



Canadian Journal of Chemistry

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Journal:	<i>Canadian Journal of Chemistry</i>
Manuscript ID	cjc-2023-0166.R1
Manuscript Type:	Research Article
Date Submitted by the Author:	22-Nov-2023
Complete List of Authors:	Lyczywek, Viktor; Cape Breton University, Chemistry Kaliaperumal, Rajendran; Cape Breton University, Chemistry Zuieva, Volodymyra; Cape Breton University, Chemistry; Memorial University of Newfoundland, Chemistry Titcombe, Sarah; Cape Breton University, Chemistry; Occidental College, Chemistry Bierenstiel, Matthias; Cape Breton University, Chemistry
Is the manuscript for consideration in a Special Issue or Collection?:	Highlighting Excellence in Canadian Undergraduate Chemistry Research
Keyword:	Indigenous medicine, analysis, cresol, birch bark, natural product

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Qualitative and Quantitative Analysis of Cresols Found in Maskwio'mi (Birch Bark Extract)

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ABSTRACT

Maskwio'mi (Mi'kmaw language for "oil made from birch bark") is a traditional topical skin medicine of the Mi'kmaq people of Atlantic Canada and is produced in a torrefaction process by heating birch bark (*Betula papyrifera*, paper birch) inside a metal container in a fire. The resulting viscous, oily mixture is traditionally mixed with goose fat or bear grease to create a topical salve that is subsequently applied to affected skin areas. When birch bark is exposed to high temperatures, pyrolytic chemical processes produce a complex mixture of pharmacologically active compounds including potentially harmful side products such as *ortho*-, *meta*- and *para*-cresol. This study discusses the qualitative and quantitative GC-MS analysis of cresols found in maskwio'mi and the challenges of the complex organic matrix. Using caffeine as an internal standard, *ortho*-, *meta*-, and *para*-cresol in birch bark extract were determined to be in the approximate order of 50 to 1,500 ppm range with $3.24 \pm 0.09 \times 10^2 \text{ ng mg}^{-1}$ (324 ppm), $8.7 \pm 1.0 \times 10^2 \text{ ng mg}^{-1}$ (87 ppm) and $12.4 \pm 1.6 \times 10^2 \text{ ng mg}^{-1}$ (1,240 ppm), respectively, and thus suitable by Health Canada and FDA cosmetics regulation standards when the extract is co-formulated in creams to concentrations of 0.1 to 5 wt% for topical use.

Key words

Indigenous medicine, analysis, cresol, birch bark, natural product

INTRODUCTION

Traditional natural folk remedies are at risk of being lost, particularly in oral-based cultures when their Indigenous languages are declining and vanishing. Maskwio'mi is a natural medicine of the Mi'kmaq people who are located in Atlantic Canada and the north-eastern United States.¹ a Maskwio'mi translates from the Mi'kmaq language as generated oil, or gathering, (o'mi) made from birch bark (maskwi), herein referred to as "birch bark oil" or "birch bark extract", even though direct translation into English, a noun-based language, loses its connotations from the action-based language of the Mi'kmaq.² The story on how maskwio'mi has been re-discovered is available in the Supplementary Material section.

Birch has been known to many cultures around the globe to provide pharmaceutically active compounds in roots, leaves and bark.³ Birch consists of 57 different species and grows in moderate climate zones mostly in the Northern hemisphere.⁴ There is an abundance of literature about medicinal uses and those typically involve solvent extraction processes of non-polar solvents or water steam distillation.³ In fact, one of the earliest reported natural products is the secondary metabolite of betulin, derived from the Latin word "betula" for birch.⁵ Betulin, approximately up to 25 wt% in dry birch bark, and one of its derivatives of betulinic acid have shown promising pharmaceutical properties such as anti-inflammatory, antioxidant, hepatoprotector, antitumor, and even anti-HIV.⁶⁻¹⁹ However, betulin's very low water solubility with 0.08 mg/L makes it difficult for applications as a medical drug for humans.¹⁸ Transformation and extraction processes using destructive distilling, dry distillation, and pyrolytic torrefaction processes are not often used even though ice-age Otzi was found to have had residue of birch bark tar in its luggage.^{20,21} Siberian farmers have used pyrolytic birch bark for tanning of hides in leather making using polyphenolic compounds.²² Reports from Scandinavia stated use of *Betula pendula* made tar was used successfully as a topical skin ointment. Natural health products are defined by species, part of plant and preparation.^{23,24} It is important to remind chemists not familiar with natural products that extraction results are species-dependent and thus can vary little or completely depending on a

specific case. While some *Betula* species are well researched, *Betula papyrifera* the paper birch species of Eastern Canada is not much reported and there is only one wide-ranging category of the International Nomenclature Cosmetic Ingredient (INCI) database for *Betula papyrifera* bark extract with non-defined extraction method.²⁵ There is currently no entry for *Betula papyrifera* under Health Canada natural health products regulation. Torrefaction and pyrolysis processes of organic matter can yield aromatic compounds. Depending on reaction conditions, there is potential for the development of cresols in the extract given that Health Canada is regulating cresols for use in cosmetics applications and natural health products.²⁶

Traditionally, maskwio'mi is made by heating a metal container filled with birch bark over an exposed fire for about 2 to 3 h in which a viscous oil drips through a hole in the bottom of the metal container to a receptacle container that is placed underneath the hole (for schematic drawing see Supplementary Information). As the maskwio'mi process is a pyrolysis reaction, exposing the lignocellulosic biomass to heat generates cresol compounds among other products. According to Okuda *et al.*²⁷, the lignin found in bark is pyrolyzed to give *ortho*-, *meta*-, and *para*-cresol at 400 °C. However, analysis of aqueous lignin samples from catalytic pyrolysis experiments by Mullen *et al.* revealed that cresols were not found within detection limits.²⁸ This suggests cresols can be present in trace amounts in maskwio'mi.

According to the *Canadian Environmental Protection Act*²⁹, cresols that enter the environment are deemed not hazardous to human life or health as they are of natural and ecological origin. Although many species exposed to cresols can metabolize and excrete them, they were seen to be minutely to moderately toxic in lab testing.³⁰ Uzhdavini *et al.* found the dermal LD₅₀ values of *ortho*-, *meta*-, and *para*-cresol in rats to be 620 mg/kg, 1,100 mg/kg, and 750 mg/kg respectively.³¹ Meanwhile, Vernot *et al.* reported the following dermal LD₅₀ values for the three cresol isomers in rabbits to be 890 mg/kg, 2,830 mg/kg, and 300 mg/kg.³² Therefore, this study aims to quantify cresols in

- 80 birch bark extract so that its relative safety can be assessed for use in skincare products and potential
- 81 future use as recognized natural health product.

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MATERIALS and METHODS

Ethics Approval

Co-PIs Tuma Young and Matthias Bierenstiel have been jointly researching maskwio'mi supported by CIHR Catalyst and Project Grant funding. Young is Mi'kmaq and involved the local Mi'kmaq community of Membertou First Nation, Sydney, Nova Scotia early with his project. The joint overall project was approved by REB at Cape Breton University and Membertou Ethics Watch oversights. It is based on *etuaptmumk* (Mi'kmaw language for 2-Eyed Seeing), developed by Elder Albert Marshall Jr.³³, balancing Indigenous Knowledge and science.

Materials

The cresol isomer standards (*ortho*-cresol, *meta*-cresol, *para*-cresol), tetramethyl silane (TMS), caffeine, and high purity LC-MS grade solvents were obtained from Sigma-Aldrich (MilliporeSigma Canada Ltd., Ontario, Canada). New solvent bottles were used and the solvent was not further purified. The purity of compounds was confirmed by analysis with GC-MS of dilute solutions in MeOH. 3.8 L (1 US gal) steel metal cans (empty paint containers) and 250 mL mason jars (Bernadin brand) were purchased from Home Depot and Walmart. The bark of *Betula papyrifera* (paper birch) was collected from fallen trees which were identified by botanical classification.³

Maskwio'mi (birch bark extract) was produced by the reported traditional method in a can-over-can method.¹ About 1 kg of bark of the paper birch (*Betula papyrifera*) was washed in water to remove dirt, then dried and cut with scissors into about 1 to 2 cm length pieces and filled inside a 3.8 L steel can which has a punctured hole (approx. 2 mm diameter) on the bottom. The top of the can was sealed tightly with a metal lid. At a suitable site outside, a small receptacle jar (250 mL mason jar) was placed inside a small hole in the ground and ground carefully placed around so as to not dirty the inside of the jar. The metal can is then carefully placed over the receptacle jar in alignment with the opening of the hole on the bottom. Some rocks or dirt are carefully placed on

the outside of the metal can to keep the container in place. Small and large pieces of firewood are stacked around the metal container and ignited. After keeping a fire for about 2 to 3 h, the warm ash and wood residues are carefully moved away from the metal container with a stick and the can is allowed to cool for 15 to 45 min. The metal container is taken away, and the receptacle jar is carefully removed from the ground without contaminating dirt falling inside. Approx. 50 to 80 mL of a dark viscous extract layer (maskwio'mi) is obtained atop about 50 mL of condensed water. The extract is obtained by decanting or pipetting off of the water layer and then stored in a sealed jar.

Bierenstiel developed a proprietary extractor equipment that mimics the campfire conditions by using an electric oven for maskwio'mi production. The obtained extract is similar to the extract obtained in the traditional can-over-can method. The extractor equipment is currently kept under trade secret as IP protection is currently explored.

Internal Standard

Internal standard analysis was required for the quantification of cresol isomers in birch bark extract to compensate for the difference in detector response. TMS and caffeine were chosen as non-interfering and non-contained substances with other compounds in the extract.

Sample Preparation

All cresol isomer standard solutions were made in methanol, forming approximate exact concentrations in the range of 20, 50, 100, 200, and 500 ng mg⁻¹ (ppm). These solutions were used for obtaining the calibration curves. The 20 ng mg⁻¹ (ppm) caffeine internal standard solution was prepared in a co-solvent mixture of water and methanol from a stock solution of 1,000 ng mg⁻¹ (ppm) caffeine in 1:1 water: methanol by volume and was diluted to 20 ng mg⁻¹ (ppm) by addition of methanol. The bark extract sample was prepared by diluting 0.4357 g in 4 mL of methanol.

GC-MS conditions

This study utilized two GC-MS instruments. An Agilent Technologies 5973 GC-MS system (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with an inert capillary J&K Scientific 2130-250252 NSP5 Inert column (J&K Scientific Inc., NS, Canada) of 30.0 m length, diameter of 0.250 mm and a film thickness of 0.25 μm . The method included an injection volume of 1.0 μL with a split ratio of 50:1. The carrier gas flow had a constant rate of 0.6 mL/min. The temperature began at 28 $^{\circ}\text{C}$ for 2 min and then increased by 15 $^{\circ}\text{C}/\text{min}$ until reaching 280 $^{\circ}\text{C}$. The final temperature was held for 5.00 min giving a total run time of 23.80 min. The other instrument was a Thermo Scientific Trace 1310 Gas Chromatograph (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a Restek Rtx-5MS column (Restek Corp., Centre County, PA, USA) of 30.0 m length, diameter of 0.250 mm and a film thickness of 0.25 μm . The gas chromatograph was coupled with a Thermo Scientific TSQ 9000 Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The optimized method included an injection volume of 1.0 μL with a split ratio of 50:1. The carrier gas flow had a constant rate of 10.0 mL/min. The temperature started at 50 $^{\circ}\text{C}$ for 1 min. This was increased by 20 $^{\circ}\text{C}/\text{min}$ until reaching 250 $^{\circ}\text{C}$ for another 1 min hold. Again, this was increased by 20 $^{\circ}\text{C}/\text{min}$ until reaching the final temperature of 310 $^{\circ}\text{C}$ which was held for 15.00 min giving a total run time of 30.00 min. Electron impact ionization was used for MS analysis. The MS transfer line and ion source temperatures were 250 $^{\circ}\text{C}$ and 230 $^{\circ}\text{C}$, respectively.

RESULTS and DISCUSSION

Method Development using TMS

The initial study to qualitatively and quantitatively analyze the cresol isomers in the birch bark extract utilized an Agilent Technologies 5973 GC-MS system equipped with an NSP5 Inert column. For method development, an injection volume of 1.0 μL dilute samples of bark extract

and cresols were analyzed. Cresols are relatively small molecules with 106 g/mol molecular mass and are eluting as one of the first compounds in the highly complex mixture of the bark extract (Figure 1). It was important to balance suitable GC thermal conditions to have maximum signal separations of the cresols while avoiding lengthy elution times. With conditions of a temperature ramp of 15.00 °C/min to 250 °C with a 4:1 split ratio, *ortho*-cresol signal occurred at 5.385 min while *meta*-cresol and *para*-cresol elute at 5.586 min (see Figures S1 – S4 in the Supplementary Material). This correlates directly with the weaker intermolecular forces of *ortho*-cresol shown also by its lower boiling point, at ambient pressure, of 191.0 °C while *meta*- and *para*-isomers have very close boiling points of 202.0 °C and 201.9 °C. There is a clear GC window within the first 5 min as it has few signals while the proverbial forest of peaks of the highly complex birch bark extract matrix occurs from 5 min onwards (Figure 1). This prompted the use of suitable internal standard that is eluting very early and not included in the bark extract matrix. TMS was chosen as a suitable standard as silanes are not present in natural organic products. It is inert and non-polar and highly volatile and eludes at 4.607 min with the chosen method. For quantification of the amount of *ortho*-cresol in a sample, the response factor for standard solutions were measured. First, 50 mL of both 3.88×10^4 ng mg⁻¹ (ppm) TMS stock solution and 6.30×10^4 ng mg⁻¹ (ppm) *ortho*-cresol stock solution were prepared in methanol and subsequently a series of three standard solutions of *ortho*-cresol 3.15×10^3 , 9.45×10^3 and 18.90×10^3 ng mg⁻¹ (ppm) were prepared by using 0.5, 1.5 and 3 aliquots of *ortho*-cresol stock solution. Analysis of the raw extract with TMS standard was measured in triplicates. 0.37 ± 0.09 mg of *ortho*-cresol was detected in the birch bark extract sample with a mass of 1.1830 g (see Table S1 in Supplementary Material). Therefore, $3.1 \pm 0.7 \cdot 10^2$ ng mg⁻¹ (w/w) *ortho*-cresol, or 310 ppm is present in the birch bark extract.

While these GC conditions and methodology were capable of quantifying *ortho*-cresol it was insufficient for the quantitative analysis of *meta*- and *para*-cresols as their signals overlapped.

Therefore, full cresol quantification required switching to the conditions and methodology described above permitted quantitative analysis of all cresol isomers.

Qualitative Identification of Isomers of Cresol

The identification of the isomers was then performed using full scan monitoring on a different GC-MS instrument of a Thermo Scientific Trace 1310 Gas Chromatograph coupled with a TSQ 9000 Triple Quadrupole MS detector. The GC was installed with a Restek Rtx-5MS column which did not allow the use of TMS due to chemical interactions of the column surface as described by the manufacturer. A new internal standard was required, and caffeine was chosen as most suitable for its inertness and absence in the bark extract matrix. A new GC separation methodology was developed with chosen conditions of 50 °C initial temperature, ramp of 20 °C/min until reaching 250 °C for another 1 min hold and continued ramp of 20 °C/min until reaching the final temperature of 310 °C for a total run time of 30.00 min (Figure 2 and S5 in Supplementary Material). The triple quadrupole mass spectrometer scanned the range of 50.000 to 550.000 Da, providing the mass spectra of cresol isomers (see Figures S6 – S8 in the Supplementary Material). The isomers eluted at the following retention times of *ortho*-cresol at 5.194 min, *meta*-cresol at 5.358 min, and *para*-cresol at 5.355 min. *Meta*-cresol eluded slightly after *para*-cresol which is similar to the only slightly higher boiling point of *meta*-cresol.

Calibration and Validation

For calibration, serial dilutions ranging 20 – 500 ng mg⁻¹ for each cresol isomer were prepared with the addition of 40 ng mg⁻¹ caffeine. The base peaks of each compound were chosen for quantification (108.1 *m/z* for *ortho*-cresol, 107.1 *m/z* for *meta*- and *para*-cresol). Calibration plots for cresol isomers were determined from the ratio of isomer and internal standard peak areas versus the sample concentrations (see Figure S9 in Supplementary Material). The graph for *ortho*-cresol expressed linearity in the 20 – 200 ng mg⁻¹ range as $f(x) = 222901.0077x + 3465580.8001$ ($R^2 =$

0.9262). The curve for *meta*-cresol expressed linearity in the 20 – 500 ng mg⁻¹ range as $f(x) = 232593.6937x - 3673107.1616$ ($R^2 = 0.9995$). The curve for *para*-cresol expressed linearity in the 20 – 500 ng mg⁻¹ range as $f(x) = 153723.661x - 2880621.2820$ ($R^2 = 0.9980$). Following the calculations described by Apostol *et al.*³¹, the assessments of the limit of detection (LOD) and limit of quantification (LOQ) for each isomer were based on the Standard Deviation (SD) of the y-intercept from the regression of their respective calibration curve. The LOD values for *ortho*-, *meta*-, and *para*-cresol are reported as 20.6 ng mg⁻¹, 23.6 ng mg⁻¹, and 60.9 ng mg⁻¹. Similarly, the LOQ values for *ortho*-, *meta*-, and *para*-cresol were determined to be 62.6 ng mg⁻¹, 71.4 ng mg⁻¹, and 184.6 ng mg⁻¹ (Table 1).

Cresol Isomers Detected in Birch Bark Extract

Following full-scan analysis by GC-MS, the quantities of *ortho*-, *meta*-, and *para*-cresols in birch bark extract were determined as $3.24 \pm 0.09 \times 10^2$ ng mg⁻¹ (324 ppm), $8.7 \pm 1.0 \times 10^2$ ng mg⁻¹ (87 ppm) and $12.4 \pm 1.6 \times 10^2$ ng mg⁻¹ (1,240 ppm), respectively. Given that the hydroxy group is a +M organic functional group, resulting *ortho* and *para* isomers are most dominant in the sample over the *meta*-isomer. Additionally, there is a steric interaction of the adjacent methyl group making the *para*-isomer most abundant. While the separation of *meta*- and *para*-cresols is very tight, we were able to selectively identify the two isomers for quantitative analysis in the newer model of Thermo Scientific Trace 1310 GC coupled with a TSQ 9000 over the Agilent Technologies 5973 GC-MS instrument. Nevertheless, the detected quantitative determination of 310 ± 7 ppm with the Agilent system overlaps widely with 324 ± 9 ppm ranges of the Thermo Scientific instrument showing that there is congruency between the two different methods and confirming quantification.

Safety Assessment of Cresol Isomers in Birch Bark Extract

The *Int. J. Toxicol.*³⁵ reports that the concentrations of *ortho*- and *meta*-cresol up to 0.5 wt% in cosmetics appear to be safe. Concentrations of the order of 50 ppm to 1,500 ppm, or 0.005 wt% and 0.15 wt%, have been detected in the birch bark extract samples. Furthermore, the typical concentration of active pharmaceutical ingredients in topical skin ointments is between 0.1 wt% to 5 wt% in the compounded cream, thus the bark extract is diluted by the base cream of a factor of 20 to 1,000, or one to three orders of magnitude, making final concentrations of cresols in the creams on the order of maximum 75 ppm, or 0.0075 wt% to just trace amounts and thus deeming the extract safe for cosmetic regulation in Canada. As a comparison, roasted coffee typically contains 10 to 50 ppm cresols concentrations which is ingested internally and in much larger amounts when drinking several cups of coffee.^{30,36,37}

CONCLUSIONS AND FUTURE WORK

We developed a GC-MS method for the detection and quantitation of three cresol isomers in the traditional Mi'kmaq skin medicine of maskwio'mi (birch bark extract) using caffeine as an internal standard. Separation of *meta*- and *para*-cresol is difficult due to their similar intermolecular forces. *Ortho*-, *meta*-, and *para*-cresol in birch bark extract were determined to be in the 50 to 1,500 ppm range of the order of magnitude as the first step of content analysis of maskwio'mi. This range is sufficiently low to determine that maskwio'mi is safe for topical skin treatments with regard to cresol content. We are cautious with a conservatively wider reported range of cresols than by the determined results of each compound as the next step of the analysis is a deeper study regarding the variations of cresols and other compounds given natural variances in a campfire for pyrolytic conditions. Bierenstiel developed a proprietary extract equipment that mimics the campfire can-over-can method of the traditional Mi'kmaw method. This equipment has multiple sensors and with electric heat can modify pyrolytic reaction conditions. Future analysis will include analysis of cresol levels given wider processing conditions.

Currently, anecdotal stories of maskwio'mi having beneficial medical effects on a variety of acute and chronic skin conditions are being collected and analyzed. Studies are underway to explore the pharmacological properties of the extract. These properties are then correlated with future studies of isolating and identifying pharmacologically active compounds in the highly complex matrix of birch bark extract.

ACKNOWLEDGEMENTS

We thank the members of the Mi'kmaq community of Membertou First Nation, Sydney, Nova Scotia for their collaboration and support for this project in particular Tuma Young, KC who rescued and rediscovered the maskwio'mi story and recipe. Funding was provided by the Canadian Institutes of Health Research (CIHR), and Cape Breton University. Initial cresol determination was by VL as part of his BSc Chemistry (Honours) project that was suddenly cut short by the initial Covid-19 lockdown. ST is an undergraduate BSc student and wants to thank Mitacs Globalink and US Fulbright for their financial support.

FUNDING

This research was supported by Cape Breton University (CHEM 4900 project, Viktor Lyczewek), Canadian Institutes of Health Research (Project Grant 420598), Fulbright Canada Student exchange program (Sarah Titcombe), and Mitacs Globalink (Project # 30805, Sarah Titcombe).

Competing Interests Statement

Tuma Young and Matthias Bierenstiel hold a financial stake by co-founding an independent commercial business (Maskwiomin Inc.) in 2020 from this project for the ethical commercialization of an L'nuk (Mi'kmaw) medicine with the support of the community of Membertou. The IP of the proprietary extractor equipment has been assigned to the Maskwiomin

company. Maskwiomin Inc. is producing and selling maskwio'mi creams and soap products under cosmetic licensing regulation of Health Canada.

Data Availability Statement

Data generated or analyzed during this study are provided in full within the published article and its supplementary materials.

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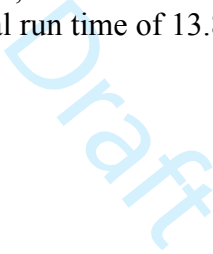
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Table 1. Retention time, linear range, correlation coefficient (R^2), limits of detection (LOD), and limits of quantification (LOQ) for *ortho*-, *meta*- and *para*-cresol.

Compound	Retention time, min	Linear range, ng mg ⁻¹	R ²	LOD, ng mg ⁻¹	LOQ, ng mg ⁻¹
<i>ortho</i> -cresol	5.194	20 – 200	0.9262	20.6	62.6
<i>meta</i> -cresol	5.358	20 – 500	0.9995	23.6	71.4
<i>para</i> -cresol	5.355	20 – 500	0.9980	60.9	184.6

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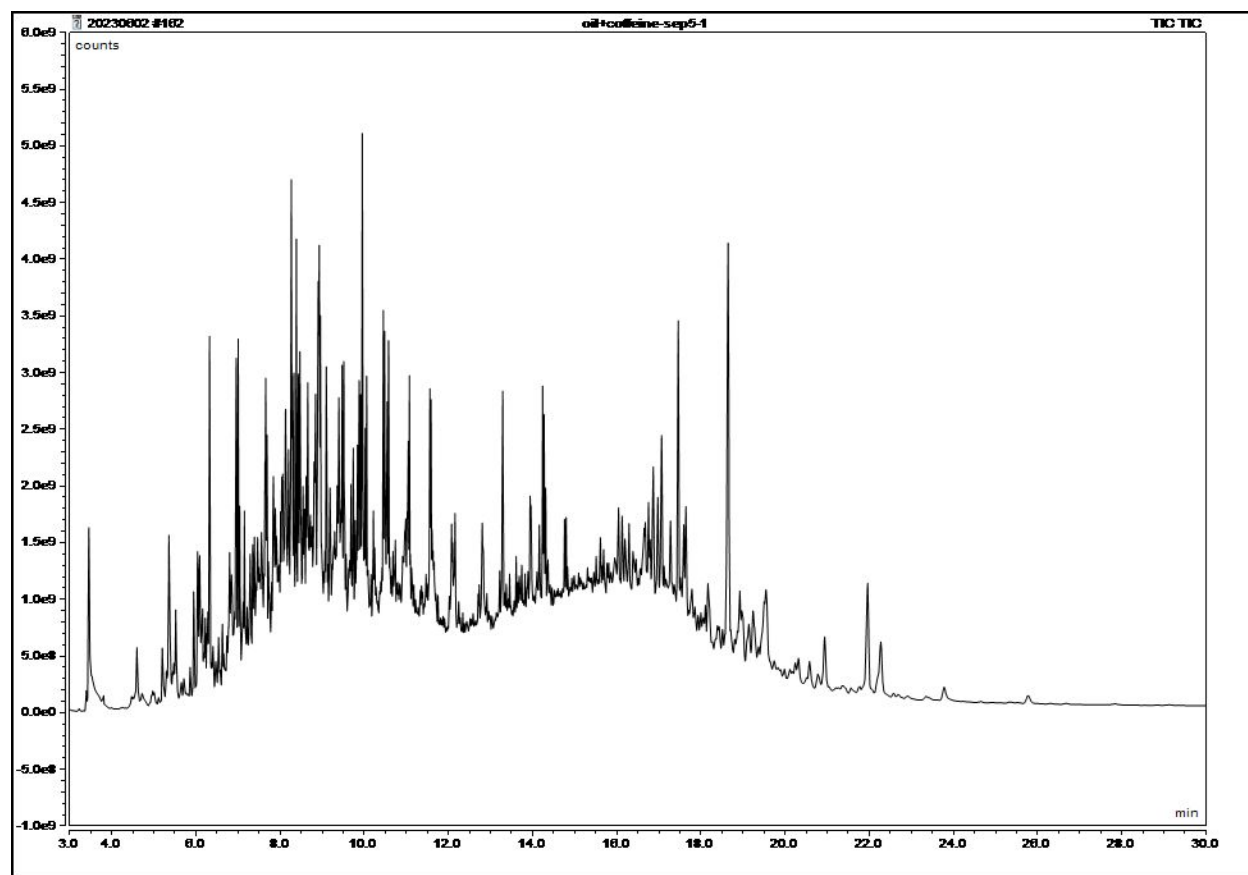


Figure 2. Analysis of birch bark extract in methanol with Thermo Scientific Trace 1310 Gas Chromatograph equipped with a Restek Rtx-5MS column and TSQ 9000 Triple Quadrupole MS detector; elution of *ortho*-cresol at 5.194 min, *para*-cresol at 5.355 min, and *meta*-cresol at 5.358 min.