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Authors	Chen, Kangni;Madadlou, Ashkan;De Pascale, Sabrina;Scaloni, Andrea;Fogliano, Vincenzo;Troise, Antonio Dario
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Spatial distribution of glucose and amino acids within all-aqueous emulsions directs the Maillard reaction and oxidation pathways

Corresponding Author: Dr Antonio Dario Troise

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This is a very good manuscript covering a quite current aspect of analytical chemistry. However, the authors should elaborate more on how their approach is linked to food production.

Then, generally, are the partition coefficients of the reactants in both aqueous phases known?

I only have some further minor comments

l.260 – please use subscripts.

l.291 – any idea for the formation of 3-amino-2,3-dideoxy-scylo-inosose? Has this compound ever been reported in glucosamine degradation?

l.298 – delete 'in'

l.444 – delete 'finally' once

l.554 – 'molecular'

fig. 3 – the structure at the lower left is '3-hydroperoxyproline indole carboxylic acid'

fig. 4 – what is meant by 'carboxylic acid'?

Reviewer #2

(Remarks to the Author)

The manuscript explores the role of spatial distribution in all-aqueous emulsions, specifically how the organization of glucose and amino acids within such systems directs both the Maillard reaction (MR) and oxidation pathways. The authors use untargeted metabolomics and molecular networking to analyze over 500 compounds, revealing how the location of reactants—whether co-encapsulated or segregated in different phases—affects the chemical diversity of reaction products. Their findings provide a new framework for controlling glycation reactions in food-relevant conditions. Based on the innovative approach and comprehensive analysis presented, this manuscript provides valuable insights into the control of complex chemical reactions in biphasic systems. Given the quality of the research and its potential impact on the field, I recommend publication in Communications Chemistry, subject to addressing the following comments:

1. The manuscript mentions the preparation of all-aqueous droplet reactors using Na₂SO₄ and PEG solutions. However, the concentrations of these solutions and their impact on the Maillard reaction (MR) products are not clear. This manuscript should provide exact concentrations of Na₂SO₄ and PEG used in the final emulsion system and explain their roles in modulating the MR pathway (Section 2.2, "Preparation of all-aqueous droplet reactors").

2. The principal component analysis (PCA) in Figure 1A shows sample distribution over time. While the variance explained is mentioned, the interpretation of the first and second principal components is insufficient. This manuscript should provide more details on how the variance correlates with the observed reaction pathways and the significance of the clustering in

different quadrants (Figure 1A).

3. The manuscript mentions the annotation of over 500 compounds using metabolomics. However, the methods of quantifying these compounds are not fully explained. This manuscript should clarify how the compound quantities were measured in each phase and what standards were used for comparison (Section 2.4, "Liquid chromatography high resolution mass spectrometry").

4. In the Discussion, the manuscript highlights the formation of oxidation products such as β -carbolines and N-formylkynurenine in the PEG phase. However, the formation pathways for these products are not adequately discussed. This manuscript should elaborate on the chemical mechanisms leading to their formation, and how the phase-specific microenvironment influences this process (Page 12, "Results and Discussion").

5. In Section 2.3, control experiments with PEG and Na₂SO₄ are used to validate the findings. However, the manuscript does not clearly explain how these controls support the interpretation of the data. This manuscript should expand on the role of the control samples and how they confirm the observed differences in reaction pathways (Section 2.3, "Running the MR in droplet reactors and single-phase solutions").

6. Figure 3 presents the reaction pathways of tryptophan and glucose, but the molecular network diagram does not clearly differentiate between the different reaction types (e.g., condensation vs oxidation). This manuscript should revise the diagram to explicitly indicate which compounds are involved in each reaction pathway and provide a clearer distinction between oxidation and condensation products (Figure 3, "Molecular network of reaction products").

7. In Figure 5, a volcano plot is presented comparing compounds in the Na₂SO₄ and PEG phases at 5 hours. However, the specific compounds that are over-represented in each phase are not fully listed. This manuscript should include a table or supplementary information listing the compounds that were identified as being significantly enriched in each phase and their corresponding concentrations (Figure 5, "Composite volcano plot").

8. There is a discrepancy in the way reaction products are named between different sections. For example, "N-formylkynurenine" is mentioned in the Results but referred to as a different compound in the Discussion section. This manuscript should ensure consistent terminology throughout to avoid confusion (Page 10, "Results and Discussion").

9. While the manuscript discusses how reactant localization influences the Maillard reaction, it could provide a deeper analysis of how these localized reactions specifically affect the formation of Amadori products and other intermediates. This manuscript should include additional data or references to support the claim that the PEG phase is more conducive to Amadori rearrangement (Page 8, "Results and Discussion", and Figure 3).

Reviewer #3

(Remarks to the Author)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

l.69, l.128 – after the bracket, a space is missing.

l.72 – what do you mean by “thereby subsequent pathways”?

l.139 - there is a formatting issue “Na SO”

l.204 – please correct “;.”

l.218 – what do you mean by “that consider involve broad dynamic ranges”?

l.227 – please correct “studying , and study”

l.232 and following – why bold letters?

l.451 – do you mean “dioxetane”?

Reviewer #2

(Remarks to the Author)

The authors have addressed all my concerns satisfactorily. I recommend the manuscript for publication as it is.

Reviewer #3

(Remarks to the Author)

All of my concerns have been adequately addressed in the revised manuscript, and I recommend the manuscript for publication.

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Dear Editor and Reviewers,
we are very much thankful for all the amendments and suggestions. We understand that the Reviewers do a voluntary job, they indeed help us to improve our paper. We provide here a point-to-point rebuttal to the Reviewers' constructive comments. All the changes are reported here in blue and can be tracked in the revised manuscript following the line numbering.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This is a very good manuscript covering a quite current aspect of analytical chemistry. 1. However, the authors should elaborate more on how their approach is linked to food production.

The introduction section has been expanded outlining the implications of chemical reactions in all aqueous two-phase systems for food production (Lines 49-58).

2. Generally, are the partition coefficients of the reactants in both aqueous phases known?

Yes, the partitioning behavior of the reactants has been extensively discussed in our previous study¹. Specifically, the partition coefficients for glucose and asparagine are 0.206 and 0.080, respectively, while the partition coefficient for tryptophan is 6.17. This information has been incorporated into the final paragraph of the introduction section (Lines 85-86).

3. l.260 – please use subscripts.

The subscripts have been revised and corrected throughout in the manuscript

4. l.291 – any idea for the formation of 3-amino-2,3-dideoxy-scylo-inosose? Has this compound ever been reported in glucosamine degradation?

We apologize for the incorrect nomenclature as in the case of 3-amino-2,3-dideoxy-scylo-inosose and in the case of the compound with chemical formula C₁₂H₂₃NO₁₀. We take now the chance to update the manuscript, tables, figures and discussion.

Even if 3-amino-2,3-dideoxy-scylo-inosose is a known chemical entity often observed as an intermediate in aminoglycoside biosynthesis², its formation is not reported in glucosamine degradation. Indeed, the compound with formula C₆H₁₁NO₄, annotated and putatively identified in this study as 6-amino-4,5-dihydroxyhexan-2,3-dione can be viewed as an analog of 1-deoxy-2,3-hexodiulose, arising from glycosylamine. While 1-deoxy-2,3-hexodiulose arises from the dehydration of the Amadori product or glucose at alkaline pH, in our conditions further reactions as dehydration could lead to the

formation of 6-amino-4,5-dihydroxyhexan-2,3-dione here observed.

In a similar way, the compound with molecular formula C₁₂H₂₃NO₁₀ is putatively formed from 1-glycosylamine. Indeed, 1-glycosylamine (or one of its isomers) is unstable in the pH range 1.5-9 and in the case of local accumulation and high concentration of reactants, it can react with reducing sugar to form compounds as threalosamine or lactosamine according to the specific kind of precursor.³ This reaction has been already observed in the case of long chain amines and reducing sugars.⁴

The manuscript and the results have been changed accordingly (Figures 5 and 8, supplementary tables, lines 346-351).

5. l.298 – delete ‘in’

The word “in” has been deleted (Line 353).

6. l.444 – delete ‘finally’ once

The word “finally” has been deleted (Line 505).

7. l.554 – ‘molecular’

The term has been corrected to “molecular” (Line 625).

8. fig. 3 – the structure at the lower left is '3-hydroperoxypyrrole indole carboxylic acid'

The Reviewer is correct: we missed the carboxylic acid moiety in the name of the structure. Now figure 3 includes the correct name 3-hydroperoxypyrrole indole carboxylic acid and the corresponding panels have been reorganized.

9. fig. 4 – what is meant by ‘carbossilic acid’?

Figure 4 has been corrected.

Reviewer #2 (Remarks to the Author):

The manuscript explores the role of spatial distribution in all-aqueous emulsions, specifically how the organization of glucose and amino acids within such systems directs both the Maillard reaction (MR) and oxidation pathways. The authors use untargeted metabolomics and molecular networking to analyze over 500 compounds, revealing how the location of reactants—whether co-encapsulated or segregated in different phases—affects the chemical diversity of reaction products. Their findings provide a new framework for controlling glycation reactions in food-relevant conditions. Based on the innovative approach and comprehensive analysis presented, this manuscript provides valuable insights into the control of complex chemical reactions in biphasic systems. Given the quality of the research and its potential impact

on the field, I recommend publication in Communications Chemistry, subject to addressing the following comments:

1. The manuscript mentions the preparation of all-aqueous droplet reactors using Na₂SO₄ and PEG solutions. However, the concentrations of these solutions and their impact on the Maillard reaction (MR) products are not clear. This manuscript should provide exact concentrations of Na₂SO₄ and PEG used in the final emulsion system and explain their roles in modulating the MR pathway (Section 2.2, "Preparation of all-aqueous droplet reactors").

The exact concentrations of Na₂SO₄ and PEG, as well as their respective roles in influencing the Maillard reaction pathways, have been included in section 2.2 of the amended manuscript (Lines 115-127).

2. The principal component analysis (PCA) in Figure 1A shows sample distribution over time. While the variance explained is mentioned, the interpretation of the first and second principal components is insufficient. This manuscript should provide more details on how the variance correlates with the observed reaction pathways and the significance of the clustering in different quadrants (Figure 1A).

This part has been rephrased emphasizing the implications of model system composition and of reaction time. New comments based on the 2D distribution over PC1 and PC2 were introduced (Lines 233-253).

3. The manuscript mentions the annotation of over 500 compounds using metabolomics. However, the methods of quantifying these compounds are not fully explained. This manuscript should clarify how the compound quantities were measured in each phase and what standards were used for comparison (Section 2.4, "Liquid chromatography high resolution mass spectrometry").

We agree with this comment and material and methods section have been updated accordingly. The results were presented in a semiquantitative data, considering that some of the end-products monitored are not available in the market or require specific synthetic protocols for an accurate quantification. We specified that the number of scans below each peak (> 8), the filtering procedure and the consequent correction of the signal based on quality control and normalization can improve the robustness of the metabolomic workflow and the response of chemically different compounds, even when semi-quantitative results are presented as in this manuscript. Regarding the annotation and identification procedures, the available standards were reported in bold in the supplementary tables (sections 2.4 and 2.5).

4. In the Discussion, the manuscript highlights the formation of oxidation products such as β -carboline and N-formylkynurenine in the PEG phase. However, the formation

pathways for these products are not adequately discussed. This manuscript should elaborate on the chemical mechanisms leading to their formation, and how the phase-specific microenvironment influences this process (Page 12, "Results and Discussion").

The discussion has been expanded according to this specific comment highlighting how temperature and pH can favor the formation of *N*-formylanthranilic acid from *N*-formylkynurenine. We also included comments on the reactivity of dioxoetane intermediate and the consequent formation of dioxindolylalanine and hydroxy and hydroperoxy-derivatives starting with the reactivity of indole rings. Based on the improved annotation of structural isomers, we introduced comments also on other two products of tryptophan oxidation as 3-hydroperoxytryptophan and 3-hydroperoxypyrroloindole carboxylic acid. Specifically for these two products we spotted the possibility that they are formed at the interface and then migrate into the PEG phase in the presence of emulsions, while Na₂SO₄ phase alone notably favored their formation. This specific behavior further reinforced the concept of aqueous two-phase systems as a complex, yet dynamic environment. In all the cases here mentioned, we would remark that specific kinetic or multiresponse experiments able to model distribution in different phases are required for establishing mechanistic insights of the formylation, oxidation, peroxidation reactions, as well as peptide bond formation. (Lines 301-314)

4. In Section 2.3, control experiments with PEG and Na₂SO₄ are used to validate the findings. However, the manuscript does not clearly explain how these controls support the interpretation of the data. This manuscript should expand on the role of the control samples and how they confirm the observed differences in reaction pathways (Section 2.3, "Running the MR in droplet reactors and single-phase solutions").

The explanation has been added to section 2.3.

5. Figure 3 presents the reaction pathways of tryptophan and glucose, but the molecular network diagram does not clearly differentiate between the different reaction types (e.g., condensation vs oxidation). This manuscript should revise the diagram to explicitly indicate which compounds are involved in each reaction pathway and provide a clearer distinction between oxidation and condensation products (Figure 3, "Molecular network of reaction products").

We accepted this comment and we decided to add specific comments on the targets of the molecular network in the Material and methods section (Lines 179-180). The molecular network provides information on the spectral similarities between different compounds and how specific compounds are connected according to chemical transformations included within the metabolomic workflow. Here, the outcome is to enlarge the number of annotated and putatively identified compounds as bases to infer on the reaction mechanisms (Lines 260-276 and Lines 367-381).

6. In Figure 5, a volcano plot is presented comparing compounds in the Na₂SO₄ and PEG phases at 5 hours. However, the specific compounds that are over-represented in each phase are not fully listed. This manuscript should include a table or supplementary information listing the compounds that were identified as being significantly enriched in each phase and their corresponding concentrations (Figure 5, "Composite volcano plot").

We included two new Supplementary Tables ST3 and ST4 reporting the compounds over-represented and down-represented shown in the volcano plots for the two model systems. We would here specify that not all the discriminating molecules provided sufficient responses in terms of annotation and identification.

8. There is a discrepancy in the way reaction products are named between different sections. For example, "N-formylkynurenine" is mentioned in the Results but referred to as a different compound in the Discussion section. This manuscript should ensure consistent terminology throughout to avoid confusion (Page 10, "Results and Discussion").

We accept the comments and we reorganized this specific section aiming at improving the clarity of the logical flow used for annotation, identification and reaction pathway construction. The formation of *N*-formylkynurenine is a consequence of oxidizing conditions along with the concomitant formation of isomers as dioxindolylalanine and hydroxypyrrroloindole.⁵ According to our conditions, alkaline micro-environment and temperature higher than 70°C we hypothesized that the main products with molecular formula C₁₁H₁₂N₂O₄ corresponds to NFK. We have adjusted the discussion in section 3. New comments on dioxindolylalanine and hydroxy and hydroperoxy-derivatives were as well introduced.

9. While the manuscript discusses how reactant localization influences the Maillard reaction, it could provide a deeper analysis of how these localized reactions specifically affect the formation of Amadori products and other intermediates. This manuscript should include additional data or references to support the claim that the PEG phase is more conducive to Amadori rearrangement (Page 8, "Results and Discussion", and Figure 3).

The higher accumulation of Amadori compounds in the PEG phase is consistent with our previous findings, where the macromolecular crowding and reduced water activity of PEG-rich environments were shown to accelerate the Schiff base-to-Amadori rearrangement and stabilize the corresponding intermediates (*Chen et al., Food Chemistry, 2025*)¹. In that study, both Fru-Asn and Fru-Trp were detected at significantly higher levels in PEG-containing systems compared to Na₂SO₄ phases,

supporting the notion that the PEG phase provides a more favorable microenvironment for the Amadori rearrangement.

Regarding intermediates, we are aware that the formation of dicarbonyls, hydroxycarbonyls, keto acids and other degradation products can provide valuable insights into the definition of the hypothesized reaction pathways. New set of experiments will be directed to answer these questions and demonstrate the active role played by carbohydrate degradation products when in the presence of aqueous two-phase systems.

Reviewer #3 (Remarks to the Author):

Comments: The manuscript presents an insightful and well-structured study on how the spatial distribution of amino acids and sugars in all-aqueous (PEG/Na₂SO₄) emulsions modulates the Maillard and oxidation reactions. The authors successfully integrate untargeted metabolomics, molecular networking, and multivariate data analysis to reveal mechanistic insights into the compartmentalized reactivity of glucose - amino acid systems. The topic is timely and relevant for the fields of food chemistry and metabolomics. Besides, the study is methodologically sound and employs advanced analytical tools (HILIC–HRMS coupled with molecular networking) to characterize reaction products. Overall, the paper is of high scientific quality and is recommended for publication, but a few issues should be addressed before acceptance.

1. This article shows similar approach with the author's previous work (ref 12). The authors mentioned that they envisioned this work in order to address two critical questions which remained unanswered in their previous work, one of which is 'how end-product formation and migration through different phases depend on amino acid partitioning behavior'. However, ref 12 already demonstrates that several end-products such as β -carboline derivatives and Strecker aldehyde exhibited greater affinity toward the PEG phase. Considering these previous observations and the fact that both studies utilize the same model reactants (glucose, asparagine, and tryptophan), the authors should clearly articulate how the current work advances beyond their earlier study and what new mechanistic insights or methodological innovations it provides.

We accept this comment and apologize for the lack of clarity on this specific point. This paper and our previous manuscript (*Chen et al., Food Chemistry, 2025*)¹ differ in the analytical context used to decipher the chemical nature of the end-products formed and (possibly) to evaluate their distribution in different phases in the *milieu* of aqueous two-phase systems.

- In Chen et al, the key focus was the study of the early stage of the Maillard reaction with Amadori rearrangement products partitioning into different phases. Next, β -carbolines and Strecker aldehyde (decarboxylation reactions) were measured following chemical structures already reported in literature through a targeted analysis. Finally, we spotted an intense brown color development indicative of a broad spectrum of *de novo* formed compounds. In our former

manuscript for brown pigments, we only used a colorimetric approach.

- Our aim in this second manuscript was to disentangle the kind of molecules formed upon thermal treatment and that were part of the brown color development.
- The untargeted metabolomics workflow was based on the annotation of degradation compounds potentially involved in polymerization reactions (i.e. indole rings, pyrrole rings and aminobenzoyl-derivatives).
- We did not limit our study to indole or pyrrole rings, but we considered aqueous two-phase system a reactor potentially linked also to prebiotic conditions. Indeed, we envisaged that multiple condensation reactions can be involved in the formation of amide bond typical of dipeptides. This objective has been added to the last paragraph of introduction (Line 99-105)

2. Furthermore, it would strengthen the manuscript if the authors elaborated on the rationale behind selecting glucose, asparagine, and tryptophan as model precursors for this study. A brief explanation of how these specific compounds represent distinct chemical behaviors would help contextualize their relevance to the investigated systems.

This has been added to the last paragraph of introduction (Lines 80-88)

3. In addition, while the manuscript provides mechanistic interpretations of oxidation and condensation pathways, several of the proposed reactions such as the formation of β -carboline, N-formylkynurenine, and peptide bonds remain speculative due to the lack of direct structural confirmation. Clarifying which compound identifications were experimentally confirmed versus putatively assigned would considerably improve the scientific rigor of the work

We are aware that NMR or the matching with pure reference standards is the prerequisite for the unequivocal identification of target analytes. We would here remark the untargeted nature of our approach and that in some cases pure reference standard are not accessible. Anyway, for the three compounds mentioned we provided fragmentation spectra with the putative chemical structures arising from a coherent retention time with HILIC, formula matching isotope distribution spectra overlapping with publicly available database and a screening of mass signals through Fragment Ion Search (FiSH) score. The results are reported in the Supplementary file section.

References added to this point-to-point rebuttal

- 1 Chen, K. *et al.* Compartmentalization vs. segregation of reactants: Accomplishment of the Maillard reaction at the water-water interface. *Food Chemistry* **465**, 142089 (2025).
- 2 Kudo, F. & Eguchi, T. Biosynthetic genes for aminoglycoside antibiotics. *The Journal of Antibiotics* **62**, 471-481 (2009).
- 3 Fischer, N. H., & Pedersen, C. M. (2025). Chemical Glycosylations in Water

and Aqueous Media. *Chemical Reviews*.

- 4 Erickson, J. G. Reactions of Long Chain Amines. V. Reactions with Sugars. *J. Am. Chem. Soc.* 1955, 77 (10), 2839–2843.
- 5 Ronsein, G. E., Oliveira, M. C., Miyamoto, S., Medeiros, M. H., & Di Mascio, P. (2008). Tryptophan oxidation by singlet molecular oxygen [$O_2(^1\Delta_g)$]: mechanistic studies using ^{18}O -labeled hydroperoxides, mass spectrometry, and light emission measurements. *Chemical research in toxicology*, 21(6), 1271-1283.

Comments:

The manuscript presents an insightful and well-structured study on how the spatial distribution of amino acids and sugars in all-aqueous (PEG/Na₂SO₄) emulsions modulates the Maillard and oxidation reactions. The authors successfully integrate untargeted metabolomics, molecular networking, and multivariate data analysis to reveal mechanistic insights into the compartmentalized reactivity of glucose - amino acid systems. The topic is timely and relevant for the fields of food chemistry and metabolomics. Besides, the study is methodologically sound and employs advanced analytical tools (HILIC–HRMS coupled with molecular networking) to characterize reaction products. Overall, the paper is of high scientific quality and is recommended for publication, but a few issues should be addressed before acceptance.

This article shows similar approach with the author's previous work (ref 12). The authors mentioned that they envisioned this work in order to address two critical questions which remained unanswered in their previous work, one of which is '***how end-product formation and migration through different phases depend on amino acid partitioning behavior***'. However, ref 12 already demonstrates that several end-products such as β-carboline derivatives and Strecker aldehyde exhibited greater affinity toward the PEG phase. Considering these previous observations and the fact that both studies utilize the same model reactants (glucose, asparagine, and tryptophan), the authors should clearly articulate how the current work advances beyond their earlier study and what new mechanistic insights or methodological innovations it provides.

Furthermore, it would strengthen the manuscript if the authors elaborated on the rationale behind selecting ***glucose***, ***asparagine***, and ***tryptophan*** as model precursors for this study. A brief explanation of how these specific compounds represent distinct chemical behaviors would help contextualize their relevance to the investigated systems.

In addition, while the manuscript provides mechanistic interpretations of oxidation and condensation pathways, several of the proposed reactions such as the formation of β-carbolines, N-formylkynurenine, and peptide bonds remain speculative due to the lack of direct structural confirmation. Clarifying which compound identifications were experimentally confirmed versus putatively assigned would considerably improve the scientific rigor of the work.