LAS: A Lipid Annotation Service Capable of Explaining the Annotations It Generates☆

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A B S T R A C T

The Lipid Annotation Service (LAS) is a representational state transfer (REST) application programming interface (API) service designed to aid researchers performing lipid annotation. It assigns certainty levels (very unlikely, unlikely, likely, and very likely) to the putative annotations received as input and explains the rationale of such assignments. Its rules, obtained from the Centre for Metabolomics and Bioanalysis (CEMBIO) and from a literature review, enable LAS to extract evidence to support or refute the annotations automatically by checking the inter-rule relationships.

LAS is the first metabolite annotation tool capable of explaining in natural language (English) the evidence that supports or refutes the annotations. This facilitates the understanding of the results by the user and, thus, increases the user’s confidence in the results. Concerning its performance, in an evaluation of blood plasma samples whose compounds had previously been identified using well-established standards, LAS yielded an F-measure higher than 80%.

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1. Introduction

Untargeted metabolomics aims to identify as many of the metabolites present in a sample as possible. The most popular approach is the detection of the changes between two or more experimental groups over the complete metabolic profile without any prior hypothesis. The metabolites associated with these changes are potential biomarkers [5]. Untargeted metabolomics is ideal for novel discoveries because the coverage of the metabolome is only limited by the sample preparation and the analytical techniques employed. Nevertheless, the huge amount of data generated makes metabolite identification a cumbersome and time-consuming task.

In untargeted metabolomics, the main bottleneck is still compound annotation and identification [22]. Liquid chromatography coupled to mass spectrometry (LC-MS) is a powerful analytical approach [6]. However, the annotation of metabolites is limited because of the variety of methodologies. Ideally, the putative annotations are confirmed using hyphenated setups (LC-MS/MS) by comparing the experimental spectra with those of commercially available standards (identification confidence level 1 (if available)) or with the spectra available in metabolomic databases (identification confidence level 2) [4]. However, the standards are limited, and, in some cases, the amount of sample or the time available makes MS/MS analysis unfeasible [12]. In these cases, researchers must extract as much information from other analyses, such as MS analysis, retention times (RTs), in-source fragmentation patterns, and isotopic patterns, as possible.

Several software tools have been developed to annotate features using MS² information [9,21,24,25], but the number of MS¹ metabolite annotation tools remains limited. At the same time, the number of experimentally measured and in silico generated compounds in the databases has quickly increased. This increase

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**Abbreviations:** API, Application Programming Interface; CEMBIO, Centre for Metabolomics and Bioanalysis; CER, Ceramide; CMM, Ceu Mass Mediator; CMM-ES, CMM Expert System; CS, Composite Spectrum; DG, Diradylglycerols; EM, Experimental Mass; FA, Fatty Acid; HMDB, Human Metabolome Database; InChI, IUPAC International Chemical Identifier; LAS, Lipid Identification System; LPC, Lysoglycerophosphocholine; LPE, Lysoglycerophosphoethanolamine; LPG, Lysoglycerophosphoglycerol; LPS, Lysoglycerophosphoserine; MB, MassBank; MZ, MZedDB; MG, Monoradylglycerols; PA, Glycerophosphate; PC, Glycerophosphocholine; PE, Glycerylphosphoethanolamine; PI, Glycerophosphoinositol; PG, Glycerophosphoglycerol; PS, Glycerophosphoserine; REST, REpresentational State Transfer; RT, Retention Time; SM, Phosphosphingolipid; ST, Sterol (Cholesterol ester); TG, Triradylglycerols.

☆ Fully documented templates are available in the elsarticle package on CTAN.

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makes MS\(^1\) metabolite annotation tools even more important because the number of candidates for a unique m/z has increased correspondingly. Some of the most popular databases devoted to metabolomics that are being actively maintained include the Cyc collection [8], KEGG [17], HMDB [31], LipidMaps [29], MassBank [15], the Metabolomics Workbench [28], Metlin [26], MINE [16], and mzCloud [21].

Parallel to this growth in metabolomic databases and software tools for metabolite annotation and identification, a number of tools for lipid identification have been launched. There are currently several software tools that enable the accurate identification of lipids, but all require the use of tandem mass spectrometry. LipidSearch Software (Thermo Scientific, San Jose, CA), LipidPro [1], Liquid [19] MSDial [30], and LipidMatch [18] are some examples of tools devoted to the identification of lipids using tandem mass spectra. The information about the fragmentation of lipids is key to their identification. However, the first step of annotation is commonly performed using just MS\(^1\) data and, sometimes, unequivocal identification is not necessary in untargeted metabolomics. Furthermore, even when unequivocal identification is necessary, a previous filter based on MS\(^2\) data to find which features represent likely lipids reduces the time necessary for the MS\(^2\) step of metabolite identification and helps to avoid incorrect assignments. LipidXplorer is another tool for annotation devoted to shotgun lipidomics using data-dependent MS/MS, so information from chromatography cannot be used to annotate the features [14]. VaLiD (Visualization and Phospholipid Identification) is a search engine for metabolite annotation with LC/MS data [3]. Its purpose is to identify lipids by m/z information searching of all theoretically possible phospholipids. It addresses the need of providing a database for all possible phospholipids because some are not present in the general databases. However, VaLiD does not assist the researcher in annotating features for which there is no prior knowledge of the compound type.

Tools to aid lipid annotation are of great value as a first step in lipid identification because in untargeted metabolomics studies researchers have no prior knowledge about the obtained features. The Lipid Annotation Service (LAS) is a representational state transfer (REST) application programming interface (API) service designed to aid researchers during lipid annotation based on MS\(^1\) data. It provides certainty levels for the putative annotations using three types of rules: (1) the ionisation likelihood depending on the type of compound and the modifier used, (2) the relationships between different features corresponding to the same compound, and (3) the RT relationships between lipids within the same class (the hydrophobicity of the lipids pertaining to the same class can be used to support or refute annotations).

Before LAS, the Ceu Mass Mediator Expert System (CMM-ES) [11] provided similar functionality, but it lacked the capability of providing explanations of the evidence supporting or refuting the annotations in natural language. These natural language explanations not only contribute to increasing the users’ confidence in the results of the tool because the user can understand the evidence supporting or refuting the annotations but also act as a pedagogical tool for novice researchers who might not know the details of how the evidence was generated. Implementing LAS functionality directly in CMM-ES would have been hampered by technical hurdles because the inference engine used by CMM-ES does not provide explanations about the inferences made. Therefore, it was necessary to reimplement from scratch the complete expert system using technologies that offer this functionality.

The core of LAS is a Prolog knowledge base published through a REST API service running in a Docker container. Both the LAS source code and the Docker container are available and have been documented in detail\(^1\) to provide a ready-to-use platform for the metabolomics community.

2. Methods

Statistical techniques play an important role in metabolite annotation, for example, when looking for correlations between the different signals arising from the same feature for grouping [2]. However, once these statistical techniques have been applied, a significant amount of manual work remains for the researcher [11]. Therefore, a rule-based system representing the knowledge that researchers use in this manual process was built as the core of LAS.

The phases of LAS development were analysis, design, implementation and testing, and performance evaluation. During the analysis phase, the problem was studied independently of the technology used to implement it. During the design, the architecture of the system was elaborated, technical decisions concerning programming languages and virtualisation were made, and tests were designed. During the implementation and testing, the system code and the tests were written in parallel. Thus, both have been debugged at the same time, and large deviations in the code with respect to its intended working have been prevented. The analysis (Section 2.2) and the design and implementation (Section 2.3) are presented below.

2.1. Functionality Borrowed from CMM-ES

The following knowledge and data have been borrowed from CMM-ES during the development of LAS:

- Knowledge to annotate lipids: the knowledge represented in the rules was already acquired from CEMBIO experts along the development of CMM-ES.
- Compounds data: the data on compounds were already available.

Nevertheless, as will be shown in the following sections, LAS has been implemented from scratch, and the way in which it represents both knowledge and data are completely different from that of CMM-ES. This has been the basis for LAS to contain new features like explanation of results, modular architecture, availability as a stand-alone service, or dockerization.

2.2. Analysis

LAS takes as its input a set of putative annotations. For example, each row of Table 1 is a putative annotation. Each putative annotation consists of an identifier (e.g. 3), an empirical compound (m/z and RT observation) (e.g. 400.3432@18.842525), an adduct (e.g. M+H), and a theoretical compound (e.g. L-palmitoylcarinute with formula C23H45N4O4 and lipid class fatty acyl carnitines [FA0707]). The lipid classification is performed using LipidMaps classification [10]. This classification has four levels: category, main class, subclass, and class level 4, and each compound can only belong to a single level. LAS applies the rules based on the compound subclass. The first number of the empirical compound represents its m/z, and the second number is its RT. The interpretation of the first putative annotation in Table 1 is that ‘empirical compound 400.3432@18.842525 may be theoretical compound L-palmitoylcarnitine with adduct M+H’.

LAS uses three sets of rules to assign certainty levels to the putative annotations. These sets of rules will be presented in Sections 2.2.1, 2.2.2, and 2.2.3. The possible certainty levels (CLSs) are very unlikely, unlikely, not applicable (N/A), likely, and very likely. The certainty order is very unlikely < unlikely < not applicable (N/A) < likely < very likely.

For example, Table 2 shows, for the putative annotations of Table 1, the CLS assigned according to the three sets of rules (ionisation rules, adduct intensity rules, and retention time rules). Thus, the meaning of the first row in Table 2 is “the CL of putative annotation 3 is ‘N/A’ according

\(^1\) https://marianofl1971@bitbucket.org/marianofl1971/mediatorkbservice.git.
to the ionisation rules, ‘very likely’ according to the adduct intensity rules, and ‘very likely’ according to the retention time rules”.

LAS provides, for each CL assignment, an explanation in English.

Along with the following subsections, the set of rules that generate multiple explained CL assignments for lipids will be presented. Each section includes the rationale and the definition of its rules and the linguistic patterns used to generate the explanations. The current version of LAS generates explanations in English. To generate explanations in other languages, more linguistic patterns should be added.

### 2.2.1. Ionisation Rules

#### 2.2.1.1. Rationale and Definition of Ionisation Rules

The majority of molecules are ionisable in both polarity modes, some of them ionise PCs, a formate or an acetate adduct is needed. Although the majority of molecules are ionisable in both polarity modes, some of them cannot be ionised by protonation, $[M + H]^+$; form $[M-H]$ ester (ST) according to the LipidMaps classiﬁcation: TG, ceramide (CER), phosphosphingolipid (SM) and cholesterol monoradylglycerols (MG), diradylglycerols (DG), triradylglycerols (LG), lysoglycerophosphoglycerol (LPG), glycerophosphoserine (PS), glycerophosphoinositol (PI), glycerophosphoglycerol (PG), glycerophosphoethanolamine (PE), lysoglycerophosphoethanolamine (LPE), glycerophosphoglycerol (GP), and the adduct is M + 2H; thus, the putative annotation is very unlikely.

The modiﬁer is not mentioned because it is indistinct. The CL assignment ‘putative annotation 78 is very unlikely’ in Table 2 has been deduced by applying this rule.

The explanation of the execution of an ionisation rule must contain information about the empirical compound, the adduct, the theoretical compound, if the ionisation is positive or negative, and the levels in the lipid hierarchy, as well as the CL of the rule. An impersonal sentence is used in the explanation: ‘it is likely that empirical compound (...) is theoretical compound (...)’.

A deﬁnition obtained considering what has been said in the previous paragraph is presented below.

### Definition 1

An ionisation rule is that whose antecedent has the following parts:

- **Modifier**: NH$_2$, HCOO, CH$_3$COO, HCOONH$_3$, CH$_3$COONH$_3$, or indistinct.
- **Ionisation**: positive or negative.
- **Levels in the lipid hierarchy**: a sequence of classes of lipids ordered from the most general to the most particular.
- **Adduct**: the assumed adduct in the putative annotation.

Two examples of ionisation rules are presented below.

#### Example 1

If the ionisation is negative, the levels in the lipid hierarchy are GP and GP01 and the adduct is M + 2H; thus, the putative annotation is very unlikely.

The modiﬁer is not mentioned because it is indistinct. The putative annotation of 211 as very likely in Table 2 has been deduced by applying this rule.

The complete list of the 109 ionisation rules is available in transformer/resources/lipids_ionization_type_rules.ods in the repository.

#### 2.2.1.2. How Ionisation Rules Are Explained

The explanation of the execution of an ionisation rule must contain information about the empirical compound, the adduct, the theoretical compound, if the ionisation is positive or negative, and the levels in the lipid hierarchy, as well as the CL of the rule. An impersonal sentence is used in the explanation: ‘it is likely that empirical compound (...) is theoretical compound (...)’ or ‘it is highly unlikely that empirical compound (...) is theoretical compound (...)’.

A deﬁnition obtained considering what has been said in the previous paragraph is presented below.

### Definition 2

The linguistic pattern followed by the explanation of each ionisation rule is as follows:

- **CertaintyLevel** that empirical compound m/z@RT (in an experiment with ionisationInEnglish ionisation and ModifierInEnglish is theoretical compound ThName with adduct Adduct, which belongs to the hierarchy CategoryU, MainClassU, SubClassU).

Here,

- **CertaintyLevel** is CL except not applicable,
- m/z@RT is the empirical compound of the putative annotation,
- ionisationInEnglish can be positive or negative,
- ModifierInEnglish is a modiﬁer,
- ThName is the name of the theoretical compound of the putative annotation.
• Adduct is the adduct of the putative annotation, and
• CategoryU, MainClassU, and SubClassU is a sequence in the lipid hierarchy to which the theoretical compound belongs.

Example 3. An explanation according to the pattern defined in Example 2 is presented below:

| Putative annotation 211 is very likely according to ionisation rules. |
| --- |
| **Rule type:** Ionisation. |
| **Core information:** It is very likely that empirical compound 281.24765@28.2695 (in an experiment with positive ionisation and no modifier) is theoretical compound 14,17-octadecadienoic acid with adduct M + H, which belongs to the hierarchy FA, FA01. |

2.2.2. Adduct Intensity Rules

A list of possible, impossible, and preferred ions for distinct compound types can be established, as well as relationships between the expected intensities of the different ions. For example, PCs can be ionised in positive mode by protonation ([M + H]+), but they can also be ionised with sodium ([M + Na]+) and potassium ([M + K]+). However, the main signal is [M + H]+ and all others have a lower intensity. Hence, a putative annotation for [PC + Na]+ can be right only if the signal corresponding to [PC + Na]+ is also present, otherwise it is an incorrect assignment.

For the current version of LAS, only the intensity pattern of adducts M + H > M + Na > M + K have been considered. More intensity patterns will be added in the future. Adduct intensity rules are defined as follows.

Definition 3. An adduct intensity rule checks whether there are pairs of compounds putatively identified as the same theoretical compound, and with adducts satisfying some of the following conditions.

1. The adduct of the first compound is M + H and the adduct of the second is M + Na, or vice versa.
2. The adduct of the first compound is M + H and the adduct of the second is M + K, or vice versa.
3. The adduct of the first compound is M + Na and the adduct of the second one is M + K, or vice versa.

The CL of the consequent will be very likely in every case.

Example 4. For instance, ‘putative annotation 3 is very likely according to the adduct intensity rules’ is inferred using relationship 1 (M + H, M + Na) with putative annotation 80.

The results of the adduct intensity rules are used in some cases to increase the CL of the ionisation rules. Thus, if an ionisation rule concludes that putative annotation p is likely, but an adduct intensity rule concludes that p is very likely, then the CL of the ionisation rule applied to p is increased to very likely, and the adduct intensity rule is considered as additional evidence. For example, an ionisation rule infers the CL assignment ‘putative annotation 312 is likely’. However, as shown in Table 2, it is transformed into ‘putative annotation 312 is very likely according to the ionisation rules’ because ‘putative annotation 312 is very likely according to the adduct intensity rules’.

2.2.2.1. How Adduct Intensity Rules Are Explained. The explanation of an adduct intensity rule has to include the empirical compound and the adduct and the theoretical compound of each putative annotation involved in the relationship between adducts.

Definition 4. The linguistic pattern followed by the explanation of each adduct intensity rule is as follows:

It is very likely that empirical compound m/z1@RT1 is theoretical compound ThName with adduct Adduct1 because empirical compound m/z2@RT2 with adduct Adduct2 may also be theoretical compound ThName.

where

• m/z1@RT1 is the empirical compound of the first putative annotation.
• ThName is the name of the theoretical compound of both putative annotations.
• Adduct1 is the adduct of the first putative annotation.
• m/z2@RT2 is the empirical compound of the second putative annotation.
• Adduct2 is the adduct of the second putative annotation.

Example 4 is explained below.

| Putative annotation 3 is very likely according to adduct intensity rules. |
| --- |
| **Rule type:** Adduct intensity. |
| **Core information:** It is very likely that empirical compound 400.3432@18.8425 is theoretical compound L-palmitoylcarnitine with adduct M + H because, for example, empirical compound 422.32336@18.8425 with adduct M + Na may be theoretical compound L-palmitoylcarnitine as well. |

If an adduct intensity rule causes an increase in the CL of an ionisation rule, the explanation of such ionisation rule will include a reference to the explanation the corresponding adduct intensity rule.

2.2.3. Retention Time Rules

The RT reflects the time that a particular molecule spends in the column because it is retained by the stationary phase. This time depends on the mechanisms of retention, column geometry and temperature, instrument dwell volume, mobile phase, modifier, and gradient. In the case of reverse-phase (RP) chromatography, the most common type of chromatography, polar analyte molecules interact little with the non-polar bed, and, thus, they elute very early. On the other hand, non-polar molecules will be retained for longer, thus eluting later. Although the flexibility of LC and the possibility to modify many experimental parameters (mobile phase, gradient, modifiers, flow, temperature, and type of column, as well as its length and diameter) make this technique very powerful, they also make obtaining reproducible RTs impossible. This is probably why there is only one proposal for metabolite annotation where dynamic RT prediction based on the chromatographic linear solvent strength for RP-LC data is used to support steroid identification Randazzo et al. [23]. The behaviour of molecules inside a chromatographic column under set chemical conditions is well defined, especially for compounds belonging to the same class Godzien et al. [12]. In consequence, although the absolute RT is very difficult to predict (even though it is not impossible Cao et al. [7]; Hagiwara et al. [13]; Stanstrup et al. [27]), the prediction of the relative order of elution is feasible for certain compounds belonging to the same chemical class that are analysed under the same analytical conditions. This can be a valuable aid in the analytical process, and the RTs for some types of compounds can be compared. This is especially interesting for lipids belonging...
to the same class because their backbones are the same, and this RT prediction is based on two relationships: the length of the carbon chain and the degree of unsaturation (number of double bonds) [12]. As the chain length increases, the hydrophobicity of a lipid molecule also increases, so the lipid will be retained longer in a RP column. On the other hand, double bonds increase the polarity of lipids, thus reducing the RT.

Consequently, the notional RT rule can be defined as follows.

**Definition 5.** Let \( p_1 \) be a putative annotation that establishes that a particular empirical compound, \( m/z_1 \), is theoretical compound \( p_1 \) having \( c_1 \) carbons and \( d_1 \) double bonds. Let \( p_2 \) be a putative annotation that establishes that a particular empirical compound, \( m/z_2 \), is theoretical compound \( p_2 \) having \( c_2 \) carbons and \( d_2 \) double bonds. Let us suppose that both \( p_1 \) and \( p_2 \) are lipid type \( p \). Then, there is evidence supporting \( p_1 \) because of RT if and only if some of the following conditions are satisfied.

1. \( c_1 < c_2 \) and \( d_1 = d_2 \) and \( r_{t1} > r_{t2} \)
2. \( c_1 = c_2 \) and \( d_1 < d_2 \) and \( r_{t1} > r_{t2} \)

The first condition means that, if the number of double bonds is the same for two lipids of the same class, that with a longer chain will have a higher RT. The second condition means that, if the length of the chains is the same for two lipids of the same class, that with a longer chain will have a higher RT.

**Definition 6.** Let \( p_1 \) be a putative annotation that establishes that a particular empirical compound, \( m/z_1 \), is theoretical compound \( p_1 \) having \( c_1 \) carbons and \( d_1 \) double bonds. Let \( p_2 \) be a putative annotation that establishes that a particular empirical compound, \( m/z_2 \), is theoretical compound \( p_2 \) having \( c_2 \) carbons and \( d_2 \) double bonds. Let us suppose that both \( p_1 \) and \( p_2 \) belong to lipid type \( p \). Then, there is evidence against \( p_1 \) because of RT if and only if some of the following conditions are satisfied.

1. \( c_1 < c_2 \) and \( d_1 = d_2 \) and \( r_{t1} > r_{t2} \)
2. \( c_1 = c_2 \) and \( d_1 < d_2 \) and \( r_{t1} > r_{t2} \)

**Definition 7.** Let \( p_1 \) be a putative annotation; the following RT rules can be applied.

1. If there is only evidence supporting \( p_1 \), then putative annotation \( p_1 \) is **very likely**.
2. If there is only evidence against \( p_1 \), then putative annotation \( p_1 \) is **unlikely**.
3. If there is both evidence supporting and against \( p_1 \), then putative annotation \( p_1 \) is **likely**.

**Example 5.** Putative annotation 95 is O-decanoyl-R-carntine, a CAR lipid with 10 carbons and no double bond. Its RT is 8.1449. Moreover, putative annotation 3 is L-palmitoylcarnitine, another CAR lipid with 16 carbons and no double bond. Its RT is 18.8425. Therefore, condition 1 of Definition 5 is satisfied and, consequently, there is evidence supporting both putative annotations.

**2.2.3.1. How Retention Time Rules Are Explained.** As shown in the following definition, the explanation of a retention time rule includes the empirical compound and the theoretical compound with the number of carbons atoms and double bonds. If there is evidence supporting or refuting the putative annotation, it will be explained as well, showing the information about the corresponding putative annotations.

**Definition 8.** The linguistic pattern followed in the explanation of each RT rule is presented below; moreover, explanations of the supporting or refuting evidence are provided.

It is **CertaintyLevel** that empirical compound \( m/z_1 \) is theoretical compound \( ThName \) with adduct \( Adduct \). The theoretical compound is a **LipidType** lipid with \( NCarbons \) carbons and \( NDDBE \) double bonds.

Here,

- \( m/z_1 \) is an empirical compound,
- \( ThName \) is the name of a theoretical compound,
- \( Adduct \) is an adduct,
- **LipidType** is a type of lipid,
- \( NCarbons \) is a number, and
- \( NDDBE \) is another number.

If there are no double bonds or there is only one, the linguistic pattern for this part of the sentence must be **no double bond or one double bond**, respectively.

**Example 6.** Example 5 is explained below.

| Putative annotation 95 is very likely according to the retention time rules. |
| --- |
| • **Rule type**: Retention time. |
| • **Core information**: It is very likely that empirical compound 316.24945@8.1449 is theoretical compound O-decanoyl-R-carntine with adduct \( M + H \). The theoretical compound is a CAR lipid with 10 carbons and no double bond. |
| • **Evidence supporting**: |
| 1. It is very likely that compound 400.3432@18.8425 is theoretical compound L-palmitoylcarnitine with adduct \( M + H \), another CAR lipid with 16 carbons and no double bond. |
| 2. It is very likely that compound 422.32336@18.8425 is theoretical compound L-palmitoylcarnitine with adduct \( M + Na \), another CAR lipid with 16 carbons and no double bond. |

2.3. Design and Implementation

The logic of the system is supported by a knowledge base (KB) in SWI-Prolog\(^2\) (see Fig. 1). Its most basic component is the **CL module**, which allows the representation of knowledge through a multi-valued logic according to the values specified at the beginning of Section 2.2. Using this multi-valued logic, the **meta-ontology** implements frame-oriented primitives: **instance of**, **subclass of**, etc. The **Compound Ontology** represents the CMM database of compounds based on the lower layers. The **module of ionisation rules** are generated from a spreadsheet that contains the rules. The **main module of the KB** loads the ionisation rules and implements the rest of the rules. This module builds a trace for explanations, which is natural language independent. That is, the formalization of the trace has been thought so that generators for different natural languages (English, Spanish, Polish, etc.) can be developed. Finally, the generator of **explanations in English** obtains the explanations from the trace built by the main module.

\(^2\) www.swi-prolog.org.
The REST API (also written in Prolog) eases the shareability of LAS as a Web resource and its integration in different systems. The whole system has been dockerised so that it can be installed on any machine regardless of the configuration, such as library version and environment variables. All the software is publicly available and documented in detail (see Footnote 1).

3. Results and Discussion

This section presents the evaluation of LAS considering its precision, recall, F-measure, the role of each group of rules (ionisation, adduct relationship, and RT), and the performance in terms of execution time, as well as the quality of the explanations obtained.

The performance of LAS in terms of precision, recall, and F-measure was determined using a gold standard provided by a CEMBIO expert. The gold standard was made up of 30 experimental mass signals (plasma samples, 3) previously identified by MS/MS or using commercially available standards (Sigma–Aldrich and Fluka Analytical). Plasma samples were prepared by simple deproteinisation with a cold methanol/ethanol (1/1) mixture. Standards were prepared in methanol with concentrations between 2 and 10 ppm. To obtain these data, analysis of the samples was carried out using a high-performance LC (HPLC) system (1200 series, Agilent Technologies) connected to an Agilent quadrupole time-of-flight QTOF (6520) MS detector. Data were collected in positive and negative electrospray ionisation (ESI) ion modes in separate runs on a QTOF MS operated in the m/z range of 50 to 1000 with an acquisition rate of 1 scan/s. The capillary voltage was set to 3000 V for positive and 4000 V for negative ionisation mode; the nebuliser gas flow rate was 10.5 L/min. Accurate mass measurements were obtained by means of an automated calibrant delivery system using a dual-nebuliser ESI source that continuously introduces a calibrating solution. All 30 experimental masses correspond to lipids.

We searched for putative annotations for the 30 experimental masses using the Ceu Mas Mediator tool, which returned a total of 891 putative annotations. These annotations were provided as input to LAS. The set of annotations generated by the LAS service, together with their corresponding evidence, was compared with the gold standard provided by CEMBIO.

3.1. Previous Definitions

Definition 9. Let \( p_1 \) and \( p_2 \) be two putative annotations. It is said that \( p_1 \) is supported by more evidence than \( p_2 \) if and only if the following conditions hold.

1. There is some set of rules (ionisation rules, adduct intensity rules, or RT rules) that supports \( p_1 \) as likely or as very likely.
2. There is at least a set of rules that supports \( p_1 \) with more evidence than \( p_2 \).
3. There is no set of rules that supports \( p_2 \) with more evidence than \( p_1 \).

Example 7. In Table 2, putative annotation 80 is supported by more evidence than 78 because 80 is very likely for both adduct relationship and retention time rules; meanwhile, 78 is N/A for both. Moreover, 78 is very unlikely according to the ionisation rules.

Let us note that there may be non-comparable putative annotations. For example, if the ionisation rules assign more evidence to \( p_1 \) but the RT rules assign more evidence to \( p_2 \).

Definition 10. Let \( p_1, \ldots, p_n \) putative annotations refer to the same empirical compound. \( p_1 \) is the putative annotation with the strongest evidence if and only if there is no putative annotation (\( p_2, \ldots, p_n \)) supported by more evidence than \( p_1 \).

Example 8. In the set of putative annotations made by 78 and 80, both referring to empirical compound 422.32336@18.8425, 80 is the putative annotation supported by the strongest evidence.

Definition 11. Two theoretical compounds are considered to be the same if and only if some of the following conditions are true.

1. Both have the same name.
2. Their names are synonyms according to Pubchem.
3. They belong to the same type of lipid with the same number of carbons and the same number of double bonds.

Example 9. The compounds MG(18:1(11Z)/0:0/0:0) and MG(0:0/18:1 (9Z)/0:0) are considered the same because they satisfy condition 3.
Fig. 3. Presentation of the results of the LAS service.
**Fig. 4.** Input form for the LAS service.
3.2. Calculation of the Performance in Terms of Precision, Recall, and F-Measure

Given the definitions in the previous section, the numbers of true positives (TP) and false positives (FP) are calculated as follows.

1. The KB is queried with the set of putative annotations generated by querying CMM. For each annotation the multiple certainty level assignment with the strongest evidence is taken.

2. Then, each annotation of the gold standard is compared to the putative annotation with the strongest evidence proposed by LAS. The following cases are possible.

(a) All the putative annotations with the strongest evidence refer to theoretical compounds considered to be same and match the gold standard. In such a case, the set of putative annotations with the strongest evidence are counted as TPs.

(b) Some putative annotations with the strongest evidence refer to theoretical compounds considered to be the same and match the real identification criteria, whereas others do not. In that case, the set of putative annotations with the strongest evidence are counted as both TPs and FPs.

(c) There are some putative annotations with the strongest evidence, but there is no putative annotation with the strongest evidence matching the gold standard. In such a case, we count an FP.

The precision, recall, and F-measure have been calculated, yielding 0.76, 0.93, and 0.84, respectively. In the next section, we will analyse in detail the contribution that each of the three groups of rules has played to achieve these results.

3.3. Contribution of Each Group of Rules

For the 30 compounds that made up the gold standard, the following rules of each type were executed.

- For 23 compounds, ionisation rules were executed; this was the only type of rule executed for 2 compounds.
- Some adduct intensity rules were executed for 10 compounds. Adduct intensity rules were always executed in conjunction with other rules. However, there is one case (400.3432@18.8425 with m/z 422.32336 and adduct M + Na) where the application of this type of rule was decisive in achieving the correct annotation.
- Some RT rules were executed for 25 identifications. RT rules were the only type of rule executed for three compounds.

Based on these results, it can be concluded that the RT rules were the most often executed and, thus, contributed most to the results, followed by the ionisation rules and the adduct intensity rules.

3.4. Execution Time Performance

Given that both the adduct intensity rules and RT rules check conditions with all pairs of putative annotations, the time required for LAS is quadratic with respect to the number of the putative annotations. Concerning real execution times, using an HP Envy 15 with an Intel i7, 16 GB RAM, and a 1 TB hard disk to host the service, LAS provided an answer in approximately 3 s for an input of 1300 putative annotations.

3.5. Integration Into the Ceu Mass Mediator On-line Tool

Before the implementation of LAS, the CMM tool also had an expert system based on knowledge that used rules similar to those presented in this paper. However, there are significant differences between the functionality previously provided by this expert system and LAS (see Fig. 2). CMM cannot provide an explanation of the evidence supporting or refuting the annotations. LAS explanations, which are provided in natural language, will increase the users’ confidence in the results of the tool because they can understand the evidence supporting or refuting the annotations. Furthermore, the LAS results could be used as a pedagogical tool for novice researchers who might not know how the evidence was generated. Providing this functionality required the reimplementation of the expert system from scratch because the technology used in CMM does not have an explanatory capability.

Furthermore, LAS formalises the CLs through a meta-ontology and an ontology of chemical compounds with the purpose of obtaining a modular, extensible, and theoretically sound system. In fact, both the meta-ontology and the ontology could be reused in other systems. CMM-ES was designed in an ad hoc manner and, thus, cannot be easily reused. Finally, LAS is accessible via a REST service. In contrast, CMM-ES is embedded in a larger Java system, and it cannot be used in an independent way. Nevertheless, LAS has been integrated as a CMM service in the on-line tool accessible through the web page [http://ceumass.eps.uspceu.es/prolog_batch_advanced_search.xhtml] (see Figs. 3 and 4). The service takes advantage of all the previous features that CMM offers for the identification of compounds and the automatic detection of adducts based on composite spectra, as well as the possibility of restricting the search to specific databases or specific compound types, the usage of a chemical alphabet, and the selection of different adducts, thus reducing the number of putative annotations for each feature. LAS has been used internally in CEMBIO since late 2018. This has allowed us to debug and provide feedback about the tool before its public launch.

4. Conclusions and Future Work

In this paper, we have presented LAS, a publicly available service that aids in the identification of lipids. The core of the system is a KB in Prolog that executes rules acquired from a group of experts belonging to CEMBIO. These rules can be divided into three large groups: ionisation rules, adduct intensity rules, and RT rules. A unique feature of the system is that it provides explanations in natural language (English) for the evidence supporting or refuting the annotations. In the evaluation carried out on a set of 30 compounds that had been identified with the use of standards or MS/MS, the LAS expert system obtained an F-measure of 0.84. LAS has been made available as a REST API service, which facilitates sharing and integration in different systems. The service has been dockerised to make it platform independent.

Regarding future work, the KB can be improved by taking into account intensities and relationships between more types of adducts in the adduct intensity rules. Concerning the explanations, they can be extended to other natural languages (Spanish, Polish, etc.) by adding linguistic patterns. Finally, content negotiation could be a useful functionality so that the results of the service can be presented in different formats (e.g. HTML).

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