conditions. This chromatogram demonstrates both the benefits and challenges of adding selectivity to a chromatographic system by using the second dimension column. It is clear that all nine compounds are separated from each other in the first dimension alone, however, they may not be fully separated from matrix interferences (there are a number of additional, un-identified peaks in this chromatogram) and, as drugs often have low volatility, resulting in longer retention times and higher elution temperatures, there may be column or septum bleed, which would also interfere with a single-dimension separation. The additional selectivity afforded by the second dimension column will also reduce interferences due to additional endogenous compounds that may be extracted from a true biological sample.

Examining some peak pairs in Figure 6, both the benefits and difficulties of adding the second dimension column are seen. For peaks 1 and 2, which represent aspirin and ibuprofen respectively, are well-separated in the first dimension and not so well separated in the second. This is not surprising as the main structural difference between the two molecules are the presence of additional hydrocarbons on ibuprofen. There is, however, some separation from the unidentified matrix components. Peaks 3 and 4, naproxen and ketoprofen were discussed above; the presence of the ketone in ketoprofen, with lone electron pairs interacting with the fluorine moieties in the Rtx-200 stationary phase increases its second dimension retention significantly. Peaks 6 and 7, mefenamic acid and tolfenamic acid differ primarily by the presence of chlorine on tolfenamic acid. Stronger retention on Rtx-200

would be expected for tolfenamic acid but this is not observed on the chromatogram. However, tolfenamic acid is wrapped-around, as is peak 8, diclofenac. While wrap around is generally avoided, in this case, as the two peaks appear within the useful separation space, they do not co-elute with any of the other analytes or interferences, so this does not present a problem and actually simplifies the separation, allowing a shorter second dimension time.

Finally, the extraction and separation of these nine NSAIDs was performed without derivatization, which is most commonly performed for many of them. This greatly simplifies sample preparation. The added selectivity and sensitivity provided by GCxGC-ToFMS coupled with optimized SPME increases the range of compounds on which GC can be performed without derivatization, which often significantly complicates the sample preparation. Although the underivatized NSAIDs are generally polar compounds, they exhibit satisfactory resolution and peak shapes. While there may be some degradation or adsorption of the NSAIDs in the inlet of the gas chromatograph, there was sufficient recovery of the analytes onto the column to allow separation and detection.

4. Conclusions

Solid-phase microextraction coupled to comprehensive two-dimensional gas chromatography-time of flight mass spectrometry (SPME-GCxGC-ToFMS) was used to extract nine non-steroidal anti-inflammatory drugs from water and separate them without derivatization. This analysis provides insight into the many variables that can affect selectivity in multi-dimensional chromatographic methods. In the extraction, selectivity was affected by fiber phase selection, extraction temperature and pH. The added selectivity provided by adding the moderately polar second dimension column both reduces the possibility of problems with matrix interferences and provides added separation space for additional analytes or interferences.

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