

RESEARCH ARTICLE

Season-Resolved Polarity and Compound-Origin Analysis of LC-HRMS Lettuce Rhizosphere Profiles Under Reclaimed-Water Irrigation

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Abstract

This article examines whether reclaimed-water irrigation leaves a detectable anthropogenic chemical signal in the lettuce (*Lactuca sativa* L.) rhizosphere when seasonal biology, root-zone matrix complexity, and ionization selectivity are evaluated separately. Eighteen rhizosphere samples were collected from tap-water, reclaimed-water 1, and reclaimed-water 2 treatments during fall 2021 and summer 2022, with three biological replicates for each treatment–season combination. The analytical sequence combined four specific evidence classes: acquisition-domain feature retention, designed-factor variance partitioning, polarity-specific annotation confidence, and compound-origin prioritization. Across positive MS1, positive MS2, negative MS1, and negative MS2 acquisition domains, 12,513 initial regions of interest yielded 10,046 retained features after blank and quality-control filtering, corresponding to 80.28% overall retention. Negative ionization retained 90.36% of its features, compared with 72.19% in positive ionization, indicating a root-zone chemical field enriched in negative-mode-compatible constituents. Designed-factor partitioning attributed 42.16% of total variance to season, 15.34% to irrigation-water type, 15.92% to the season–water interaction, and 26.58% to residual variation. Within the structured experimental fraction, season accounted for 57.42%, while irrigation-water type and the interaction contributed 20.89% and 21.68%, respectively. The annotation set comprised 37 tentative compounds: 31 endogenous or rhizosphere-associated constituents and six anthropogenic indicators, namely bisphenol A, fenoprofen, methylhexahydrophthalic anhydride, naproxen, terephthalic acid, and imiquimod. Reclaimed-water irrigation therefore produced a chemically visible indicator layer, but the broader rhizosphere profile was governed mainly by seasonal endogenous chemistry. These findings establish a season-resolved interpretation of reclaimed-water effects in which trace anthropogenic indicators are retained as monitoring targets without being allowed to dominate the biological reading of the rhizosphere profile.

Keywords: LC-HRMS; non-target screening; lettuce rhizosphere; reclaimed water

1. Introduction

Agricultural water reuse is increasingly important in regions where freshwater availability is constrained by climate variability, population growth, and competition among urban, industrial, and agricultural users. Treated municipal wastewater can contribute nutrients and maintain irrigation supply, but it also carries a chemically heterogeneous residue burden. Pharmaceuticals, plastic-associated additives, industrial intermediates, surfactants, personal-care-product residues, and

transformation products may persist after conventional treatment and enter soil–plant systems at trace levels [1–3]. The analytical challenge is therefore not limited to measuring whether a known compound is present. A defensible evaluation must determine how trace anthropogenic chemistry appears within a much larger background of plant, microbial, and soil-derived metabolites.

Leafy vegetables are a demanding test case for this problem. Lettuce has a shallow root system, a short production cycle, and direct consumer relevance as a fresh edible crop. Prior reclaimed-water studies

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have shown that contaminant uptake and plant exposure depend on chemical structure, irrigation regime, soil composition, cultivation period, and plant physiology [4–6]. These variables make a single-residue interpretation incomplete. A low-abundance xenobiotic may be analytically meaningful even if it contributes little to total metabolic variance, whereas an abundant endogenous metabolite may dominate a statistical model without representing reclaimed-water input. The crop therefore requires an analytical strategy that preserves both broad metabolic organization and focused contaminant evidence.

The rhizosphere adds another layer of complexity because it is not a passive sampling compartment. It is a chemically active interface shaped by root exudation, microbial turnover, nutrient mobilization, soil organic matter, redox conditions, and stress-responsive secondary metabolism. Root exudates include sugars, amino acids, organic acids, phenolics, flavonoids, terpenoids, and other specialized metabolites that influence microbial recruitment, nutrient acquisition, allelopathy, and defense [7–10]. Seasonal shifts in temperature, light, water demand, and microbial activity can therefore change the rhizosphere profile even when the irrigation treatment remains constant. This biological variability can conceal or distort the interpretation of water-borne trace residues unless the analytical design separates season, water type, and their interaction.

High-resolution mass spectrometry offers the chemical breadth required for this task. LC-HRMS enables the detection of polar and semi-polar compounds across a wide mass range, while data-independent acquisition (DIA) can collect precursor and product-ion evidence for complex samples. Environmental non-target screening relies on exact-mass measurement, isotope pattern information, MS/MS evidence, retention behavior, suspect lists, and confidence-level classification [11–14]. Nevertheless, rich LC-HRMS profiles require strict control of feature detection, blank filtering, peak grouping, chromatographic alignment, and MS1–MS2 linkage, because these operations determine which molecular signals remain available for interpretation [15–17]. Analytical conclusions must therefore combine compound identity, acquisition-domain retention, and annotation support rather than rely on compound names alone.

Polarity is especially consequential in rhizosphere LC-HRMS. Positive electrospray ionization can emphasize protonated or adducted nitrogen-containing compounds, many pharmaceutical residues, and several plant-defense metabolites. Negative electrospray ionization often strengthens oxygenated organic acids, phenolics, acidic xenobiotics, and microbial or plant-derived compounds that form deprotonated ions. Simultaneous interpretation of positive and negative modes is therefore more than a coverage improvement; it is a chemical-origin safeguard. Recent ROIMCR work has shown the value of connecting MS1 and MS2 signals across positive and negative ionization modes for untargeted metabolomics [18, 19]. A lettuce rhizosphere study that ignores polarity may underrepresent exactly the compound classes most relevant to either endogenous seasonality or reclaimed-water exposure.

Chemometrics provides the second safeguard. Designed multivariate methods, including analysis of variance–simultaneous component analysis (ASCA), decompose complex metabolomic matrices according to experimental factors and their interactions [20–22]. Such partitioning is valuable because reclaimed-water irrigation competes with seasonal physiology, microbial activity, and root-zone matrix effects. In this study, the central distinction is among three explanations: a dominant seasonal profile, a direct irrigation-water profile, or a season-dependent water effect. Only after these alternatives are separated can compound-origin evidence be interpreted without overstating the role of either biological seasonality or trace xenobiotic detection.

Annotation confidence must also remain explicit. The Schymanski confidence system and subsequent quality-assurance discussions emphasize that exact mass, spectral similarity, library evidence, retention behavior, and reference-standard confirmation represent different lev-

els of support [12, 13, 17]. Tentative annotations can guide prioritization, but they cannot by themselves establish absolute concentration, exposure, uptake, or toxicological risk. This distinction matters for reclaimed-water studies because public-health and environmental interpretations can be overstated when tentative non-target features are treated as confirmed quantified residues.

This study asks a specific analytical question: after season, ionization behavior, and compound origin are separated, does reclaimed-water irrigation produce a detectable exogenous signature in lettuce rhizosphere LC-HRMS profiles, or is the profile governed mainly by endogenous seasonal rhizosphere chemistry? The analysis answers this question through four linked evidence layers: acquisition-domain retention, designed-factor dominance, polarity-specific annotation confidence, and origin-resolved follow-up priority. The aim is not to replace targeted residue quantification, but to determine how reclaimed-water indicators should be read when they coexist with a stronger seasonal metabolic signal.

2. Materials and methods

2.1. Research question and analytical material

The analytical material consisted of lettuce rhizosphere samples from three irrigation treatments: tap water, reclaimed water 1, and reclaimed water 2. Sampling was conducted in fall 2021 and summer 2022, with three biological replicates for each treatment–season combination. The design therefore contained 18 rhizosphere samples. The numerical evidence comprised acquisition-domain feature counts, designed-factor variance contributions, and 37 tentative compound annotations described by ionization mode, compound-origin class, mass error, spectral similarity, and evidence-ranking descriptors. LC-HRMS/DIA measurements of the rhizosphere extracts were interpreted at the levels required to distinguish seasonal endogenous chemistry from reclaimed-water-relevant trace indicators.

The research question was defined before the evidence layers were interpreted: does reclaimed-water irrigation create an exogenous chemical signal that remains analytically visible after seasonal variation and ionization-mode behavior are accounted for? This question is narrower than a food-safety or uptake question and broader than a simple detection question. It asks whether the rhizosphere LC-HRMS profile contains a treatment-relevant anthropogenic component while recognizing that the strongest multivariate structure may arise from endogenous plant–microbe chemistry.

The sampling context is shown in Figure 1. The image documents the lettuce root zone, the plot context, and the irrigation-water materials used to define the comparison among tap-water and reclaimed-water conditions.

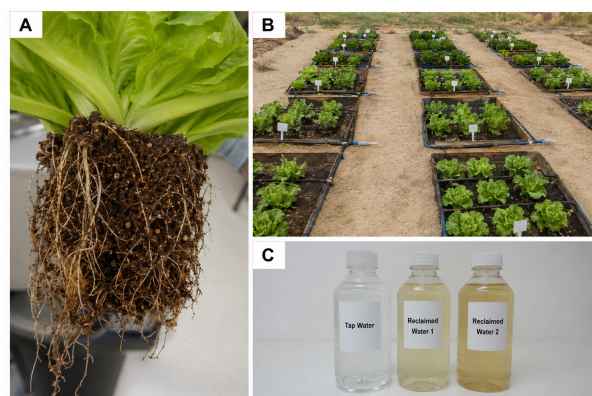


Figure 1: Lettuce rhizosphere sampling context.

This visual context is important because the manuscript interprets rhizosphere chemistry, not clean hydroponic solution or edible leaf tissue. The adhering root-zone matrix contains plant exudates, microbial products, soil organic matter, and irrigation-derived residues. The image therefore supports the central methodological choice: compound annotations must be read through an origin filter rather than treated as a uniform list of detected chemicals.

2.2. Season-gated polarity-origin analysis

The analytical sequence combined four evidence layers. The first layer was acquisition-domain feature retention, calculated for each LC-HRMS domain from the initial and retained region-of-interest counts after blank and quality-control filtering. This layer quantified how much measurable chemical information remained in positive MS1, positive MS2, negative MS1, and negative MS2 acquisition. The second layer was designed-factor partitioning, where season, irrigation-water type, season–water interaction, and residual variation were compared. The structured share of each designed factor was interpreted after excluding residual variation, because residual variance is not itself an experimental treatment.

The third layer was polarity-specific annotation confidence. Positive and negative ionization annotations were compared using mean and median mass error, mean spectral similarity, and the number of annotations with similarity score of 800 or higher. These descriptors were interpreted together because mass accuracy and spectral similarity do not measure identical forms of support. The fourth layer was compound-origin prioritization. Annotations were separated into endogenous/rhizosphere-associated candidates and exogenous indicators. Endogenous annotations were used to explain seasonal biological structure, while exogenous indicators were used to define a targeted confirmation panel for reclaimed-water monitoring.

The LC-HRMS platform context is shown in Figure 2. The panels summarize the sample sequence, instrumental bench, and acquisition environment underlying the feature audit and annotation ranking.

The instrumental images reinforce that the later numerical comparisons are not abstract compound-list summaries. Feature survivorship depends on how samples are prepared, sequenced, ionized, filtered, and linked between MS1 and MS2 acquisition. For this reason, the method treats acquisition-domain behavior as a result in its own right rather than as a hidden preprocessing detail.

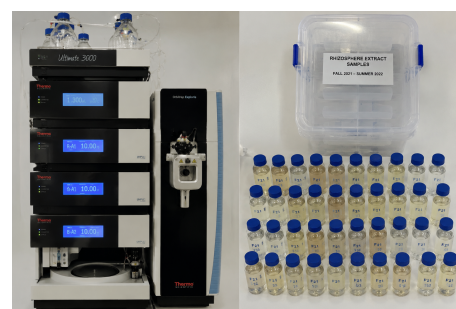
2.3. Interpretive boundaries

Chemical interpretation used four measured descriptors: feature counts, factor-effect contributions, polarity descriptors, and tentative annotation scores. Absolute concentration, plant-tissue uptake, replicate-level peak-area modelling, and reference-standard confirmation were outside the analytical scope. The article therefore presents evidence prioritization for LC-HRMS screening rather than quantitative exposure assessment. Tentative compound identities remain candidates for confirmation unless reference standards, retention-time agreement, and calibrated quantification are obtained [12, 17, 23].

3. Results and discussion

3.1. Acquisition-domain survivorship defines the usable chemical space

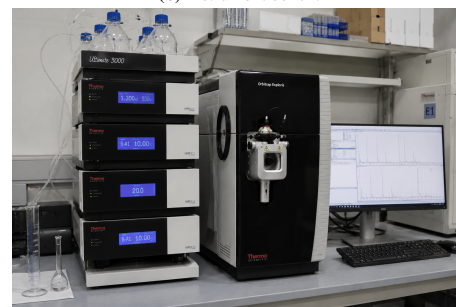
The feature audit produced 12,513 initial regions of interest across four acquisition domains and retained 10,046 after blank and quality-control filtering, giving an overall survivorship of 80.28% (Table 1). Positive-mode MS1 retained 3,010 of 4,276 initial features, positive-mode MS2 retained 2,001 of 2,665, negative-mode MS1 retained 3,114 of 3,377, and negative-mode MS2 retained 1,921 of 2,195.



(a) Sample sequence.



(b) Instrument bench.



(c) Workstation view.

Figure 2: LC-HRMS acquisition context.

Table 1: Feature survivorship by acquisition domain.

Acquisition domain	Threshold	Initial	Retained	Retained share (%)
MS1 positive ionization	1.0e6	4276	3010	70.39
MS2 positive ionization	2.5e5	2665	2001	75.08
MS1 negative ionization	1.0e6	3377	3114	92.21
MS2 negative ionization	2.5e5	2195	1921	87.52
Total	–	12513	10046	80.28

The feature audit shows that negative ionization was the more stable contributor to the retained rhizosphere feature space. Combined negative-mode survivorship was 90.36%, whereas combined positive-mode survivorship was 72.19%. The difference was not a minor technical fluctuation: positive acquisition lost 1,930 features during filtering, while negative acquisition lost 537. This pattern suggests that the lettuce rhizosphere matrix contained a large pool of negative-mode-compatible compounds, plausibly including organic acids, phenolic constituents, oxygenated lipids, and microbial or plant-derived acidic metabolites. Positive ionization remained necessary because several important defense-associated and xenobiotic candidates were represented there, but the retained feature landscape was clearly tilted toward negative-mode chemical coverage.

The near balance between acquisition levels also matters. MS1 domains together retained 80.02% of their initial features, and MS2 domains retained 80.70%. This similarity indicates that the filtering process did not selectively collapse the fragment-evidence layer. Consequently, the retained LC-HRMS profile can support a combined

feature-and-fragment interpretation, which is essential in non-target screening where precursor exact mass alone is insufficient for confident annotation.

3.2. Designed-factor partitioning identifies season as the main gate

The variance partitioning separated four components of structure: season, irrigation-water type, season–water interaction, and residual variation (Table 2). Season explained 42.16% of total variance and was statistically strong. Irrigation-water type explained 15.34%, while the interaction between season and water type explained 15.92%. Residual variation accounted for 26.58%.

Table 2: Designed-factor variance partitioning.

Factor	PCs	Variance (%)	p-value	Structured share (%)
Season	1	42.16	0.0003	57.42
Irrigation-water type	2	15.34	0.0288	20.89
Season–water interaction	2	15.92	0.0063	21.68
Residual variation	–	26.58	–	–

These values answer the first half of the research question. The main organizing force in the LC-HRMS profile was seasonal rhizosphere chemistry, not irrigation-water type alone. After residual variation was excluded, season represented 57.42% of structured experimental variance. The direct water-type contribution was smaller, at 20.89%, and the interaction term was similar, at 21.68%. This distribution indicates that reclaimed-water influence cannot be interpreted as a simple treatment shift detached from growing season. A meaningful portion of water-related chemistry was season-conditioned.

The factor visualization in Figure 3 translates the numerical partitioning into three complementary views: total variance, structured experimental share, and relative dominance among designed factors. The visual comparison prevents an over-simplified conclusion. The seasonal effect was approximately 2.75 times larger than the direct water-type effect, but the water-type and interaction terms were not negligible. Reclaimed water should therefore not be dismissed merely because season was stronger. The evidence supports a hierarchy: season defines the broad rhizosphere state, water type adds a smaller direct chemical contribution, and the interaction term shows that the water contribution partly depends on the cultivation period.

A practical sampling implication follows from this hierarchy. Single-season monitoring would be vulnerable to misclassification because it could either inflate a water effect during a responsive season or miss it during a season with stronger endogenous background chemistry. Reclaimed-water monitoring in lettuce rhizosphere studies should therefore retain at least two cultivation periods or explicitly justify why a single season is chemically representative.

3.3. Polarity-specific annotation confidence separates mass accuracy from spectral support

The annotation set contained 37 tentative compounds, with 21 detected in positive ionization and 16 in negative ionization (Table 3). Six annotations were classified as exogenous indicators, divided evenly between positive and negative ionization. The remaining 31 belonged to endogenous, plant-associated, microbial-associated, or rhizosphere-derived chemistry. Across the full set, mean mass error was 2.50 ppm and median mass error was 2.40 ppm.

The polarity comparison shows that confidence is multi-dimensional. Positive-mode annotations had lower mean and median mass error, but negative-mode annotations had higher average spectral similarity and three times as many compounds with similarity scores of at least 800. Thus, positive ionization gave stronger exact-mass compactness, whereas negative ionization gave stronger library/spectral

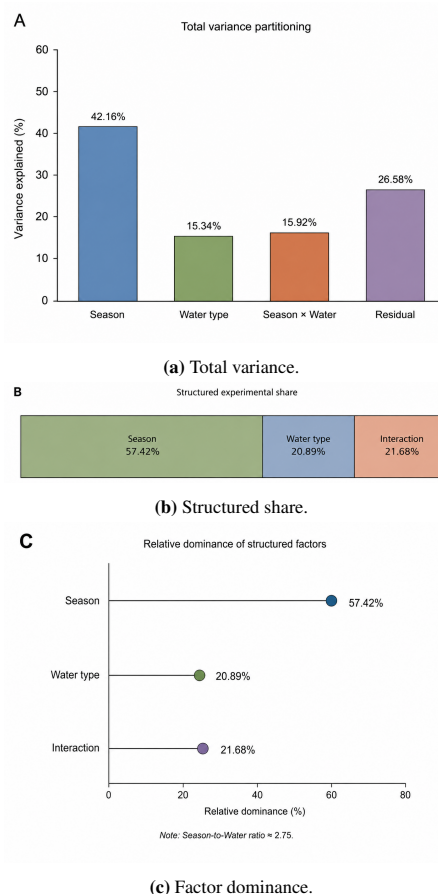


Figure 3: Designed-factor partitioning.

Table 3: Annotation summary by polarity.

Descriptor	Positive mode	Negative mode	Total
Annotated compounds	21	16	37
Exogenous indicators	3	3	6
Mean mass error (ppm)	1.97	3.20	2.50
Median mass error (ppm)	1.60	3.10	2.40
Mean similarity score	722.43	848.13	776.78
Similarity score $\geq$ 800	4	12	16

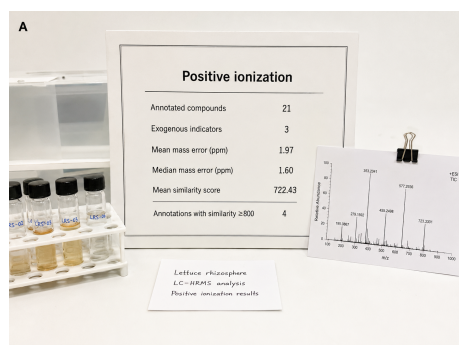
support for many compounds. A ranking system based on only one descriptor would obscure this trade-off. Polarity is therefore treated as an interpretive axis rather than as a simple instrument setting.

The ionization panels in Figure 4 show how positive and negative acquisition domains frame different parts of the annotation space.

The two-panel comparison supports a central analytical decision: both polarities should remain in future acquisition sequences. Negative mode gives a larger and spectrally strong retained chemical field, while positive mode contributes several high-priority endogenous and exogenous annotations. Omitting either polarity would change not only the number of detected features but also the biological and contaminant interpretation of the rhizosphere.

3.4. Origin-resolved ranking shows endogenous dominance with a distinct exogenous panel

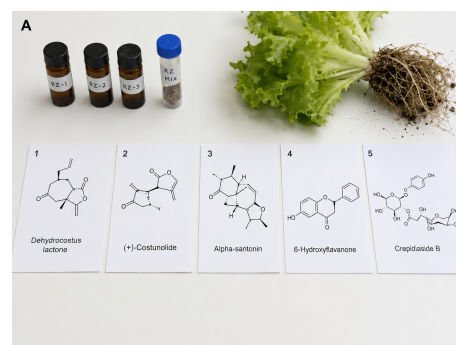
The highest-ranked compound evidence was led by endogenous and rhizosphere-associated candidates (Table 4). The top entry, 3-hydroxydodec-6-enedioic acid, was detected in negative mode with 0.8 ppm mass error and a spectral similarity score of 998. Dehydrocostus lactone, costunolide, alpha-santonin, 6-hydroxyflavanone, crepidiaside



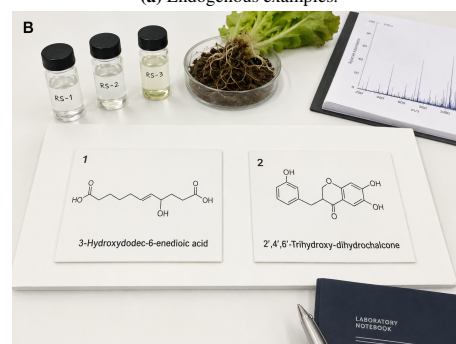
(a) Positive mode.



(b) Negative mode.

Figure 4: Polarity-resolved annotation evidence.

(a) Endogenous examples.



(b) Exogenous examples.

Figure 5: Origin-specific compound signatures.

B, and 2-prime,4-prime,6-prime-trihydroxydihydrochalcone also appeared among the strongest signatures. Bisphenol A and fenoprofen were the exogenous candidates that entered the highest-ranked group.

The ranking provides the most direct explanation for the seasonal dominance observed in the factor analysis. Most of the strongest candidates were endogenous metabolites or rhizosphere-associated compounds. Sesquiterpene lactones such as dehydrocostus lactone, costunolide, santanin-related chemistry, crepidiaside B, lactucin, and vulgarolide are compatible with defense-associated specialized metabolism in Asteraceae plants [24]. Flavonoid- and phenylpropanoid-related entries are consistent with antioxidant chemistry, redox balance, and plant-microbe signaling. These biochemical classes can reasonably vary with season because root growth, exudation, microbial activity, temperature, and water demand change between fall and summer.

Bisphenol A and fenoprofen require a different interpretation. Their presence among the top ten does not make the total rhizosphere profile exogenous, but it does identify an anthropogenic evidence layer that merits confirmation. Bisphenol A is a plastic-associated compound with environmental and toxicological relevance [25], whereas fenoprofen and naproxen belong to pharmaceutical residue classes often considered in wastewater-impacted systems. The rank table therefore produces a balanced result: the strongest broad chemical signal is endogenous, while selected exogenous indicators are strong enough to justify targeted monitoring.

The compound signature panels in Figure 5 illustrate the origin split between biological candidates and reclaimed-water-relevant indicators.

The separated origin display clarifies why compound class must be retained throughout interpretation. Plant-associated and microbial-associated metabolites answer a question about rhizosphere state, while exogenous indicators answer a question about irrigation-derived chemical input. Combining these groups without origin labels would blur two different kinds of evidence and could lead either to unnecessary risk alarm or to under-recognition of trace anthropogenic residues.

3.5. Exogenous indicators define a targeted confirmation route

The six exogenous indicators formed a compact monitoring panel (Table 5). Bisphenol A, fenoprofen, and methylhexahydrophthalic anhydride were observed in positive ionization. Naproxen, terephthalic acid, and imiquimod were observed in negative ionization. Positive-mode indicators had the highest evidence and irrigation-alert values, while the negative-mode indicators retained high spectral similarity but lower combined ranking because of larger mass errors.

The exogenous panel answers the second half of the research question. Reclaimed-water-related chemistry was analytically visible, but it appeared as a focused indicator layer rather than as the dominant structure of the profile. Bisphenol A, fenoprofen, and methylhexahydrophthalic anhydride should receive the first targeted confirmation priority because they combined comparatively high evidence values with the strongest irrigation-alert scores. Naproxen, terephthalic acid, and imiquimod should remain in the panel because their spectral similarity scores were high, making them plausible candidates even though their mass errors reduced the combined ranking.

For a confirmatory study, these six compounds should be measured with authentic standards, isotope-labelled internal standards where available, retention-time matching, matrix-matched calibration, and explicit blank correction. The same panel should be quantified in irrigation water, rhizosphere extracts, bulk soil, porewater, and lettuce tissues. Such a design would determine whether rhizosphere occurrence reflects current irrigation input, historical soil background, matrix carryover, or plant-associated retention.

The priority plates in Figure 6 separate endogenous and exogenous candidates as two different follow-up routes.

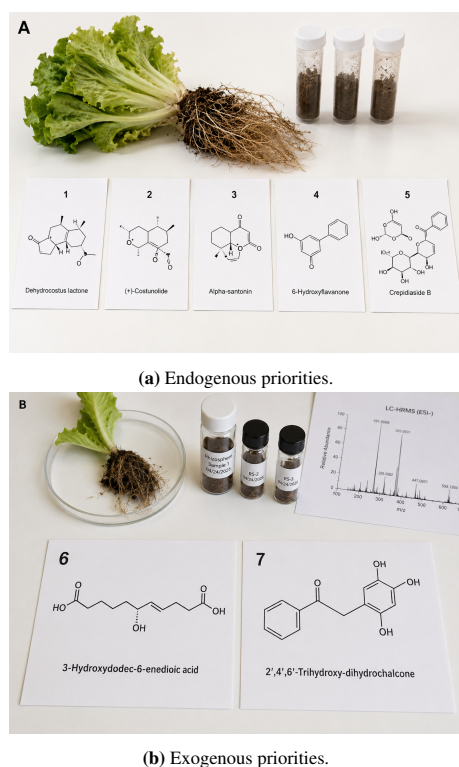
The separated priority layout is useful because analytical follow-up is not identical for the two origin groups. Endogenous compounds require biological interpretation, season-resolved comparison, and possibly root-exudate or microbial validation. Exogenous compounds require targeted quantification, recovery correction, and exposure-pathway evaluation. The same LC-HRMS run therefore yields two

Table 4: Top-ranked annotations by evidence.

Compound	Mode	Origin	Error (ppm)	Similarity	Evidence
3-hydroxydodec-6-enedioic acid	Negative	Endogenous	0.8	998	0.851
Dehydrocostus lactone	Positive	Endogenous	0.4	883	0.816
(+)-Costunolide	Positive	Endogenous	0.5	816	0.739
Alpha-santonin	Positive	Endogenous	0.2	751	0.722
6-Hydroxyflavanone	Positive	Endogenous	0.6	792	0.703
Crepdiaside B	Positive	Endogenous	0.3	708	0.667
2-prime,4-prime,6-prime-Trihydroxy-dihydrochalcone	Negative	Endogenous	0.6	698	0.620
Bisphenol A	Positive	Exogenous	0.5	670	0.607
1-(3-ethyl-2,4-dihydroxy-6-methoxyphenyl) butan-1-one	Negative	Endogenous	0.9	725	0.606
Fenoprofen	Positive	Exogenous	2.6	999	0.595

Table 5: Exogenous indicators for confirmation.

Compound	Mode	Error (ppm)	Similarity	Evidence	Irrigation alert
Bisphenol A	Positive	0.5	670	0.607	0.127
Fenoprofen	Positive	2.6	999	0.595	0.124
Methylhexahydro-phthalic anhydride	Positive	0.2	616	0.592	0.124
Naproxen	Negative	3.2	832	0.439	0.092
Terephthalic acid	Negative	4.0	965	0.434	0.091
Imiquimod	Negative	4.6	999	0.399	0.083

**Figure 6:** Compound-priority panels.

distinct outputs: a biological rhizosphere-state interpretation and a reclaimed-water contaminant-priority list.

3.6. Biochemical interpretation of the endogenous seasonal layer

The endogenous layer is coherent with known rhizosphere chemistry. Lettuce is a member of Asteraceae, a family known for sesquiterpene lactones and related specialized metabolites. In the annotation set, dehydrocostus lactone, costunolide, alpha-santonin, crepidiaside B, lactucin, and vulgarolide form a defense-associated axis. These molecules are not simply background noise; they provide a plausible biochemical reason for why season explained the largest variance share.

Between fall and summer, changes in root development, evapotranspiration, temperature, microbial activity, and oxidative pressure can all alter the balance of specialized metabolites in the root zone.

The flavonoid and phenylpropanoid-related candidates add a second explanation. 6-Hydroxyflavanone and 2-prime,4-prime,6-prime-trihydroxydihydrochalcone are compatible with antioxidant and signaling functions. The highest-ranked compound, 3-hydroxydodec-6-enedioic acid, points toward oxygenated lipid or organic-acid chemistry that is well suited to negative ionization. Fungal-associated or microbial-linked entries, including cyclocalopin F and isomarasmane in the broader annotation narrative, indicate that microbial chemistry likely contributed to the observed profile. The endogenous seasonal signal is therefore not a single-compound phenomenon; it is a composite of plant defense, root exudation, microbial metabolism, and matrix chemistry.

3.7. Treatment and matrix context prevents overinterpretation

The treatment and matrix panels in Figure 7 reconnect the chemical ranking to the actual sample types: irrigation water, lettuce root/rhizosphere material, and chromatographic evidence.

The sample-context panels illustrate why a compound detected in a rhizosphere extract should not automatically be assigned to a single origin. The same molecule may reflect irrigation-water input, soil background, microbial transformation, plant release, laboratory background, or a mixture of these pathways. Origin assignment must therefore remain probabilistic until confirmed by targeted multi-compartment measurements.

The replicate extract series in Figure 8 provides a final quality-context view of the analytical material.

The vial series should be read as documentation of matrix and treatment context, not as quantitative evidence. Visible sample differences can guide laboratory traceability and quality review, but the manuscript's conclusions come from feature survivorship, designed-factor partitioning, polarity-specific annotation confidence, and origin-resolved compound prioritization. This distinction prevents visual sample appearance from being overinterpreted as chemical separation.

3.8. Analytical significance, limitations, and validation needs

The season-gated polarity-origin analysis contributes to analytical chemistry by fixing the order of interpretation. Feature retention is evaluated before factor effects; factor effects are evaluated before origin claims; origin claims are separated before follow-up priorities are assigned. This order reduces two common errors in reclaimed-water LC-HRMS interpretation. The first error is treating seasonal metabolomic separation as proof that water treatment is irrelevant. The second error is treating a small set of tentative xenobiotic annotations

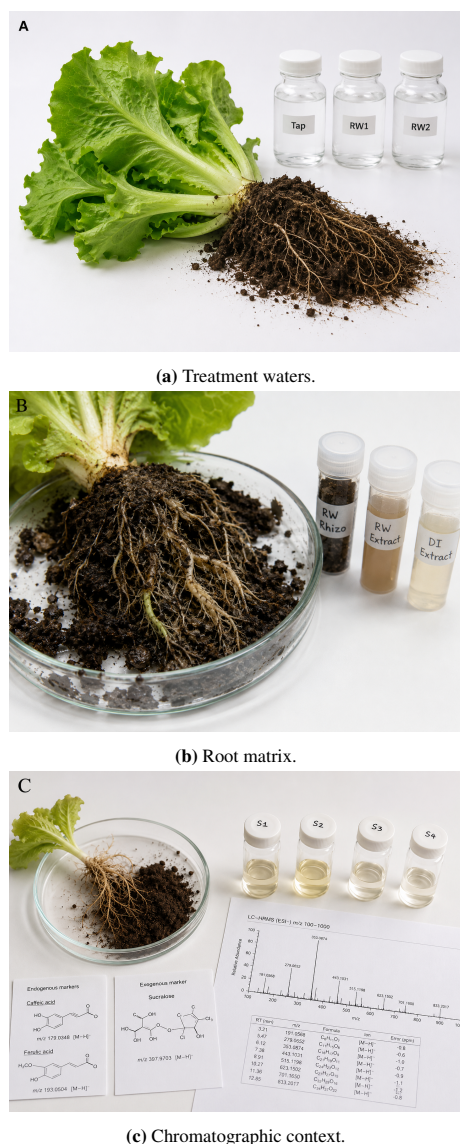


Figure 7: Treatment and matrix context.

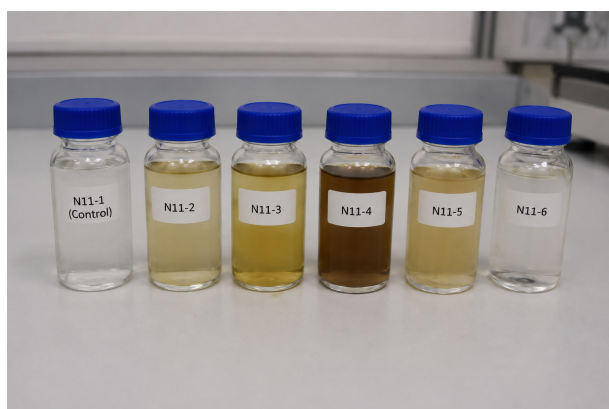


Figure 8: Replicate extract series.

as proof that the whole rhizosphere profile is contaminant-driven. The present evidence supports neither extreme.

The main limitation is that the LC-HRMS evidence does not provide absolute concentrations, replicate-level peak-area modelling, or reference-standard confirmations. The compound identities therefore remain tentative and should be treated as prioritized candidates. The

factor contributions describe organized variance in the rhizosphere profile, but they do not identify causal mechanisms by themselves. The annotation-origin categories also retain uncertainty, because some compounds can plausibly arise from more than one environmental or biological route. These boundaries should be addressed through targeted confirmation.

Confirmation should quantify the six exogenous indicators across irrigation water, rhizosphere extracts, bulk soil, porewater, root tissue, and edible leaf tissue. The endogenous panel should be evaluated with season-resolved metabolomics, microbial profiling, and root-exudate characterization. Independent LC-HRMS processing with alternative peak-picking, alignment, and MS1–MS2 linking settings would test whether negative-mode retention, annotation ranking, and exogenous-indicator priority remain stable across processing choices [16, 17, 19, 26]. Such measurements would provide a stronger exposure and mechanism assessment.

4. Conclusions

This study asked whether reclaimed-water irrigation produces a detectable exogenous signature in lettuce rhizosphere LC-HRMS profiles after season, ionization behavior, and compound origin are separated. The answer is yes, but with an important qualification: the exogenous signature is analytically visible as a focused indicator panel, whereas the dominant structure of the rhizosphere profile is seasonal and endogenous. The distinction is central to the paper's conclusion. Reclaimed-water irrigation should not be judged only by the total variance structure, because a low-share exogenous panel can still be monitoring-relevant. At the same time, the irrigation effect should not be overstated, because season explained the largest portion of the chemical profile.

The acquisition-domain evidence showed that 10,046 of 12,513 initial features survived blank and quality-control filtering, producing an 80.28% retained share. Negative ionization contributed the more stable filtered chemical field, with 90.36% survivorship compared with 72.19% in positive ionization. This finding demonstrates that dual-polarity acquisition is necessary for lettuce rhizosphere LC-HRMS because positive and negative modes carry different chemical evidence. The designed-factor partitioning then showed that season accounted for 42.16% of total variance and 57.42% of structured experimental variance. Irrigation-water type accounted for 15.34% of total variance, while the season–water interaction accounted for 15.92%. These values indicate that reclaimed-water effects were secondary and partly season-dependent rather than absent.

The compound-origin results explain the variance pattern. Endogenous and rhizosphere-associated compounds, including 3-hydroxydodec-6-enedioic acid, dehydrocostus lactone, costunolide, alpha-santonin, 6-hydroxyflavanone, crepidiaside B, and related specialized metabolites, provided the strongest biochemical evidence for a seasonally organized plant–microbe matrix. Six exogenous indicators—bisphenol A, fenoprofen, methylhexahydrophthalic anhydride, naproxen, terephthalic acid, and imiquimod—defined the reclaimed-water-relevant monitoring panel. Among these, bisphenol A, fenoprofen, and methylhexahydrophthalic anhydride are the highest-priority candidates for targeted confirmation. The conclusion is therefore specific: lettuce rhizosphere chemistry under reclaimed-water irrigation is best interpreted as a season-gated biological profile containing a narrower but detectable anthropogenic indicator layer. A treatment-label-only interpretation would miss this structure. The analytical route should retain dual-polarity LC-HRMS acquisition, partition season and water effects before compound claims are made, separate endogenous and exogenous annotations, and confirm the six exogenous indicators with reference standards and multi-compartment sampling. This resolves the research question and defines the next experiment needed to move from LC-HRMS screening to quantitative exposure assessment.

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